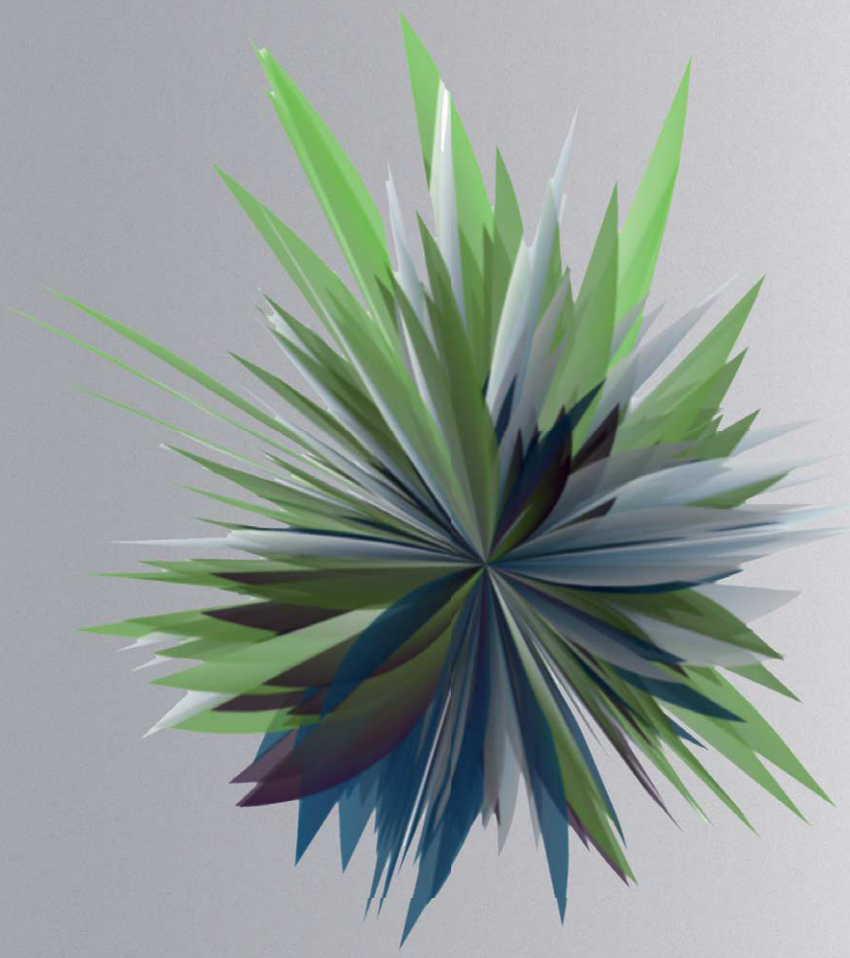
ADVANCES IN ALZHEIMER'S DISEASE 7

Handbook of Traumatic Brain Injury and Neurodegeneration



Edited by
Rudy J. Castellani

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ADVANCES IN ALZHEIMER'S DISEASE 7

Series editors: George Perry Ph.D and J. Wesson Ashford, MD, PhD

Handbook of Traumatic Brain Injury and Neurodegeneration

Holbourne's theory that rotational head movement and shear strains were limiting factors in producing acute parenchymal brain damage was a watershed moment in understanding traumatic brain injury (TBI). Long term effects, and in particular neurodegenerative proteinopathy subsequent to TBI, remain theoretical, notwithstanding the poorly understood 'punch drunk' syndrome of the early and mid-20th century, and the 21st century concept of chronic traumatic encephalopathy.

This book, the *Handbook of Traumatic Brain Injury and Neurodegeneration*, has as its theme the marriage between neurodegenerative disease and neurotrauma through TBI surrogates such as sport, military service, and experimental models, and the legitimacy of that marriage. In the 32 contributions included here, this handbook not only explores the deleterious effects of genuine TBI, but also, and more importantly, the relationship between TBI and neurodegeneration. Controversy notwithstanding, there is much to be learned about the biological effects of TBI, substrates for long-term sequelae, the relationship between TBI and diverse neuropsychiatric disorders, and targets for therapy.

The overall message to the neuroscience community from these papers may be a cautionary tale. The null hypothesis, that there is no causal relationship between TBI and progressive neurodegenerative disease, appears to be very much in play, and the book will be of interest to all those working in the field.

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Handbook of Traumatic Brain Injury and Neurodegeneration

Edited by

Rudy J. Castellani, MD

Professor and Vice-Chair of
Pathology Research & Section Chief of Neuropathology,
WVU Department of Pathology,
Morgantown, WV, USA

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Dedication

This book was made possible by the efforts and work of Beth Kumar, Managing Editor of the Journal of Alzheimer's Disease, and Dr. George Perry, Editor in Chief of the *Journal of Alzheimer's Disease*. Commendation is given to Rasjel van der Holst, a publisher at IOS Press, who conceptualized the idea of this book, participated in the recruitment of the editors, and worked diligently to make this book a reality. A special note is made here to honor the memory of Dr. Mark A. Smith, the late Editor in Chief of the *Journal of Alzheimer's Disease*, who continues to be a source of inspiration for efforts such as these, and beyond.

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Handbook of Traumatic Brain Injury and Neurodegeneration
R. Castellani (Ed.)
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Preface: Traumatic Brain Injury and Neurodegenerative Disease: A Marriage Made in Sport?

High school football in the 1940s

The watershed moment in understanding of traumatic brain injury (TBI) occurred with Holbourne's theory that rotational head movement and shear strains were limiting factors in producing parenchymal brain damage [1]. He based this on physical properties of the brain, including its extreme incompressibility and lack of rigidity. Holbourne's theory was substantiated and elaborated upon in primate experiments, in which coronal plane rotation of sufficient magnitude and pulse duration rendered subjects vulnerable to diffuse axonal injury [2, 3], while sagittal plane rotation and relatively short pulse duration predisposed to bridging vein rupture and subdural hematoma [4]. Related concepts have been invoked to explain the contrecoup contusion phenomenon [5].

Although subsequent modifications were inevitable, the initial theory coupled with experimental observations provided the biomechanical underpinnings for cardinal traumatic brain lesions—namely, subdural hematoma, contusion, and diffuse axonal injury. These same concepts have since been exploited to improve neuroprotection in motor vehicle accidents, military service, and sport, and are still relevant today. Myriad biochemical cascades in TBI have been elaborated, along with advances in diagnosis and acute management of a multiplicity of lesions. It is perhaps noteworthy that the foundational knowledge was acquired in the absence of computer technology, modern molecular biology, and immunohistochemical analysis of autopsied brain tissue.

A parallel line of inquiry into the enigmatic condition known initially as "punch drunk" [6] and later dementia pugilistica (DP) [7] was somewhat different and has been a source of confusion since its description. Punch drunk was called to attention in 1928 not because of acute injury, but because neurological signs were observed in boxers over the course of their boxing career and afterwards. DP was also a stationary condition in most cases [8], a feature distinct from classical neurodegenerative diseases. This may in part explain why no autopsy information on boxers was reported until 1954 [9], despite considerable interest in the topic in the 1930s and 1940s. It is also interesting, though largely unrecognized, that the index autopsy report in boxers was in fact a case of early-onset Alzheimer's disease, a condition of genetic etiology having nothing to do with boxing. The largest case series of DP to date emphasized a spectrum of brain lesions [10], some clearly trau-

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matic in line with Holbrourne's theory (e.g., septal fenestrations), but the focus since has been on the neurofibrillary tangle as a bridge between acquired neurotrauma and neurodegeneration.

The third line of inquiry is genuine neurodegenerative disease. Unlike neurotrauma, no acquired etiologies have been verified scientifically aside from spongiform encephalopathies, there is no stationary condition once initiated, and there is often highly selective cell type vulnerability. In all major neurodegenerative disease phenotypes, an invariably progressive and fatal neurological deterioration is associated with consistent neuropathological lesions and anatomically distinct neurodegeneration. None of these features are found consistently in the DP literature or modern case series using the term "chronic traumatic encephalopathy" (CTE). Indeed, neurodegeneration in the true sense, that is, loss of neurons, is neither a required nor supportive criterion in the current consensus iteration of CTE pathology [11].

A bewildering superstructure has nevertheless been assembled for TBI-neurodegeneration theory, which may highlight enthusiasm for hypothesis confirmation rather than hypothesis testing. The search for biomarkers in line with the preferred theory, including PET scanning for putative tau surrogates [12], serum and cerebrospinal fluid protein analyses [13], and examination of a broad array of neuropsychiatric endpoints [14], all emphasize sensitivity over specificity. Sophisticated experimental mechanisms have been invoked to explain disease progression [15], while progression *in vivo* has not been demonstrated. Constructs hypothesizing *causality* between TBI and neurodegeneration have been suggested [16], while evidence of marginal risk or no risk in large scale epidemiological surveys [17–24] essentially preclude causality. Sport-neurodegeneration theory is embedded in medical science, yet a quality evidence base is entirely lacking. The TBI-neurodegeneration hypothesis apart from sport is also broadly accepted, yet relies on a literature replete with methodological weaknesses [25]. Remarkably, it has become customary to view sport as a surrogate for environmental TBI exposure [26], yet modern athlete case series are devoid of empirical manifestations of TBI (e.g., contusions, subdural collections, post-traumatic epilepsy). Clearly, more research, and perhaps more skepticism, is needed.

The interested reader should keep in mind that this handbook does not simply explore the deleterious effects of genuine TBI, which are substantial, but rather the relationship between TBI and neurodegeneration. It was inevitable that some articles would touch upon the hypothetical CTE construct given the controversy and media exposure, although the interest in *critical* review on the part of multiple authors of diverse background was noteworthy. Casson and Viano, for example, bring to bear decades of experience and expertise in contact sport, and review in copious detail the stark differences between boxing and American football, not only clinically, but radiographically, and pathologically [27]. They appropriately highlight that neurological sequelae from boxing has traditionally been defined by clinical examination, whereas the putative condition described in football is purely pathological, or more precisely immunohistochemical, with no discernible clinical substrate. The totality of their review casts doubt, in a definitive sense, on the common rhetorical claim that "repetitive head impacts" from whatever sport is DP by another name.

Brett et al. [28] critically examine neuropathological and clinical criteria for CTE, and identify a noticeable lack of probabilistic assessments, which are otherwise customary when attempting to characterize ill-defined and hypothetical entities. They also expose the problematic emphasis on sensitivity over specificity. Zuckerman et al. [29] describe a number of limitations in CTE research, including ascertainment bias, recall bias, lack of generalizability of samples of convenience, lack of accounting for substance abuse, lack of adequate controls, and a CTE definition that has no lower limit. Schwab and Hazrati [30] further point out pervasive flaws in the CTE studies, including a decided lack of an evidence base as noted, insufficient samples, pathological inconsistencies, unreliable clinical data, and flawed study design. They raise the legitimate possibility of unintended consequences on broader society from promoting a fatalistic view of an uncharacterized process. The problem of amending public policy prematurely is also raised, and may warrant more attention than that afforded by a medical science community immersed in patient care and research.

Iverson et al. [31] provide a thorough evaluation of clinicopathological correlation in the CTE construct, and suggest that CTE neuropathology might be disambiguated from hypothesized clinical features in order to better understand each component in future research. The collective works from Castellani and Perry [32, 33], Castellani et al. [34], and Tripathy et al. [35] suggest a number of additional unresolved questions, such as the kinetics of progression in DP, the existence of a TBI-neurodegenerative

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proteinopathy construct in general, the role of tau as a driver of disease, the reliability of postmortem diagnostic interpretation in an emotionally charged environment, and the existence, if any, of CTE in military service members.

Overall, the articles consist of a roughly equal proportion of reviews and primary data papers. The majority discuss human disease, either in review form or as original research, with a few articles exploring TBI in animal models [36–39]. The human studies span a spectrum of endpoints, including PET imaging of putative tau surrogates [40], perfusion neuroimaging [41], potential biomarkers such as MCP-1 [42] and BDNF polymorphism [43], and postmortem proteinopathy as noted above.

The overarching theme of this Handbook is thus the marriage between neurodegenerative disease and neurotrauma by virtue of sport or military service, and the legitimacy of that marriage. Overall, it may be gleaned from these pages that, controversy notwithstanding, there is much to be learned about the biological effects of TBI, the presence and extent of genuine TBI in athletes and military service members, pathogenic mechanisms and substrates for long-term sequelae, the relationship between TBI and diverse neuropsychiatric disorders, and targets for therapy. If there is any broad message to the neuroscience community from the collective contributions, it is that the null hypothesis—that there is no relationship between TBI and neurodegenerative proteinopathy—is still very much in play.

Rudy J. Castellani MD
Professor of Pathology and Neuroscience
West Virginia University and
Rockefeller Neuroscience Institute

DISCLOSURE STATEMENT

The author's disclosure is available online (https://www.j-alz.com/manuscript-disclosures/19-1269).

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Long-Term Neurological Consequences Related to Boxing and American Football: A Review of the Literature

Ira R. Casson[a,*] and David C. Viano[b,*]
[a]*Department of Neurology, Zucker School of Medicine at Hofstra-Northwell, Hempstead, NY, USA*
[b]*ProBiomechanics LLC, Bloomfield Hills, MI, USA*

Abstract. The long-term effects of repetitive head trauma on the brain have often been studied in boxers and American football players. The medical literature on this topic was reviewed in order to compare the findings related to boxing with those related to football. The evidence gathered from this review indicates that there are significant differences between the clinical and neuropathological descriptions of the chronic brain damage reported in retired boxers compared to those reported in retired football players. Differing biomechanics of head impacts in the two sports may help explain the different clinical and neuropathological consequences of participation in boxing versus football.

Keywords: Boxing, brain injury, concussion, football, head injury, neuropathology

The athletic field has long served as a type of clinical laboratory for the study of head trauma. Due to the nature of their two sports, boxers and American football players have been frequent subjects of investigations of the effects of repetitive head injuries on the human central nervous system (CNS). The purpose of this paper is to review the salient medical literature in order to compare and contrast the long-term neurological syndromes seen in ex-boxers with those in ex-American football players. Many of the studies of living retired boxers included routine EEGs. None of the studies of living retired American football players included routine EEGs. Since we therefore cannot compare the results of this test in the two groups, we have decided to omit the EEG results from our reviews of the boxing literature.

The medical literature regarding the long-term neurological consequences related to boxing and American football was reviewed. The diagnostic methods and key results are presented and compared for the two sports. The biomechanical literature on head impacts in boxing and American football was summarized and compared.

BOXERS

In 1928, Martland [1] reported on a condition known as "punch drunk" that was well known to occur in older boxers. A fight promoter acquaintance gave Martland the names of 23 fighters that he considered to be "punch drunk". Dr. Martland examined 5 of them in person and reported in detail his findings in one case. This 38-year-old ex-boxer had a staggering, propulsive gait and a mask-like facies similar to that

*Correspondence to: Ira R. Casson, MD, 484 Arizona Ave, Rockville Centre, NY 11570, USA. E-mail: iradocdad@gmail.com and David C. Viano, Dr. med, PhD, 265 Warrington Road, Bloomfield Hills, MI 48304-2952, USA. E-mail: dviano@comcast.net.

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seen in parkinsonism, stammering, hesitant speech, and a fine tremor of both hands. His intelligence was normal. Martland gave abbreviated information on the other 4 cases he had personally examined: one had parkinsonian signs, one dragged his legs, another dragged his legs and had slow speech, and the fourth dragged his legs, had slow speech, and slow thinking. Based upon these 5 cases and the descriptions of other "punch drunk" cases by fight promoters, trainers, and fans, Martland stated that the early clinical features began in the extremities with "flopping" of one or both lower extremities and mild unsteadiness occasionally accompanied by "slight mental confusion". These signs might then progress to distinct dragging of the lower extremities, slowing down of all movements, hesitant speech, and tremors. In severe cases, parkinsonian signs, ataxia, and tremors resulted in a "staggering, propulsive gait". In end stages, marked mental deterioration may result in commitment to an asylum. Although Dr. Martland was a pathologist, no brain pathology of any boxers was presented. Martland hypothesized that this condition was the result of the accumulation of numerous traumatic brain microhemorrhages sustained while boxing. He presented the pathology results of a case of multiple brain hemorrhages resulting from head trauma in a non-boxer from a large series of such cases he had seen in his medical examiner practice.

Over the next 40 years, a number of clinical case and small series reports of a chronic neurological syndrome seen in retired boxers appeared in the literature. These are well summarized in a table in AH Roberts' 1969 monograph [2]. These consistently described a clinical neurological pattern of ataxia, slurred speech, cerebellar, extrapyramidal (parkinsonian), and pyramidal signs. There was no mention of suicidality and only occasional mention of depression. Personality changes such as euphoria, aggressive behavior, and sometimes violence occurred on occasion. Memory impairments and dementia also were reported in some cases. It is important to note that when depression, other personality changes, memory problems, or dementia occurred, they never did so in isolation; in other words, these only were seen in cases with coexisting abnormal patterns of clinical neurological signs. To illustrate these points, some of the well-known reports of the neurological abnormalities in retired boxers in the literature between 1928 and 1968 are described in detail.

In 1937, Winterstein [3] reported on the clinical findings of 50 professional boxers that he had examined. He did not detail the individual results but gave his impressions of the group as a whole. He noted a chronic neurological pattern which began with disequilibrium progressing to an unsteady ataxic gait, a "positive" Romberg sign, slurred, hesitant speech and a "vacant look" He also reported that "most of those badly affected" showed bad memory, impaired intelligence, and "mental dullness." Some of them were "euphoric, some paranoid and suspicious". There was no mention of depression or suicidality.

In 1957, Critchley [4] reported on 69 cases of chronic neurological disease in boxers he had personally examined. He described the clinical features of what he termed "chronic progressive traumatic encephalopathy of boxers". He described a gradual evolution of a "fatuous or euphoric dementia with emotional lability" with little insight, progressively slower speech and thought processes, and memory deterioration. The mood was commonly cheerful but "sometimes there is depression". Irritability and sometimes violent behaviors were noted in some cases. All of these cognitive and/or behavioral issues occurred in boxers with accompanying neurological abnormalities—"almost any combination of pyramidal, extrapyramidal and cerebellar signs". Dysarthria and tremors were commonly present. Critchley reported that within this "punch-drunk state" there were 4 commonly recurring syndromes: 1) signs reminiscent of neurosyphilis, 2) signs resembling multiple sclerosis, 3) signs resembling those of a frontal brain tumor, and 4) pallidal and striatal signs resembling parkinsonism. Critchley's impressions were that this syndrome occurred more frequently in professional fighters than in amateurs, more often in 2nd or 3rd rate fighters than in champions, more often in sluggers than in scientific skilled pugilists and more often in smaller compared to bigger men. He believed that the total number of fights and the number of times the fighter had been knocked out were also related to the development of this disease.

In 1959, Neubaerger et al. [5] reported on 2 cases of their own with clinical and neuropathological findings in former boxers and referenced 2 other cases [6, 7] with clinical and neuropathological findings in former boxers. Clinically the cases had neurological signs of extrapyramidal, pyramidal, and cerebellar dysfunction with dementia. Pathologically, three of the brains had increased large astrocytes, neuronal loss, widespread "fibrillary" changes, and cerebral atrophy, and the fourth brain [6] showed extensive

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plaque formation and cerebral amyloid angiopathy which may have represented early onset Alzheimer's disease (AD) unrelated to boxing.

In 1962, Spillane [8] reported neurological and pneumoencephalogram (PEG) findings in 5 former professional boxers. He found:

- Case 1 had a progressive dysarthria, urinary incontinence, spastic-ataxic gait, hyperreflexia, and a left Babinski sign without personality change or intellectual impairment; PEG showed an absence of the septum pellucidum.
- Case 2 had a progressive dysarthria, ataxia, right hemiparesis, urinary incontinence, and right optic atrophy. There was no personality change or intellectual impairment at first visit, but 2 years later there was occasional anxiety or depression with significant intellectual worsening. PEG demonstrated a cavum septum pellucidum.
- Case 3 had progressive dementia with severe memory impairments along with ataxia, tremors, and falling on Romberg testing. He was "euphoric" and "childish". PEG revealed a cavum septum pellucidum and markedly enlarged lateral ventricles.
- Case 4 complained of headaches and failing memory. Neurological examination was normal. There was no change in personality. PEG was normal.
- Case 5 had progressively worsening dysarthria, dragging of the left leg and depression resulting in psychiatric hospitalizations. Neurological examination revealed that he had poor recent memory and poor concentration but not dementia. He appeared "morose". He had dysarthria, ataxia and nystagmus. PEG was normal. He died of an myocardial infarction; autopsy revealed "softening" of the left cerebral and cerebellar hemispheres.

In 1963, Mawdsley and Ferguson [9] reported the clinical features of 8 former professional boxers. They found a pattern of dysarthria, nystagmus, limitation of upgaze, ataxia, tremors, and signs of pyramidal and extrapyramidal dysfunction. All of these cases plus 9 others were reported on by Johnson in 1969 [10], who reported on the clinical neurological and psychiatric findings of these 17 boxers. One case of an amateur boxer who was seen for transient self-resolving anxiety due to family issues was excluded from the results. The clinical neurological "manifestations" of these boxing related syndromes appeared before any psychiatric signs. These included varying combinations, of ataxia, intention tremors, dysarthria, pyramidal and extrapyramidal signs. Of the 16 cases, 11 had pyramidal signs, 7 had extrapyramidal signs, and 11 had cerebellar signs. Johnson found the following psychiatric syndromes in these 16:1) an amnestic syndrome in 11 and dementia in 3, 2) a morbid jealousy syndrome in 5, 3) psychosis in 5, and 4) a personality disorder with rage reactions in 4. In 3 of the cases with rage reactions, impulsive aggressive behavior was a lifelong trait. Johnson did not report any cases of depression or suicidality.

In 1968, Payne [11] reported on the clinical neurological findings and neuropathology of 6 former professional boxers. One case had a manic-depressive psychosis. Another had dysarthria, ataxia, a left 3rd nerve palsy, and papilledema. He had a history of compulsive gambling, sometimes became paranoid and violent, and at one point had set his house on fire. Another had a history of headaches, poor concentration, urinary incontinence, and depression. Examination revealed dementia, dysarthria, and incoordination. Another had dysarthria, ataxia, nystagmus, and incoordination along with headaches, depression, and difficulty concentrating. The 6th case had a normal neurological examination. Neuropathological findings were ventricular dilatation in all 6, cavum septum pellucidum in all 6, fenestrations of the septum pellucidum in 3, and multiple areas of micro scars in the gray matter in all 6.

The most detailed and comprehensive report to date on the clinical neurological features and the epidemiology of chronic traumatic encephalopathy of ex-professional boxers is AH Roberts' landmark 1969 monograph [2]. Before detailing his findings, Roberts reviewed more than 53 reported cases of chronic neurologic findings in former boxers in the medical literature since Martland's 1928 paper [1]. He noted that these revealed a pattern of various combinations of dysarthria, ataxia, tremors, parkinsonian signs, pyramidal signs, and cerebellar signs. The bulk of the monograph consists of neurological clinical reports on 224 randomly selected British former boxers. Working with the British Boxing Control Board, the records of 250 (representing about 1.5% of the total number of boxers registered with the board in past years) retired British boxers who had been licensed by the Board for at least 3 years in the past were selected for the study. These 250 and their wives were traced and invited by letter to participate in the study. When necessary, social workers or

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former boxing colleagues contacted the subjects to encourage their participation. Ultimately, 180 subjects came in to be examined, 43 were examined in their homes, and 1 was examined in the mental hospital where he was residing (total of 224). The examinations performed by Dr. Roberts (a neurologist) consisted of a detailed medical history, boxing history, family history, social history and educational history, and a comprehensive medical and neurological examination. Many, but not all, of the subjects were given "abbreviated versions" of psychometric tests of intellectual function. 37 of the 224 (17%) of the ex-boxers had "evidence of lesions of their central nervous systems similar to those reported and attributed to boxing by others". Not included in that 37 were subjects who had only isolated neurological signs (e.g., Babinski sign in isolation) and those whose neurological signs were indicative of clinical neurological entities seen in "routine neurology practice." Roberts presented in detail the findings in 11 characteristic cases. The clinical syndrome characteristically consisted of varying combinations of dysarthria, cerebellar, pyramidal, and extrapyramidal abnormalities with one or more of these predominating in different subjects. Dementia frequently accompanied these findings. Depression was only mentioned in one of the 11 cases described in detail. According to Roberts, this composite picture was "unlikely" to represent a "fortuitous" occurrence of neurological disease in boxers. Further evidence that these neurological abnormalities are related to boxing consists of evidence that their presence was statistically related to the subject's length of boxing career and number of professional fights in one's career (but interestingly, not to the number of times the boxer had been knocked out during his career). The limited neuropsychological test results revealed difficulties with synonym selection and poor verbal facility, but memory test results varied with age, not length of career. Sixteen of the 37 subjects with findings of traumatic encephalopathy showed "some degree of impaired intellectual function or personality change": 2 were definitely demented, 9 had severe memory impairments, 5 had "slow mentation", and 6 were irritable apathetic or uninhibited. When considering the entire cohort of 224 ex-boxers, 1 had a longstanding history of depression, 3 had minor phobias, and 5 had paranoid states (2 who were demented included in the 37 affected subjects, 2 with psychotic paranoid delusions included among the 37 affected subjects, and 1 with catatonic schizophrenia who was examined in a mental hospital and had no abnormal neurological signs). There was only 1 case among the 224 with clear evidence of a severe memory deficit without any clinical neurological abnormalities on examination.

With the clinical picture of chronic neurological disease of boxers having been well defined, Corsellis et al. [12] completed the picture with a seminal description of the associated pathology in 1973. They reported on the neuropathology of the brains of 15 ex-boxers (12 professional, 3 amateurs) which they had collected over a 16-year period. Their medical and boxing histories and results of clinical examinations were obtained posthumously from interviews with families and review of hospital/medical records. One subject had attempted suicide at age 65 (not completed) and died at age 72. He had parkinsonian features and parkinsonism documented in his medical records. A few of the other subjects had histories of aggressive behaviors and/or paranoia in conjunction with various combinations of dementia, dysarthria, cerebellar, pyramidal, and extrapyramidal signs on clinical examinations. Corsellis et al. described a tetrad of pathological findings that characterized the brains of these ex-boxers:

(1) abnormalities of the septum pellucidum (two subjects had died of intraventricular hemorrhage; in both cases the leaves of the septum were torn and separated but the authors were unsure if this had been accentuated by the bleeding so they did not include those cases in discussion of septal abnormalities). 12 of 13 had a cavum septum more than 3x the width of the small cavum septums they had seen in the brains of non-boxers; the one case without a cavum septum had fenestrations of the cavum; 11 of the 13 had gross fenestrations of the cavum;
(2) cortical scarring on the inferior surfaces of the lateral lobes of the cerebellum with associated marked loss of Purkinje cells was present in 10 of the 15 brains;
(3) gross depigmentation of the substantia nigra associated with almost complete loss of pigmented cells and neurofibrillary changes in many of the remaining cells (no Lewy bodies were seen) in 11 of the 15 cases;
(4) a regional occurrence of neurofibrillary tangles spread "diffusely through both cerebral cortex and brainstem", with very intense staining in the medial temporal gray matter, and a sparsity or total absence of senile plaques.

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The authors stated that this tetrad of abnormal neuropathological findings cannot be explained by any condition/disease other than exposure to boxing. They also pointed out that there was clinicpathological correlation between the neuropathology they were reporting and the clinical features of chronic brain disease in ex-boxers. Clinical disorders of memory/dementia correlate with neuropathology of the limbic areas (septal regions, fornix, mamillary bodies, medial temporal gray regions), rage reactions and other "abnormal affective states" correlate to these same regions, parkinsonian signs correlate to neuropathology in the substantia nigra and cerebellar signs correlate to the scarring/Purkinje cell loss in the cerebellar hemispheres. There was now a well-defined clinical picture associated with a highly correlated well-defined neuropathology of the disease that can be called chronic traumatic encephalopathy of boxers.

In the 1980 s and 1990 s, there were a few studies employing new neuroradiological techniques (i.e., CT scanning), more standardized neuropsychological testing, and newer brain microscopic staining methods to further refine knowledge of chronic brain injury in boxers. In 1982, Kaste et al. [13] reported the results of clinical neurological, neuropsychological, and brain CT scan testing on 14 Finnish retired champion boxers (8 amateurs, 6 professional) ages 19–53. One professional had apraxia, unsteadiness, and slowed mental functions on neurological examination. He and another professional reported episodes of "embarrassing inappropriate behavior". On neuropsychological testing, 12 subjects had abnormal results on Trail making tests and 2 of the professionals had abnormal results on more than 1 of the other subtests that made up the battery. On CT scans, 3 of 6 professionals and 1 of 8 amateurs had cerebral atrophy; 2 professionals and 1 amateur had a cavum septum pellucidum (CSP).

Also, in 1982, Casson et al. [14] reported on CT scans of 10 active professional boxers; 5 of the 10 had cerebral atrophy and 1 had a CSP. In 1983, Ross et al. [15] reported the results of CT scans and neurological examinations of 40 ex-boxers (amateurs and professionals) ages 21–73. Of the 38 who had CT scans, 20 had cerebral atrophy (presence or absence of CSP was not evaluated). The presence of cerebral atrophy was directly correlated with the number of total boxing matches fought by the subjects. Six of the 24 boxers who underwent clinical neurological examinations had abnormalities including memory loss, ataxia, difficulty with tandem gait, diminished deep tendon reflexes, and sensory abnormalities.

In 1984, Casson et al. [16] reported the results of neurological examinations, CT scans, and neuropsychological tests in 18 modern (all fought in the post-World War II era) boxers (13 retired professionals, 2 active professionals, 3 active amateurs) ages 18–60. The retired professionals were specifically selected from a group of 29 who were still associated with the sport in New York. Twenty-three who were age 60 or younger, had not retired from boxing for medical, neurological, or psychiatric reasons, and had no known history of neurological or psychiatric disease and no known history of alcohol or drug abuse were contacted and asked to participate in the study. Thirteen former boxers volunteered and were included in this report. All were fully employed. Such a selected group "would not be expected to have obvious symptoms or signs of neurological dysfunction". Eight of the 15 professionals had cerebral atrophy on CT scan and 3 of these 8 also had a CSP. All 3 amateurs had normal CT scans. Five of the 13 retired boxers had abnormalities on neurological examination (3 dementia including 1 who also had a unilateral Babinski sign, 1 impaired recent memory without dementia, 1 dysarthria and nystagmus). All the active professionals and amateurs had normal neurological examinations. Every subject had more than one abnormal neuropsychological subtest score (see Chart 1 for details). The group as a whole performed especially poorly on tests of recent memory, timed tests, and tests of executive function. Abnormalities on CT scan and neuropsychological testing correlated with number of professional fights but not with the number of knockouts or episodes of amnesia sustained.

In 1990, GW Roberts et al. [17] reinvestigated the neuropathological slides from Corsellis et al.'s 15 cases [12] with recently developed "immunocytochemical methods and an antibody to the beta protein present in Alzheimer Disease plaques". They reported that all of the cases "showed evidence of extensive beta protein immunoreactive deposits" that had not been detected by the staining techniques that were available to Corsellis et al. in 1973. The authors recommended that Corsellis et al's finding of tangles but no plaques be amended to acknowledge the presence of "substantial beta protein deposition". This paper was a purely neuropathological investigation with no clinical data. In the first paragraph of the paper, however, the authors mentioned 3 clinical stages of the "punch drunk" syndrome:

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1) affective and psychotic symptoms; 2) social instability, worsening psychiatric symptoms, memory loss and parkinsonism; and 3) the final stage of worsening cognition progressing to dementia and pyramidal tract disease.

There is no footnote reference for this statement and no data in the body of the paper related to this statement.

Having established the clinical, neuroradiological, neuropsychological, and neuropathological findings that characterize chronic neurological disease in boxers, we now turn our attention to American football players.

AMERICAN FOOTBALL

In 2005, Omalu et al. [18] reported a case of the brain neuropathology of a deceased 50-year-old former National Football League (NFL) player whose NFL career spanned 17 years after playing in high school and college. The gross pathology consisted of decreased pigmentation of the substantia nigra and otherwise was normal. Routine histology reportedly revealed neuronal dropout in the substantia nigra and cerebellar cortex but this was not depicted in accompanying images or quantified in any manner. No Lewy bodies were present. "The battery of immunohistochemical stains revealed frequent diffuse extracellular amyloid plaques, sparse-positive neuritic threads, and sparse intraneuronal band-shaped and flame-shaped neurofibrillary tangles (NFTs) in the frontal, temporal, parietal, occipital, and cingulate cortex and the insula." The authors wrote that these findings "met criteria for CTE", specifically citing Corsellis et al.'s 1973 [12] report on the neuropathology of chronic traumatic encephalopathy (CTE) in boxers. Omalu et al. stated that "possible symptoms of CTE may include recurrent headaches, irritability, dizziness, lack of concentration, impaired memory, and mental slowing; mood disorders, explosive behavior, morbid jealousy, and pathological intoxication and paranoia, tremor, dysarthria, and parkinsonian movement disorders". The subject's premortem medical history (obtained from posthumous telephone interviews with relatives of the deceased) of "dysthymic" mood disorder, deficits in memory and judgment and parkinsonian symptoms was noted. There was subsequently an exchange of letters between Casson et al. and Omalu et al. discussing the validity of Omalu et al.'s statements [19, 20].

In 2005, Guskiewicz et al. [21] reported the results of a study on memory impairments and AD in retired NFL players. The authors mailed self-report health questionnaires to 3,683 retired players; 2,552 participated by returning the forms. Thirty-three (1.3%) of these indicated that they had been diagnosed with AD by a physician. There was no correlation with the number of concussions reported, but the authors stated that AD occurred in younger ages in the subjects compared to the general public. A mild cognitive impairment (MCI)/memory questionnaire was filled out by a subset of 758 retired players and in 641 cases also a spouse or other close relative. Twenty-two subjects reported that they had been diagnosed with MCI by a physician. Another 77 subjects were reported to have "significant" memory impairments according to their spouses or other close relatives. The authors reported that there was a significant correlation between the prevalence of MCI/memory impairments and subjects' history of having sustained 3 or more concussions during their playing careers.

In 2006, Omalu et al. [22] reported "the second autopsy-confirmed case of chronic traumatic encephalopathy in a retired professional football player". They stated that the neuropathological features differed from those in their first case; "In contrast to the first case, the brain in this second case revealed topographically distributed sparse to frequent NFT (neurofibrillary tangles) and NT (neuritic threads) in the neocortex, hippocampus, subcortical ganglia, and brainstem. There were no diffuse amyloid plaques." There was a non-fenestrated CSP and mild pallor of the substantia nigra. Neuronal dropout was reported in the substantia nigra, the cerebellum and the brainstem. This was not quantified or depicted in accompanying images. Premortem medical history was obtained posthumously from surviving relatives and review of medical records from a psychiatric hospital. There is no mention of any objective neurological examinations. The 45-year-old subject had played football for 2 years in the military, 4 years in college, and 8 years in the NFL. He had a history of major depression, mood swings, multiple suicide attempts (cause of death was a completed suicide), multiple business failures, and being indicted for arson.

In 2007, Guskiewicz et al. [23] reported on the prevalence of depression in retired NFL players. The study data was collected from the same self-report questionnaires returned by the same group of subjects that was discussed previously [21]. Two hundred and sixty-nine of the 2,552 subjects (11%) reported that

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they had previously or currently been diagnosed with depression. The authors stated that this prevalence "is generally consistent with the lifetime prevalence in the general U.S. population". They reported that the prevalence of depression was three times greater in subjects with a history of 3 or more concussions compared to subjects with a history of fewer than 3 concussions.

In 2009, McKee et al. [24] reported the neuropathological findings in 2 retired pro boxers and 1 retired pro football player. They also reviewed the 51 cases of "neuropathologically confirmed CTE" in the literature (including the 3 cases they reported in this paper). They stated that repeated closed head injuries occur in many contact sports and that in collision sports like football and boxing "players may experience thousands of subconcussive hits during the course of a single season". The 51 cases they reviewed included 39 ex-boxers and 5 ex-professional football players. They stated that the clinical picture of "CTE" gleaned from this review began with cognitive and psychiatric/ behavioral symptoms which progressively worsened and in severe cases developed "slowing of muscular movements, abnormal gait, masked facies, vertigo, speech problems and tremors". They (incorrectly) cited Corsellis et al. [12] as reporting 3 stages of clinical deterioration as follows:

- "The first stage is characterized by affective disturbances and psychotic symptoms.
- The second stage involves social instability, erratic behavior, memory loss, and initial symptoms of Parkinson disease.
- The third stage consists of general cognitive dysfunction progressing to dementia and is often accompanied by full-blown parkinsonism, as well as speech and gait abnormalities."

McKee et al. [23] reported that 14 of the 45 cases (boxers and football players) had a mood disturbance, usually depression, early in the course and that "movement abnormalities were eventually found in 41%" of the cases. They cited Corsellis et al. [12] in the discussion of the neuropathological findings, listing the major findings (CSP and fenestrations of the cavum listed fourth). McKee et al.'s [24] review of the literature revealed a CSP in 69% of cases and fenestrations in 49%. Review of the graphic presentation of the neuropathology of the cases indicates that the brain of the new football player subject in this paper did not have a CSP or fenestrations but the brains of the new boxer cases presented in this paper both had CSPs and one had fenestrations of the septum as well. McKee et al. [24] emphasized the histological and immunocytochemical findings of widespread neurofibrillary changes and tau deposition in the superficial cortical layers (II and III), often in a perivascular distribution. They stated that these neuropathological findings, specifically the distribution of abnormalities, correlated with the clinical picture of behavioral and cognitive dysfunction and the later appearance of parkinsonism, ataxia and speech disturbances.

In 2010, Omalu et al. [27] reported on 5 cases (4 ex American football players and 1 wrestler) of neuropathologically confirmed CTE that had exhibited suicidality while alive. Two of the football players were previously reported by Omalu et al. in 2005 and 2006 [18, 22]. Clinical information on all 5 cases was obtained from retrospective, posthumous interviews with relatives and/or friends of the deceased. The authors reported that the 5 cases had demonstrated behavioral symptoms such as memory loss, abnormal executive functioning, paranoid ideation, depression, and suicidality. They concluded that "CTE refers to chronic cognitive and neuropsychiatric symptoms of chronic neurodegeneration following a single episode of severe traumatic brain injury or (more commonly) repeated episodes of mild traumatic brain injury".

In 2011, Omalu et al. [26] reported on neuropathology of 14 professional athletes (including 8 retired American football players and 1 ex-boxer (some of these cases had been previously reported by Omalu et al). Seven of the 8 football players and the boxer were neuropathologically diagnosed with CTE using the following criteria: "The fundamental neuropathologic feature of CTE is the presence of sparse, moderate, or frequent band-shaped, flame shaped, small globose, and large globose NFTs in the brain accompanied by sparse, moderate, or frequent neuritic threads "in a characteristic topographic distribution including perivascular and depths of sulci locations". Only one of the cases in this series (the 1 ex-boxer) was reported to have abnormalities of the septum (fenestrations). Unlike McKee et al.'s [24] cases, Omalu et al.'s [26] cases did not demonstrate that these neurofibrillary changes preferentially involved the superficial cortical laminae. Omalu et al. [26] also pointed out a number of other differences between the neuropathologies in this series and those described by McKee et al. in 2009 [24]. Omalu et al. [26] suggested that these differences might be related to the fact that most of the cases of CTE reported by McKee et al. [24] in that literature review and case

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report were boxers whereas most of the cases Omalu et al. were presenting were in football players with only one being a boxer. "The differences between their conclusions and ours may suggest that there may be subtle neuropathologic distinctions between CTE found in different sports professionals." The clinical features were similar to those reported in Omalu et al.'s 2010 report [27].

In 2013, McKee et al. [28] published the neuropathological findings of "CTE" in the brains of 85 athletes, 34 of whom were retired American football players. In the Introduction, the authors linked the paper to work on dementia pugilistica in boxers beginning with Martland in 1928 [1] and repeated their earlier statements [24] regarding the predominance of symptoms of behavioral/psychiatric (including suicidality) and cognitive dysfunction in cases of CTE dating back to those earlier reports in boxers. Medical histories were obtained posthumously from families and friends of the deceased. CTE was diagnosed by histological/immunocytochemistry findings of specific patterns of hyperphosphorylated tau in these brains. The authors divided up the cases into 4 stages of CTE based on extent of tau deposition, with stage 1 being the mildest and stage 4 the most severe. None of the 7 stage 1 cases had septal abnormalities, 4 of the 14 stage 2 cases had small CSPs, 5 of the 15 stage 3 cases had septal abnormalities, and "most" of the 15 stage 4 cases had large CSPs or septal perforations or absence of the septum. The authors reported the following clinical classification:

- Stage I chronic traumatic encephalopathy included headache and loss of attention and concentration;
- Stage II added symptoms including depression, explosivity and short-term memory loss;
- Stage III included executive dysfunction and cognitive impairment;
- Stage IV included dementia, word-finding difficulty and aggression.

The authors specifically noted that 13 of the 51 CTE cases in this series had suicidality (7 completed suicides and 6 with suicidal ideation). The authors also stated that the neuropathological stages of CTE correlated with number of years of football played. Finally, McKee et al. [28] stated that there was a clinicopathological correlation between the neuroanatomical areas "affected by CTE" and the behavioral/psychiatric and cognitive dysfunctions that make up the clinical picture.

In 2013, Hart et al. [29] reported the results of neurological and neuropsychological testing in 34 retired NFL players (ages 47–71) and the results of MRI imaging in 26 of these same subjects. The results were compared to those of matched controls. Results of routine anatomical MRI brain imaging were not reported; the results of advanced 3T MRI techniques including FLAIR, diffusion tensor imaging, hemosiderin scanning and arterial spin labeling were reported. On NP testing, 14 subjects (41%) demonstrated" cognitive deficits": 4 had "fixed" deficits, 8 had MCI, and 2 had dementia. Eight of the 34 (24%) were diagnosed as having depression. Overall, the NP testing revealed "mild difficulties" in naming, word finding, and episodic verbal and visual memory. Detailed results of various NP subtests are included in Table 1. The results of the clinical neurological evaluations of all the subjects are not explicitly stated. The report indicates that 7 of the NFL players complained of headaches and 1 complained of dizziness, that "no neurological abnormalities suggestive of" amyotrophic lateral sclerosis were detected and "that none of the retired players fit the reported clinical profile for CTE". The authors reported that cognitive impairments and depression in the NFL players correlated with changes in blood flow to specific brain regions and white matter abnormalities detected on advanced MRI imaging.

In 2014, Casson et al. [30] reported the results of neurological, neuropsychological, and MRI testing of 45 retired NFL players between the ages of 30 and 60. The subjects were recruited from a list of over 5,000 retired NFL players supplied by the players' union. The testing procedures were modeled after the author's 1984 study of living retired boxers [16]. Neurological examinations revealed isolated Babinski signs in 3 subjects, mild sustention-intention tremors in 2 subjects, horizontal nystagmus in 1 subject, and abnormal dynamic visual acuity testing in 3 subjects. There were no instances of dementia, dysarthria, cerebellar or extrapyramidal signs. On neuropsychological testing, there were no cases of dementia, 28 subjects (62%) were normal, 11 subjects (24%) had impairments on 1 or 2 subtests (possible MCI), and 6 subjects (13%) had borderline to mild impairments on 1 or 2 subtests confounded by low verbal IQ or lack of effort (Table 1). Anatomical MRI scans revealed 3/45 subjects with a large CSP and cerebral atrophy. Thirty-one subjects had small CSPs which the authors pointed out was similar to the prevalence of small CSPs on MRI in the general population. Advanced MRI techniques revealed 4 subjects with

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Table 1
Summary of standardized neuropsychological test findings in boxers and football players

Source	Cases	Ages (y)	Testing results
Boxers			
Kaste [13]	14 boxers, 8 amateurs, 6 pros	19–53	Trails Making test – 12/14 abnormal Abnormal overall NP tests – 2/6 pros, 0/8 amateurs
Casson [16]	13 ex-pros, 2 active pros,	18–60	Clinical mental status testing of 15 pros, 3 dementia, 1 impaired recent memory
	3 active amateurs		Trails making test – 14 pros*, 10/14 abnormal Digit Symbol – 14 pros*, 7/14 abnormal Wechsler Memory – 13 pros tested**: Verbal immediate – 13/13 abnormal Verbal delayed – 13/13 abnormal Visual immediate – 11/13 abnormal Visual delayed – 11/13 abnormal Bender-Gestalt 5 second delay – 15/15 pros abnormal
Football Players			
Hart [29]	34 retired pros	47–71	"Cognitive deficits" 14/34, 4 "fixed", 8 MCI, 2 dementia Trail making tests A and B – 22 tested – all normal CVLT T scores – 22 tested 12 from "unimpaired " subgroup, all normal 10 from "impaired " subgroup had lower mean T scores (mean 39.6) than "unimpaired group but range was 32.5 – 46.7 and T scores above 33 are normal WAIS Digit span all ss scores normal
Casson [30]	45 retired pros	<60	Trail making tests A and B T scores – 2/45 abnormal part A, 3/45 abnormal part B (no overlap) WAIS digit span – all ss (scores normal BVMT Delayed T scores (visual memory) – 8/45 abnormal CVLT Delayed T scores (verbal memory) – 12/45 abnormal
Koerte [34]	72 retired pros		Trail Making A and B- as a group, mean scores similar to controls Other tests, only group scores given, difficult to compare to other studies

*One pro not tested. **Two pros not tested.

microbleeds on susceptibility weighted imaging and mean fractional anisotropy results on diffusion tensor imaging for the entire group that correlated with number of concussions sustained in the NFL and number of years of pre-high school and high school football played. The authors concluded that "MRI lesions and neuropsychological impairments were found in some players; however, the majority of retired NFL players had no clinical signs of chronic brain damage."

In 2016, McKee et al. [31] reported on the results of a NINDS-NIBIB consensus meeting on the neuropathological criteria for the diagnosis of CTE. "The panel defined the pathognomonic lesion of CTE as an accumulation of abnormal hyperphosphorylated tau (p-tau) in neurons and astroglia distributed around small blood vessels at the depths of cortical sulci and in an irregular pattern." Other tau related pathologies and gross pathologies such as septal abnormalities were supportive of the diagnosis but not necessary for the diagnosis. The authors stated that "previous data has shown that the pathological features of CTE associated with boxing (often referred to as "dementia pugilistica") are similar to the pathological features of CTE associated with football." No clinical criteria for the diagnosis of CTE were mentioned.

In 2017, Mez et al. [32] reported on the neuropathological findings in 202 American football players, 111 of whom had played professionally in the NFL. Using the NINDS-NIBIB consensus refined neuropathological criteria for the diagnosis of CTE (i.e., at least 1 perivascular phosphorylated tau (p-tau) deposit at the depths of cortical sulci), 110 of the 111 NFL players were diagnosed with CTE. Stages 1 and 2 CTE cases were considered mild; stages 3 and 4 CTE cases were considered severe. Clinical information on the cases was obtained retrospectively (posthumously) from family and friends of the deceased. The authors stated that there were 2 general clinical presentations of CTE: 1) behavior and/or mood symptoms in those presenting at a younger age, and 2) cognitive symptoms in those presenting at an older age. Among the behavior/mood symptoms frequently seen in these cases were impulsivity, depressive symptoms, apathy, and anxiety; less

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commonly reported were hopelessness, explosivity, verbally or physical violent, and suicidality. Dementia and motor symptoms were common in severe cases. Among the 27 mild CTE NFL cases, 26 had behavioral and/or mood symptoms and 23 had cognitive symptoms. Among the 84 severe CTE NFL cases, 75 had behavioral and/or mood symptoms and 80 had cognitive symptoms. Despite claiming that in severe cases of CTE the anatomical distribution of p-tau correlated with behavioral/mood/cognitive clinical symptomatology, the authors also noted that "participants with mild CTE pathology often had these symptoms despite having relatively circumscribed cortical pathology and absence of ptau pathology in the hippocampus, entorhinal cortex or amygdala."

COMPARE AND CONTRAST THE CHRONIC NEUROLOGICAL CONSEQUENCES OF REPETITIVE HEAD INJURIES IN BOXING AND AMERICAN FOOTBALL

Although the initial reports regarding both boxers and football players were authored by forensic pathologists [1, 18], the means of investigation and research methods involved in evaluating athletes in the two sports diverged immediately following these bylines. In the 40 years following Martland's report, well over 100 cases of the clinical neurological findings in living retired boxers were described in the literature [1, 3, 4, 8, 9]. There were detailed descriptions of neurological signs in individual cases as well as descriptions of the neurological syndromes seen in large groups of retired boxers that had been personally examined by authors. Then, in 1969, AH Roberts [2] published his classic monograph in which he described the unique clinical pattern of neurological findings that defined a chronic disease of ex-boxers, based upon his personal examinations of 224 randomly selected living retired boxers (37 of the 224 exhibited signs of this disease). Although a few autopsy reports of brains of boxers had appeared over the years, it was not until Payne [11] and then Corsellis et al. [12], that a definitive, unique pattern of neuropathological abnormalities in retired boxers was described. Payne [11] reported on the neuropathological findings in the brains of subjects whom he had previously examined in life. Corsellis et al.'s [12] neuropathology reports were accompanied by clinical information obtained from posthumous interviews with relatives of the deceased as well as reviews of medical records. It is thus clear that the development of ideas regarding chronic CNS disease in retired boxers was, from the beginning, clinically driven and based largely upon neurological evaluations of living subjects.

This stands in sharp contrast to the neuropathologically driven origins and subsequent investigations into chronic CNS neurological disease in retired American football players. Both of the initial papers by Omalu et al. [18, 22] reported on a single case of the neuropathological findings in a deceased former NFL player. The only clinical information came from posthumous statements by relatives of the deceased. No objective reports of neurological examinations during life were included. Later reports of larger series of neuropathology findings in deceased NFL players by Omalu et al. [26, 27] followed this same pattern of clinical information collection. When McKee et al. [24] started reporting on neuropathology in retired football players, they included a review of the literature of "neuropathologically verified cases" of chronic brain disease related to repetitive brain trauma (mostly ex-boxers) without reviewing the large number of clinically defined cases (without neuropathology) that make up the bulk of the cases in the boxing literature. McKee et al.'s later reports [28, 31, 32] of large numbers of cases of "CTE" in retired NFL players are based on neuropathological findings in the brains of deceased subjects with clinical information obtained from more structured interviews with relatives and friends of the deceased but still without objective neurological examination findings while the subjects were alive.

In addition to these neuropathology reports, two reports of data on depression and cognition from a large number of retired NFL players have been reported; both relied solely on self-report questionnaires from the subjects and their spouses and neither include any objective neurological examinations [21, 23]. The two studies of clinical examinations of living retired NFL players in the literature found some changes on advanced MRI, some cognitive/memory dysfunction, a prevalence of depression similar to or slightly higher than the general population, and essentially unremarkable clinical neurological examinations [29, 30]—none of which seemed to correlate with the neuropathology that has been reported by Omalu et al. [18, 22, 25–27] and McKee et al. [24, 28, 31, 32].

In view of these divergent approaches to investigating chronic CNS disease, it should come as no surprise that the clinically-based research into

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boxers should result in a clinically-defined syndrome which correlates with a well-defined neuropathological picture and that the neuropathologically-based and centered research into football players has led to a purely neuropathologically-defined condition.

There are clear differences between the descriptions of the long-term clinical effects on the brain in boxers versus football players. The clinical picture in football players is predominantly mood/behavioral and memory/cognitive in nature. "Motor symptoms" including parkinsonism and gait disturbances are described as occurring late in the disease and are mentioned mostly in passing. The reports from Omalu et al. [25–27] emphasized behavioral/mood abnormalities such as suicidality, depression, and paranoid ideation along with abnormalities of memory and executive functions. In their 2009 paper, McKee et al. [24] stated that the clinical picture of "CTE" gleaned from their review began with cognitive and psychiatric/behavioral symptoms which progressively worsened and in severe cases developed "slowing of muscular movements, abnormal gait, masked facies, vertigo, speech problems and tremors". In McKee et al.'s [28] study on a large series of cases (mostly football players), McKee et al. [28] described clinical features of mild cognitive impairments, explosive behaviors, and depression in early, milder cases progressing to dementia, executive function dysfunction, and aggressive behavior in later stages. In Mez et al.'s [32] paper reporting a large series of cases of "CTE" in football players, they stated that there were two general clinical presentations of CTE: 1) behavior and/or mood symptoms in those presenting at a younger age; and 2) cognitive symptoms in those presenting at an older age.

Among the behavior/mood symptoms frequently seen in these cases were impulsivity, depressive symptoms, apathy, and anxiety; less commonly reported were hopelessness, explosivity, verbally or physical violent, and suicidality. Dementia and motor symptoms were common in severe cases.

In their reports, Omalu et al. [18, 22, 25–27], McKee et al. [24, 28, 31], and Mez et al. [32] often stated that the clinical symptoms they were reporting in American football players were consistent with, similar to or the same as those reported by the earlier studies of retired boxers. The review of the boxing literature that is the focus of this present review clearly indicates that such statements are not correct. It is possible that a methodological error may be, in part, to blame for these different points of view. In McKee et al.'s [24] review of the boxing literature, only "neuropathologically verified cases of CTE" were included. McKee et al. [24] thus inadvertently ignored the purely clinical reports of Winterstein [3], Critchley [4] (his study was referenced in the footnotes only to support the concept that the condition is progressive), AH Roberts [2], and others that laid out in great detail the clinical neurological descriptions that characterize the disease in living ex-boxers.

Beginning with Martland [1] and continuing along through Winterstein [3], Critchley [4], Spillane [8], Mawdsley and Ferguson [9], and then AH Roberts [2], the descriptions of the clinical features in boxers emphasize the neurological signs of dysarthria, cerebellar dysfunction, extrapyramidal dysfunction (parkinsonism), and pyramidal dysfunction, occurring in varying combinations. These authors emphasize the unique nature of these neurological patterns seen in boxers as being distinct (but reminiscent) from those seen in other neurological conditions. Memory impairments and dementia were seen in some cases (16 of 37 in AH Roberts' monograph [2]) but generally in the more severe cases and almost always accompanied by the abnormal neurological signs previously mentioned. For example, only 1 of the 224 ex-boxers examined by AH Roberts [2] had significant memory deficits unaccompanied by the abnormal neurological signs of chronic brain damage. Psychiatric/behavioral disorders were present in some cases, again almost always accompanied by abnormalities on the clinical neurological examination. Depression was mentioned in only a small number of cases. Suicidality was rarely reported. When Johnson [10] reported on psychiatric disorders in 16 retired boxers, he noted that in all 16 the neurological signs of disease appeared prior to the psychiatric impairments. Johnson [10] reported 4 types of psychiatric disorders in these retired boxers: amnestic syndrome, morbid jealousy, psychosis, and personality disorder with rage reactions/impulsive aggressive behaviors. There was no mention of suicidality or depression. In summary, the clinical features reported in football players are predominantly mood/behavior/cognitive in nature whereas those reported in boxers are predominantly neurological in nature; when cognitive/behavioral symptoms occur in boxers, they usually are seen late in the course, concomitantly with abnormal neurological signs and are of different types than have been reported in football players.

There are a number of possible explanations for these major differences in clinical features. A likely reason is that the chronic CNS disease of boxers is

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a distinctly different condition than the chronic CNS disease of American football players. Another possibility is that the different methods of ascertainment of the clinical features in the subjects (neurological examinations of living retired boxers versus posthumous collection of medical history from the relatives and friends of deceased football players) has resulted in widely differing information being collected. It is also possible that the selection bias in the football cases (i.e., families of deceased players who manifested behavioral/mood/cognitive problems in life may be more likely to donate their loved ones' brains to brain banks than families whose loved ones manifested only physical or no symptoms in life), may have affected the reported clinical picture. This selection bias could also result in the inclusion of some clinical symptoms that are unrelated to the effects of head trauma.

There are also a number of significant differences between the brain neuropathology reported in football players and that reported in boxers. The unique tetrad of pathology in boxers reported by Corsellis et al. [12] (septal abnormalities, cerebellar scarring and neuronal loss, gross depigmentation of the substantia nigra with near total loss of pigmented neurons and regional occurrence of neurofibrillary tangles diffusely throughout the brain with scarce or no senile plaques – amended in 1990 by GW Roberts [17] to include diffuse immunoreactivity to amyloid proteins) is not what has been reported by Omalu et al. [18, 22, 25–27] and later McKee et al. [24, 28, 31] in football players. The latter have focused their descriptions on the histopathology of neurofibrillary changes/p-tau deposition in specific topographic distributions in the brain. Septal abnormalities which were present in all 15 of Corsellis et al.'s [12] boxing cases and all 6 of Payne's [11] boxing cases have not been present in many of the "mild" football cases but are more common in the "severe" football cases. As McKee et al. [31] have refined the pathological definitions of chronic brain damage in football players, the presence of septal abnormalities and other gross abnormalities has been more and more de-emphasized. According to present NINDS-NIBIB criteria [31], the sole feature required for a diagnosis of "CTE" in football players is the presence of 1 or more foci of perivascular accumulations of p-tau in neurons and astroglia in the depths of sulci in an irregular pattern. Despite Omalu et al.'s and McKee et al.'s claims to the contrary, this is a far cry from Corsellis et al.'s description [12]. In a 2018 study of immunostaining of material from 14 of Corsellis et al.'s 15 boxing cases, Goldfinger et al. [33] found that only 7 of the 14 met the NINDS-NIBIB pathognomonic criteria for the diagnosis of "CTE" in football players; this serves as further evidence of the significant differences between the neuropathology of boxers and that of football players.

Comparison of the results of ancillary tests (e.g., neuroimaging studies, standardized neuropsychological testing) in living retired boxers [13, 17] and football players [29, 30, 34] can also shed light upon the long-term effects of participation in these sports on the brain (Table 1). On the Trails making test, which is a measure of executive functioning, 22 of 28 boxers were impaired whereas only 5 of 67 football players were impaired. In another group of retired football players [34] where individual scores were not recorded, the group of 72 NFL players performed similarly on the Trails A and B tests to a group of controls. On Wechsler Memory Scale testing of verbal memory, 13 of 13 boxers were impaired and on testing of visual memory, 11 of 13 boxers were impaired. Although it is not the same exact test, football players were administered the California Verbal Learning Test of verbal memory. In one study, 12 of 45 football players were impaired on the California Verbal Learning Test: in the other study, 10 of 22 football players had mean T scores in the low normal range but it is not clear how many were impaired. Although it is not exactly the same test as the Wechsler visual memory test, football players were administered the Brief Visuospatial Memory Tes test of visual memory. Eight of 45 football players were impaired on the Brief Visuospatial Memory Tes. The total numbers of retired boxers and football players whose equivalent standardized test results are available for comparison is small. The results do suggest that retired boxers exhibit a much higher prevalence of impaired function in the spheres of executive function and verbal/visual memory compared to retired football players.

Comparison of neuroimaging studies between boxers and football players is not straightforward, mainly because different neuroimaging studies were used in different eras. Thus, PEG studies were performed on boxers in the 1960 s [7, 8, 35], CT scans were performed on boxers in the 1980 s and 1990 s [13–15, 17, 36, 37], and MRI scans were performed on football players [29, 30, 34, 38] (and in one study on active boxers [39]) from 2000 onwards. With this in mind, Table 2 summarizes the neuroimaging find-

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Table 2
Summary of neuroradiology findings in boxers and football players

Source	Cases	Tests	Results
Boxers			
Spillane [8]		PEG	2/5 large cavum septum (CSP), 2/5 cerebral atrophy, 1/5 absent septum
Grahmann [7]		PEG	1 case with large CSP and cerebral atrophy
Isherwood [35]		PEG	9/16 CSP, 11/16 enlarged ventricles, 4/16 cerebellar atrophy, 3/16 cerebral cortical atrophy
Kaste [13]		CT scans	Cerebral atrophy 3/6 pros, 1/8 amateurs, CSP 2/6 pros, 1/8 amateurs
Ross [15]		CT scans	Ex-pros, 20/38 cerebral atrophy, did not assess for CSP
Casson [14]	10 active pros	CT scans	Cerebral atrophy 5/10, CSP 1/10
Casson [16]	3 amateurs	CT scans	All CTs normal
Casson [16]	13 ex-pros, 2 active pros	CT scans	Cerebral atrophy 7/13 ex pros, 1 of 2 active pros, CSP 2/13 ex-pros, 1 of 2 active pros
Jordan [36, 37]	338 active pros	CT scans	22/338 cerebral atrophy, 7 of these 22 had CSP, 45/338 had CSP
Jordan [36, 37]	45 active pros	CT scans	Serial CTs of – 3 developed a new CSP on followup scan, 3 developed progressive atrophy on followup scan
Aviv [39]	164 active pros	MRI scans	Some with serial scans, CSP: 81/164 – 24 medium size, 13 large size 8 boxers developed new CSP on followup scans
Football players			
Hart [29]	26 retired NFL players	MRI scans	no mention of CSPs or cerebral atrophy, FLAIR*, DTI**
Casson [30]	45 retired NFL players	MRI scans	3/45 with large CSP and cerebral atrophy, 31/45 small CSP SWI : 4/45 – 1 microbleed DTI: as a group, FA correlates with # of NFL concussions and years of pre high school and high school football played
Gardner [38]	17 retired NFL players	MRI scans	16/17 CSP – 1 large, 8 medium, 7 small
Koerte [34]	72 retired NFL players	MRI scans	CSP 66/72 – 35/72 > 6mm size (large), presence of large CSP correlated with poorer performance on some cognitive tests

*FLAIR: deep white matter lesions. **DTI: disrupted white matter integrity.

ings in the literature. It is important to note that MRI scanning is much more sensitive in visualizing anatomical structures such as the septum pellucidum than CT or PEG. Therefore, the prevalence of CSPs on MRIs may not be comparable to the prevalence on CTs due to better technology. Early generation CT scans were unlikely to detect small CSPs, so any that were visualized on those CT scans were probably large. Review of the studies summarized in Table 2 suggests that cerebral atrophy was more commonly seen on neuroimaging of retired boxers than of retired football players; however, since only one of the MRI studies of retired football players actually assessed cerebral atrophy [30], this awaits further investigations. Large CSPs on neuroimaging seem to be more common in both groups than in the general population. MRIs and CT scans have demonstrated that CSPs can develop over time in active boxers [36, 40]. It certainly appears that repetitive brain trauma is linked to large CSPs.

Based upon this review of the medical literature, Table 3 compares the main clinical and neuropathological features of the chronic CNS conditions in retired boxers and retired American football players [1–39].

BIOMECHANICS OF BOXING AND AMERICAN FOOTBALL HEAD IMPACTS

Differences between the head impacts sustained in football and boxing may partially explain the long-term neurological consequences of repetitive head injuries in players of the two sports. Football players wear hard shell helmets and faceguards during practice and games while professional boxers wear no headgear during bouts (they may wear soft headgear while sparring). Boxers frequently sustain multiple blows to the head in rapid succession often with only minimal intervals between impacts. Football players sustain head impacts in rapid succession much less frequently due to the intervals between plays that are part of the sport. The differing biomechanics of head impacts between the two sports, although less obvious to the general public, may play an even greater role.

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Table 3
Chronic CNS conditions in retired boxers and retired American football players (based upon review of medical literature).

Boxers	Football Players
Clinical picture – primarily neurological	**Clinical picture – primarily cognitive or psychiatric/behavioral**
Dysarthria	Suicidality
Cerebellar dysfunction	Mood disorders including depression
Extrapyramidal dysfunction – parkinsonism	Aggressive/erratic behavior/personality changes
Pyramidal dysfunction	Memory loss, executive dysfunction, dementia
Secondarily cognitive/or psychiatric	**Secondarily motor, parkinsonian dysfunction**
only in some cases	If present, occur late in the disease
if present, almost always in conjuction with neurological signs	
Neuropathology	**Neuropathology**
Abnormalities of septal region (large cavum and/or fenestrations of cavum)	Primary: pathognomonic finding is one or more foci of phosphorylated tau perivascularly at the depths of one or more sulci
Widespread neurofibrillary tangles with few or no plaques (but amyloid-beta present with special staining techniques)	Supportive features such as other histological findings and septal abnormalities may be present but are not necessary for the diagnosis
Neuronal loss in substantia nigra (without Lewy bodies)	
Neuronal loss in cerebellum	

The biomechanics of punches has been studied using surrogates for the opponent. Atha et al. [40] used a single boxer and an instrumented ballistic pendulum to evaluate a single straight punch. The professional boxer punched the surrogate with 4,096 N, which the author estimated translated into 6,320 N of force to a human head. This force produced peak acceleration for 53 g on the 7 kg ballistic pendulum. Joch et al. [41] placed 70 boxers into one of three categories, including 24 elite, 23 national, and 23 intermediate boxers. The force of straight right punches was measured with a water-filled punching bag fit and pressure transducer. The maximum punch force ranged from 2,932 N – 3,453 N.

Smith et al. [42] developed a boxing dynamometer to measure punch force. Twenty-three boxers were sorted into elite, intermediate, and novice boxer categories. The elite boxers had a mean rear-hand punch force of 4,800 N and a front-hand punch force of 2,847 N. The intermediate boxers' rear and front hand punch forces were 3,722 N and 2,283 N, respectively, and the novice boxers' mean rear and front hand punch forces were 2,381 N and 1,604 N.

Walilko et al. [43] studied the biomechanics of straight punches to the jaw causing translational and rotational head acceleration. Seven Olympic boxers from five weight classes delivered 18 straight punches to the compliant face of the Hybrid III dummy. The punch force averaged $3{,}427 \pm 811$ N, hand velocity 9.14 ± 2.06 m/s and an effective punch mass 2.9 ± 2.0 kg. The jaw load was 876 ± 288 N. The peak translational acceleration was 58 ± 13 g, rotational acceleration $6{,}343 \pm 1789$ r/s^2, and neck shear 994 ± 318 N. The severity of the punch increased with weight class primarily due to a greater effective mass of the punch.

Viano et al. [44] reported on the punch biomechanics of 11 Olympic boxers weighing 51 kg (112 lb) to 130 kg (285 lb). The effective mass of the arm was measured and the tests followed the methods of Walilko et al. [43]. A Hybrid III dummy with a frangible face was used to represent the response of the jaw and realistically transfer acceleration to the head. For the tests, a cork insert was used to give facial compliance for the straight jaw punches. There were straight punches to the forehead, hooks to the temple, and uppercuts jaw of the Hybrid III dummy.

Table 4 summarizes the Olympic boxer's height, weight, and effective punch mass of the hand. The hand mass increased with boxer weight and averaged 1.67 ± 0.28 kg. The average acceleration of the hand in the punch was 180.9 ± 98.8 g with an average hand force of $2{,}994 \pm 1{,}875$ N. The hook produced the greatest impact force of $4{,}405 \pm 2{,}318$ N), inertial load on the head of $3{,}107 \pm 1{,}404$) and neck load of 855 ± 537 N. It also had the highest velocity change of the hand at 11.0 ± 3.4 m/s. The hook also produced the largest head translational and rotational accelerations at 71.2 ± 32.2 g and $9{,}306 \pm 4{,}485$ r/s^2. The lowest forces occurred with the uppercut to the jaw. The punches resulted in $2.98 \pm 0.9 = 87$ change in velocity of the Hybrid III head with an average 48.9 ± 28.4 g head acceleration and $6{,}319 \pm 3{,}739$ r/s^2 for all punches.

Pellman et al. [45, 46] reported on the reconstruction of NFL game impacts causing concussion. The

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Table 4
Comparison of the biomechanics of boxing and football impacts

	Boxers [43, 44]		NFL Players [45–47]				
	Severe punch		No injury		Concussion		
	Average	sd	Average	sd	Average	sd	
Olympic Boxers							NFL Players
Height (cm)	177.2	9.2					Height (cm)
Weight (kg)	76.2	22.1	98.3	16.1	100.7	16.4	Weight (kg)
Hand weight (kg)	1.67	0.28			14.0		Eff. striking weight (kg)
Hand velocity (m/s)	9.1	2.1	7.0	2.6	9.3	1.9	Impact velocity (m/s)
Punch							Striking Player
Hand accel (g)	180.9	98.8	54.8	22.8	56.8	22.4	Resultant accel (g)
			114	103	122	107	HIC
Hand delta V (m/s)	8.87	2.73	4.00	1.24	4.13	1.28	Head delta V (m/s)
Hand force (N)	2,994	1,875	5,209	1,774	7,642	2,259	Impact force (N)
Struck Head							Struck Player
Resultant accel (g)	48.9	28.4	67.9	14.5	94.3	27.5	Resultant accel (g)
HIC	52	53	145	39	357	184	HIC
Head delta V (m/s)	2.98	0.87	5.38	0.48	7.08	1.88	Head delta V (m/s)
Rotation accel (r/s2)	6,319	3,739	4,714	1,097	6,654	1,745	Rotation accel (r/s2)
Rotational Velocity (r/s)	22.9	7.2	31.8	5.4	40.6	9.9	Rotational Velocity (r/s)
Head intertial force (N)	2,131	1,237	3,026	649	4,206	1,228	Head inertial force (N)
Neck load (N)	1,016	638			2,319	782	Neck load (N)

biomechanics of the helmet impacts were determined using Hybrid III dummies in laboratory testing. Viano et al. [47] reported on the biomechanics of the struck player experiencing concussion. Table 4 summarizes the results of the NFL reconstructions. The effective mass of the striking player was estimated at 14.0 kg. This is 8.4-times greater mass than the mass of the hand in a boxing punch. Since the speeds of impact are similar, the football impacts involve more momentum in the impact. The translational accelerations of the head are greater in football impacts with concussion than in boxing (94.3 ± 27.5 g versus 48.9 ± 28.4 g) and resulted in higher Head Injury Criterion (HIC, 357 ± 184 versus 52 ± 53). This is a result of the smaller effective mass of the hand in boxing. Boxing punches produced about the same rotational accelerations of the head ($6{,}319 \pm 3{,}739$ g versus $6{,}654 \pm 1{,}745$ r/s^2). The boxer's punch resulted in lower rotational velocity of the head than in NFL concussion (22.9 ± 7.2 r/s versus 40.6 ± 9.9 r/s) because of the lower momentum in the punch.

The force from the boxer's hook exceeded that of the non-injured NFL players and was within the statistical range for concussion. The jaw and forehead impact forces were lower and the uppercut produced the lowest inertial loads on the Hybrid III head. Boxers deliver the same or more rotational acceleration to the Hybrid III dummy head for the hook and jaw punches than occurred in NFL concussions. However, the duration of impact is shorter for the boxing punch, so the rotational velocity of the head is similar to that in NFL concussion impacts with longer duration but lower rotational acceleration. The NFL reconstruction data includes the correction for problems with the rotational acceleration package used in the testing [48].

The boxers' punches resulted in lower translational accelerations in the struck head, as compared to the football impacts. The boxers' punches applied a higher moment to the struck head than did the football impacts. This necessarily resulted in higher rotational accelerations in the head struck by the boxers' punch. Boxers sustain brain injury by two mechanisms, translational and rotational accelerations of the brain, with a preponderance of the rotational component. Professional football players sustained MTBI mostly by translational accelerations.

The super-heavy weight boxer generated punch forces of $5{,}352 \pm 2{,}775$ N, which is above the average impact force of $7{,}642 \pm 2{,}259$ N with concussion in NFL players. Since a majority of NFL players are injured by facemask or lateral impacts on the helmet, the loading direction is consistent with lateral direction of the boxing punches. Straight anteroposterior impacts are an uncommon cause for NFL concussions.

There are probably two primary means by which boxers deliver concussive blows. The first means involves the boxer delivering enough translational acceleration. The hook involves a blow to the temple, which is just above the head center of gravity (cg).

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The forehead punch delivers force frontally above the head cg and the jaw impact applies force below the head cg. These impacts translate the head, and the forces can reach levels consistent with NFL concussions. The damaging mechanism is translational acceleration where the greater the mass of the punch, the greater the HIC and translational acceleration. The second means involves rotational acceleration, which occurs with the impacts taking advantage of the offset from the head cg. During the punch, the axis of impact moves away from the head cg and introduces proportionately more rotational acceleration during the punch. The hook, for example, is always thrown with the elbow bent [49]. This necessarily results in the axis of impact moving away from the cg after impact, thus imparting a significant amount of rotational acceleration to the opponent's head.

With the use of football helmets, the striking player must line up his impacts closely with the head cg of the other player. This allows the impact to transfer energy. If the impact vector is at an angle, the blow will glance off due to the smooth plastic shell of the helmets. Players need to align their impact through the head cg to deliver a solid blow and maximize energy transfer to the other player. Severe helmet impacts causing concussion involve high translational acceleration and change in head velocity (delta V). NFL concussions involve an average impact velocity of 9.3 ± 1.9 m/s; and, the delta V is 7.08 ± 1.8 m/s for the concussed player. Since the duration of impact is nominally 15 ms, the peak head acceleration is high at 98 ± 28 g. In football, there is a strong correlation between translational and rotational acceleration due to the impact alignment and subsequent head-helmet motion.

In boxing, the punch and glove conform more to the head of the opponent allowing punches to induce high rotational acceleration without high translational acceleration. The effective mass of the boxer's fist is 1.67 ± 0.28 kg, which is more than an order of magnitude lower than the 14 kg effective mass of the helmeted football player who strikes an opponent [47]. With concussion, the striking player lines up their head, neck, and torso so their effective mass is considerable, and only the head and part of the opponent's neck resist the blow. In boxing, the most efficient energy transfer involves more rotational acceleration than translational acceleration.

The punch velocity of the boxers averaged 6.7–11.0 m/s for the four different punches. These levels are essentially similar to the impact speed in football concussions; but, the head delta V after a punch was only 2.8–3.1 m/s on average, well below half that of the head delta V with NFL concussion. This reflects the much lower effective mass of the punch. Boxers cannot deliver high translational acceleration and delta V to the opponent because of the low punch mass in comparison. Boxers deliver rotational accelerations in and above the range where NFL players are concussed.

These significant differences between the biomechanical forces impacting the head and brain in football as opposed to boxing are likely a partial explanation for the differing long-term neurological effects seen as the result of repetitive head injuries in the two sports.

CONCLUSION

Participants in boxing and American football are often subject to multiple repetitive head injuries over time. This review of the medical literature has focused on the possible long-term effects of such exposure on the CNS. The evidence indicates that there are significant differences between the clinical neurological features of chronic brain damage seen in boxers and those seen in football players. There are also significant differences in the brain neuropathology that has been described in boxers as compared to that described in football players. The clinically driven approach to studying boxers that is evidenced in reviewing the boxing literature has resulted in a well-defined clinical picture and a well-defined neuropathological picture that correlates well with that clinical picture. The pathology driven approach to studying football players has resulted in a highly refined and specific neuropathological definition of chronic brain damage in football players with uncertain correlations to a clinical picture mostly arising from the highly subjective retrospective recollections of relatives of deceased players rather than from objective clinical neurological examinations of living retired players. Differences in the biomechanical impact forces in boxing and football may be a part of the explanation for the differences between the chronic CNS disease of boxers versus football players.

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mittee. Neither author has been affiliated with the NFL since 2010. This paper is dedicated to Joshua and Benjamin.

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Dementia Pugilistica Revisited

Rudy J. Castellani[a,*] and George Perry[b]
[a]*Center for Neuropathology, Western Michigan University School of Medicine, Kalamazoo, MI, USA*
[b]*College of Sciences, University of Texas, San Antonio, San Antonio, TX, USA*

Abstract. Extensive exposure of boxers to neurotrauma in the early 20th century led to the so-called punch drunk syndrome, which was formally recognized in the medical literature in 1928. "Punch drunk" terminology was replaced by the less derisive 'dementia pugilistica' in 1937. In the early case material, the diagnosis of dementia pugilistica required neurological deficits, including slurring dysarthria, ataxia, pyramidal signs, extrapyramidal signs, memory impairment, and personality changes, although the specific clinical substrate has assumed lesser importance in recent years with a shift in focus on molecular pathogenesis. The postmortem neuropathology of dementia pugilistica has also evolved substantially over the past 90 years, from suspected concussion-related hemorrhages to diverse structural and neurofibrillary changes to geographic tauopathy. Progressive neurodegenerative tauopathy is among the prevailing theories for disease pathogenesis currently, although this may be overly simplistic. Careful examination of historical cases reveals both misdiagnoses and a likelihood that dementia pugilistica at that time was caused by cumulative structural brain injury. More recent neuropathological studies indicate subclinical and possibly static tauopathy in some athletes and non-athletes. Indeed, it is unclear from the literature whether retired boxers reach the inflection point that tends toward progressive neurodegeneration in the manner of Alzheimer's disease due to boxing. Even among historical cases with extreme levels of exposure, progressive disease was exceptional.

Keywords: Boxing, dementia pugilistica, neurofibrillary degeneration, tauopathy

INTRODUCTION

Given the necessity for self-defense in essentially all animal species, it is not hard to imagine the clenched fist appearing in human evolution alongside the act of running, or that pugilism is likely as old as humankind itself. Egyptian hieroglyphics dating from 4000 BC suggest the existence of boxing-like combat as a military expediency, with thongs wrapped around hands as a precursor to the boxing glove [1]. Similar leather-wrapped fists were noted in ancient Baghdad. Boxing as a part of various games and festivals was evident in early Greek and Roman cultures [1]. The term *boxing* is believed to have its origin in the clenched fist, or the folding of fingers and thumb into a box-like structure, its roots from the Greek and Latin being *puxos* and *buxus*, respectively. Implements such as the cestus, a form of lethal gauntlet, were used in Ancient Rome for contests among gladiators on feasts and holidays, but were eventually banned in favor of the common sense view that fights to the death, or near death, was not necessarily advantageous for warrior training [1]. Without the prospect of someone being killed, public interest in boxing declined in the later years of the Roman Empire. Emperor Theodosius the Great banned the Olympic games and therefore its boxing component in 393 AD [1]. The subsequent 1300 years saw a relative historical silence to boxing as a spectacle with greater emphasis on warfare

*Correspondence to: Rudy J. Castellani, MD, 300 Portage Street, Kalamazoo, MI 49007, USA. Tel.: +1 269 337 6173; Fax: +1 844 337 6001; E-mail: rudolph.castellani@med.wmich.edu.

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and weaponry, but resurfaced in England, the "cradle of pugilism," [1] coinciding with expansion of the proletariat of the industrial revolution. Ritualistic armed contests such as fencing that developed in the middle ages drifted into sport for the aristocracy, while boxing was something of an equalizer for the new labor class, and a diversion from the squalor of urban confinement.

By the late 19th and early 20th century, boxing gloves and rules of engagement (e.g., Queensberry Rules of 1867) had been broadly implemented, allowing more of a sporting quality compared to underground bare knuckle contests, and formal promotion of "prize fighting." Its popularity was such that quality professional boxers could occasionally subsist solely on contest proceeds, while many others could earn a worthwhile supplement to a laborer's wage [2]. This is perhaps among the many factors that led to increased exposure and increased numbers of those exposed in recent times. At any other time in recorded history, boxing appears to have been either compulsory or provided an unenviable sum to that which could be derived from the low wage labor market or the informal street economy. The increased popularity also meant that lower tier and undertrained boxers would continue fighting while their skills were in decline, and at times offer themselves up for a small stipend in professional contests, knowing full well that severe physical punishment was the likely outcome [2]. Moreover, when not participating in promoted fights, many boxers fought in carnival booths, as often as 30 to 40 times a day [3], in an unmonitored fashion, "taking on all comers." Others sparred with upper tier and more punishing fighters as a separate endeavor, or in addition to promoted contests and booth exhibitions [2]. The long term effects of these ancillary activities, potentially synergistic with promoted contests, was obliquely addressed by Roberts [2], but are largely unknown to this day and likely lost to history. The plight of such rank and file boxer is summed up well by Critchley [4]:

> "*Of special aetiological importance is that humbler side of boxing where the contestant travels in fairs in the boxing booth (or "blood-tub"), taking on all-comers at any weight. Almost as characteristic is the story of the boxer who eventually gives up the ring, having failed to make the grade, later to become what they call a "punch-bag"- that is, one of a team of sparring partners to a first-class heavyweight. A typical story is that the boxer, after a promising early career in the ring, begins to slow up; to be knocked out more often; to win fewer contests; and to be seedy for increasingly longer periods after each affray. Most characteristic of all is the admission on the part of the boxer that he finally abandoned the ring because of his wife's increasing disapproval of his career.*"

High levels of neurotrauma exposure from boxing in the early 20th century is apparent in the early case material with professional fights numbering as many as 1,000 and after a lengthy amateur career [5]. Such high numbers are not encountered today. According to one literature review, the average boxing career since the 1930 s has declined from 19 years to 5 years, and the number of fights has declined from an average of 336 to 13 [6]. The popularity of the sport at that time was such that skilled fighters would have bouts arranged and promoted almost every week, compared to once a month or less by the 1950 s [2]. Oversight of the contests also differed. Prior to World War II, there was no inclination on the part of either the referee or the competitors to stop the fight when one of the fighters was obtunded [2]. Repetitive concussive blows leveled on an incapacitated fighter "out on his feet," until he lay prostrate on the canvass, occurred as a matter of routine [4]. The presence of a physician was variably required among the state boxing commissions, mainly for purposes of emergency care [2]. It was not until 1979 that the New York State Athletic Commission specified that an assigned physician could step into the ring and stop a bout [7]. Exceptionally long contests with numerous two minute rounds were commonplace before 1940, and little care was taken in fight promotions to match evenly skilled or evenly weighted boxers [2]. Lighter boxing gloves (6 oz.) were common [5]. Thus, severe and protracting beatings, and numerous such beatings with no mandatory exclusion times, were typical over a boxer's professional career, notwithstanding any additive effects of one's career as an amateur, a sparring partner, or a booth fighter. It is perhaps not surprising, in light of the dynamics of boxing participation and boxing oversight, that a neurological condition known colloquially as 'punch drunk' emerged in the early 20th century and has since tapered.

The focus on neurological injury from boxing exposure tends to place emphasis on poor outcome, although it should be pointed out for balance that transcendent human benefits are still commented upon in the modern era, such as self-esteem, respect within

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the community, physical fitness, and avoidance of a criminal lifestyle, alcohol, and illicit drugs [8]. Boxers themselves have rejected, sometimes bitterly, the notion that boxing first and foremost encourages violence [8]. Nevertheless, heated debate among boxing advocates versus those favoring a ban on the sport have been taking place for many years and are ongoing [9]. For the purposes of this review, we do not presume to provide insight into the question of whether ordered society has the moral imperative to ban individuals from a sport like boxing, or whether the benefits of boxing outweigh the risks. We are more concerned with the nature of neurological and biological processes associated with the "model experiment" [10] of boxing, and the analysis of the largely historical entity of dementia pugilistica with the benefit of hindsight.

PUNCH DRUNK

Punch drunk appeared in the medical literature for the first time in 1928, when Martland commented on a 'pecular condition' among prize fighters [11]. The condition, plainly visible to boxing fans, promoters, and indeed the fighters themselves in advance of Martland's paper, had not been codified previously as a neurological disease state. It was said to affect lower skilled fighters of the "slugger" type who tended to take considerable punishment while sizing up their opponent for the elusive knockout blow, or who were otherwise limited in defensive skills but possessed the constitution to withstand substantial physical punishment. Parker would later comment that "Quick, agile, clever boxers who guard themselves well and take little punishment seem to escape. Chiefly affected are the less expert but courageous men who take considerable injury in the hope of wearing out their opponents" [12]. Critchley drew similar conclusions regarding the style of boxing, and also suggested that the condition was much less common in African American boxers [4], although he did not provide any statistical basis for this latter conclusion.

Twenty-three fighters with the putative punch drunk condition were reported to Martland from a single, well known, boxing promoter. 15 of these were described simply as punch drunk, four were said to be committed to an asylum and not otherwise characterized, three dragged an extremity (two of these were also dysarthric, one was in 'bad shape'), and one had a parkinsonian syndrome. Martland examined five of these subjects, although he provided no description of any of the five. Instead, he described an additional subject as *case 2* in his report – a 38-year-old man who became symptomatic at age 23 with a left hand tremor and unsteadiness in his legs, after 7 years of fighting. Martland frankly acknowledged the differential diagnosis of early-onset Parkinson disease (paralysis agitans) in this subject, although he eliminated epidemic encephalitis from the time course of clinical symptoms. (*case 1* in Martland's paper consisted of the autopsy findings in a 76-year-old man who succumbed after falling down a flight of stairs; this subject had no boxing history and was presented only to illustrate Martland's original hypothesis that punctate hemorrhages were responsible for the clinical signs of the punch drunk syndrome).

In keeping with present day discussions of chronic traumatic encephalopathy, the relationship between clinical findings, which themselves were wide-ranging, and neuropathology, was speculative. Martland described flopping of the foot or leg, unsteadiness in gate, uncertainty in equilibrium (even while actively boxing), appearance of intoxication, peculiar tilting of the head, dragging of one or both legs, a staggering or propulsive gait, parkinsonian faces, tremors, vertigo, deafness, and in some cases marked mental deterioration. The pathological substrate offered by Martland appeared more in line with acute parenchymal brain injury. His depiction of the stairway-related fall is that of diffuse axonal injury, with acute gliding contusions, hemorrhages in the corpus callosum, and small hemorrhages in the basal ganglia. Martland offered the theory that "Punch drunk bares the same relation to multiple concussion hemorrhages as do many of the post-concussion neuroses and psychoses that follow blows or falls on the head." There was no mention of neurofibrillary tangles (NFT) or Alzheimer-related changes, or otherwise a lesion that would correspond to accumulations of phosphorylated tau (p-tau) or chronic traumatic encephalopathy as it has been recently described [13]. The potential for a manufactured condition was not lost on Martland in his index report, noting:

> "*While the establishment of these facts is of enormous importance to the courts and to labor compensation boards in placing many cases of cranial injuries on a firm pathologic basis, it also will have its disadvantages. A very great field is opened for the so-called expert testimony, in which malingerers and those suffering from various forms of psychoses and neuroses*

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may claim undue compensation. The correct diagnosis during life will always be extremely difficult, as the condition can only be proved by autopsy" *[11].*

The latter concept of autopsy-proven disease has its own element of subjectivity. Martland's "proof" at the time consisted of concussion-related hemorrhages, which were never seriously considered as a lesion responsible for long term sequelae after the 1940 s.

Millspaugh introduced the term "dementia pugilistica" in 1937 [14] in a somewhat meandering discussion, commenting on Martland's findings and the possibility for microscopic ring hemorrhages. Observations by Parker, including his assertion of a multitude of individual lesions [12], and by Jokl and Gutterman [15] favoring a cerebral trauma-induced dementia, were noted. Some attention to the problems of weight differences among fighters, and rules requiring the attendance of medical officers, indicated a level of concern for serious or permanent injury at the time. Dyslalia of heterogeneous origin (laryngeal trauma, facial and dentition trauma, psychic effects), othematoma, and traumatic metacarpophalangeal arthropathies, were mentioned briefly. Psychiatric disturbances, including quasi-delusional ideation with "magnification of former prowess" was commented upon, as were gait abnormalities and Parkinsonian symptoms. Overall, dementia pugilistica was meant to convey a physical-psychic syndrome that accumulated over a lengthy boxing career, but was less derisive and less resented among boxers than 'punch drunk'.

Despite the newly introduced term encompassing 'dementia', Millspaugh tended to convey a static condition, in that steady neurological deterioration was less striking than the odd psychic characteristics, neurological signs, and speech difficulties. The morbid anatomy and associated mechanism were addressed with some specificity. Particular vulnerability of the "corpora striata, corona radiata, and the basal ganglions" was suggested. Mechanistic theory included "Traumatic punctate cerebral hemorrhages, hydrostatic disequilibrium of the spinal fluid, cerebral edema, concussion injury to the cortical cells, cerebral vasomotor imbalance, reparative gliosis or degenerative lesions of cerebral parenchyma, variation in the weight and therefore inertia between gray and white matter and tension transmission by nerves, blood vessels and musculature," which tend to suggest cumulative damage from acute injury mechanisms, as opposed to a progressive Alzheimer-like neurodegeneration.

THE NATURE OF DISEASE PROGRESSION

Modern experimental constructs implicating protein templating as an underlying mechanism [16] require *a priori* the conclusion that progressive neurodegeneration is caused by repetitive neurotrauma. A close examination of the earliest case material in dementia pugilistica, i.e., those with the most extensive exposure to repetitive neurotrauma, nevertheless indicate heterogeneity with regard to progressive disease, and that a trauma-induced, progressive proteinopathy model is overly simplistic. Martland stated unambiguously that "Many cases remain mild in nature and do not progress beyond that point" [11]. Parker similarly noted that "a pugilist may be only mildly affected, and may continue to fight to the end of his career, or he may be so disabled that he ultimately has to quit boxing and yet gets no worse in after life" [12]. On the other hand, a "progressive neurological syndrome may appear, putting an end to all fighting, and leading finally to mental or physical helplessness" was also suggested in some cases. Critchley also raised the issue of a "groggy state," [4] or a form of preclinical dementia pugilistica, in which the boxer's skills may have subtly declined, rendering him increasingly vulnerable to concussive blows to the head, from which recovery is prolonged and more likely incomplete. In this respect, an exponential, rather than linear, increase in traumatic brain damage during this era late in a boxing career may be hypothesized, emphasizing the importance of retirement once a critical point is reached (i.e., once the boxer "softens up") [17, 18], particularly if cumulative structural injury underlies clinical progression.

Three case studies by Parker indicated injuries traceable to specific fights, and a static or improved course over time [12]. In Parker's case 1, the boxer "attributed all his difficulties to his last and futile appearance in the ring. The unbroken chain of cause and effect is very evident in this case, and it is to be noted that in nearly two years the condition had not become worse nor was there much improvement beyond a certain point." He went on to say that "No specific nervous syndrome appeared, such as Parkinson's disease, but rather a medley of scattered and incomplete lesions of the brain." Parker's case 2 showed a number of neurological signs and

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deficient memory but that did not deteriorate over the 6 years prior to the report and, if anything, improved. Parker's case 3 showed progressive symptoms "up to his final, ignominious failure in the ring, and then, for eleven years thereafter, his condition remained much the same as it was when he ceased fighting," despite an earlier clinical diagnosis of lateral sclerosis.

Critchley on the other hand suggested the term chronic traumatic encephalopathy in 1949 [19] and then "chronic *progressive* traumatic encephalopathy of boxers" in 1957 [4], suggesting that lack of insight into the neurological deficits was evidence of an insidious onset, often culminating in a fatuous or euphoric dementia with emotional lability. Speech and thought became progressively slower in his experience, with mood swings, truculence, and uninhibited violent behavior. To support his views, Critchley detailed 10 cases of punchdrunkenness, although the individual case histories fit into the neurological and psychiatric spectrum of other described cases. He nevertheless favored a progressive process once initiated:

> "*Of great interest, pathological as well as practical, is the fact that this traumatic encephalopathy is a progressive condition. Once established it not only does not permit of reversibility, but it ordinarily advances steadily. This is the case even though the boxer has retired from the ring and repeated cranial traumata are at an end*" *[4].*

Mawdsley and Ferguson described a series of 10 subjects with air encephalography, documenting abnormalities in the septum pellucidum and dilated lateral ventricles [20]. The case histories likewise showed a mixture of neurological signs with gait and speech abnormalities, pyramidal and extra-pyramidal signs, memory loss, and brain atrophy. Slow progression of dysarthria, disorder of movement, and memory deficits, were noted, and it was stated that 8 out of 10 subjects had a significant disability. Many were "unemployed for many years," while the authors also commented that "Cerebrovascular lesions in ex-boxers of middle age could possibly aggravate pre-existing traumatic damage."

Without insight into prevalence of the presumed head-trauma induced condition, discussions of disease progression prior to the late 1960 s may have been premature. Indeed, misinterpretation of single cases, or failure to exclude coincidental neurological illness, could mean the difference between concluding progressive disease versus a stationary condition. To address this issue, the Royal College of Physicians of London set up a committee 'to report on the medical aspects of boxing', and appointed Dr. A. H. Roberts to carry out a large scale cross-sectional study, and the only study of its kind to date on long term effects of neurotrauma in humans [2]. This was accomplished by examining a random sample 250 boxers out of 16,781 total boxers registered by the British Boxing Board of Control between 1929 and 1955. Of those 250, 16 died, 9 emigrated, and one refused to cooperate. 224 were thus studied by a series of four neuropsychological tests, patient interview, clinical examination, and electroencephalography. The results of the studies were published in a book, 'Brain Damage in Boxers' in 1969. The remarkable yield of cooperation was only possible through the diligent and time-consuming house-to-house inquiries by social workers, many times with the help of boxers themselves, in order to facilitate dialogue. When one considers the diligence of the effort as well as the era of numerous boxers with extensive neurotrauma exposure, it is not surprising that this now 50-year-old study has never been replicated.

Roberts found that of the 224 boxers studied, 37 (17%) "had evidence of lesions of their central nervous systems, similar to those reported and attributed to boxing by others, and not typical of the clinical entities encountered in routine neurological practice." Of these, 13 (6%) were more severely disabled and appeared to present two clinical syndromes: "one predominantly extra-pyramidal and apparently more clearly progressive", and the other predominantly cerebellar. (Extra-pyramidal signs attributable to boxing have since been said to be exceedingly rare [5, 21].) The more severe nature of the thirteen cases was evident in their presentation to neurological clinics. According to Roberts, these 6% were of the kind labeled 'punch drunk' in boxing circles. The same could not be said of the other 24 cases, as these individuals "were on the whole only marginally or in no way disabled." Interestingly, all of the 13 cases with more severe clinical signs recognized as punch drunk boxed prior to World War II. Roberts commented on the issue of disease progression at some length despite the cross sectional design, as the idea of a neurodegenerative disease provoked by repetitive head trauma was already being raised. He noted that the "first evidence of the condition may follow one or a series of particularly hard fights, and that it may then regress if the boxer stops fighting." This same phenomenon appears in a number of other described

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cases [3], rather than after a period of latency. Roberts goes on to state:

> "*When there is an adequate independent account, or the individual has sufficient insight, most of these boxers appear to have developed the symptoms of the condition during their last years boxing or after a series of particularly hard fights. It seems that some degree of progression may occur which cannot be accounted for simply on the basis of normal ageing, and occurs more commonly in the extra-pyramidal lesions, but apart from a few isolated exceptions reported previously and in one case in the present series (case 2), this progression is not in general characteristic of that seen in the systems degenerations or in the commoner presenile dementias*" *[2].*

The appearance of traumatic encephalopathy from midbrain injury after a single bout supports Roberts' assertions [17, 22, 23]. Roberts also identified asymmetrical motor signs, including tremor and hyperreflexia, with an overall tendency to favor the boxer's left side [2]. Given the predominance of right-handed boxers, it has been asserted that such asymmetry is due to structural injury *per se* to the upper brainstem, from mechanical trauma *per se*. Case 2 in the selected case summaries provided by Roberts, on the other hand, suggests a progressive neurological disease. Roberts commented that the condition dated from the subject's active boxing career (as opposed to following a period of "latency"), and expressed some difficulty in excluding a co-existing neurodegenerative disease, especially given the rarity of this type of clinical progression in the series. In the final analysis, Roberts' described uncertainty on issue of progression versus the aging process superimposed on static deficits, a possibility endorsed by Jordan [24]:

> "*How far it can be assumed that a progressive degenerative process involving the neuron is mirrored in the widespread neurofibrillary changes found in some ex-boxers' brains remains unanswered. It seems certain that diffuse cellular and axonal depletion has resulted from boxing to account for the clinical syndrome related to occupational exposure found in the present study. It must therefore be assumed that cerebral trauma of a minor degree may result in permanent structural damage that is cumulative. There was good evidence in some cases that the condition had progressed for some time after retirement from boxing, particularly in those with evidence of extra-pyramidal lesions, but the information available from the study proved inadequate to settle finally the question of progression unrelated to the changes associated with ageing*" *[2].*

In short, cases with progressive disease were the exception in Roberts' series, and still lacked Alzheimer-like progression in the rare patients with extrapyramidal signs. At one point, Roberts commented that "There is a good deal of evidence in the present study to suggest that in most cases the condition remains stationary when the individual has stopped boxing, and indeed there are excellent independent accounts for a few of undoubted improvement after their retirement." The contrast between Roberts' case synthesis and classical neurodegenerative diseases is noteworthy, as the latter never remain stationary let alone improve. Only two subjects in Roberts' series were indisputably demented judging by their inability to live unaided in the community [2]. The protein cascades currently under discussion seem, therefore, to over-run the empirical observation that a progressive neurodegenerative disease, in a manner of Alzheimer's disease (AD), is exceptional at best, even in these early 20th century boxers whose exposure levels were extreme. It finally should be noted that none of the Roberts' cases were unaccompanied by pathological confirmation. The difficulty in obtaining such cases at autopsy, then and now, speaks to the lack of a cohesive neurodegenerative disease entity, and keeps viable the possibility or probability that some cases in this largest series of boxers consisted of idiopathic progressive synucleinopathies or other sporadic neurodegenerative diseases, cerebrovascular disease, and/or an undiagnosed infectious or inflammatory processes.

NEUROPATHOLOGY DESCRIBED IN BOXERS

Historically, neurodegenerative diseases were uncovered by the appearance of an unusual and inexorably progressive neurological decline, followed by neuropathological examination at autopsy. The index case of AD, for example, occurred in a middle aged woman with relentless neurologic deterioration and autopsy demonstration of hallmark lesions [25]. In contrast, Martland's original description [11], Millspaugh's article introducing the term "dementia

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pugilistica," [14] Roberts' case series [2], and many other early reports describing encephalopathy in boxers [3], were unaccompanied by autopsy neuropathology.

It is further emblematic of the complexity of the topic that the first report of boxers' encephalopathy at autopsy was not a case of dementia pugilistica or punch drunk syndrome, at all. In 1954, Brandenburg and Hallervorden described the autopsy findings in a 51-year-old man who had boxed from age 18 to 29, ultimately holding the title of amateur German middle-weight champion for 6 years [26]. At age 39, personality changes appeared, which deteriorated into insomnia, memory loss, dysphasia, parkinsonism, and frank dementia. The most remarkable finding at autopsy was extensive AD pathology, including an abundance of senile plaques of various morphological types, severe cerebral amyloid angiopathy, and numerous NFT. Noteworthy as well were numerous so-called "condensation plaques" which correspond to today's "cotton wool" plaque. The findings thus indicate rather unambiguously the presence of early-onset AD. The abundance of cotton wool plaques also suggests familial disease associated with presenilin-1 mutation [27]. Unfortunately, no family history was available or otherwise provided in this case.

The case reported by Grahmann and Ule in 1957 [28] is often considered alongside the case report by Brandenburg and Hallervorden as among the first cases suggesting a "link" between neurofibrillary change and boxing. In this case, the findings in a 46-year-old man who boxed between the ages of 15 and 25 were described. He expired after suffering hemorrhagic venous infarction from a cerebral venous sinus thrombosis, and after a documented, progressive neurological decline starting at age 36. The case history is similar to the earlier report by Brandenburg and Hallervorden in its description of progressive neurological deterioration, which clearly suggests neurodegenerative disease. It differs, however, in the absence of senile plaques and cerebral amyloid angiopathy, and with neurofibrillary changes noted largely in the brainstem and medial temporal lobe. Cerebral atrophy was present, along with cavum septum pellucidum, as was loss of Purkinje cells and granular neurons of the cerebellum. Since limited pathological illustrations were made available, it is unclear how carefully sporadic systems degenerations were excluded. From the standpoint of Roberts' epidemiological survey, this case would have represented an outlier, both in terms of the long symptom-free interval between boxing and onset of symptoms, and the inexorable disease progression. The possibility of coincidental neurodegenerative disease unrelated to boxing remains a consideration. To his credit, Roberts recognized the possibility of coincidental neurological disease in the above two cases, noting:

> "*it would appear that the kind of progression indistinguishable from Alzheimer's disease in two of the fourteen neuropathological studies of boxers reported so far must be extremely uncommon. So much so, that the fortuitous occurrence in Boxers of Alzheimer's disease in these two cases cannot be entirely ignored*" *[2].*

Neubuerger et al. reported two cases in 1959 [29]. His first case presented at age 46 initially for a coronary complaint, and four years later with a complaint of headaches. Neurological examination revealed a head and neck tremor, increased tone in the right arm, defective upward gaze, a mildly abnormal EEG, and abnormalities on neurocognitive testing. Neuropathology was limited to a brain biopsy, performed for unstated reasons, which showed gliosis. His second patient presented at age 53 with neurological decline including dementia and ataxia, eventually expiring secondary to pulmonary fibrosis and cor pulmonale. He had boxed between the ages of 18 and 24. Autopsy examination showed significant frontal cortical atrophy but no neurofibrillary degeneration or senile plaques. This case thus lacks all pathological features attributed to boxing, while frontotemporal dementia would be a consideration if not likely.

Courville (1962) published what he considered a verified case of dementia pugilistica in a 49-year-old man with alcoholism and diabetes mellitus, and a four-year boxing history [30]. Autopsy, however, showed nonspecific thickening of the leptomeninges and nonspecific changes on microscopy. Spillane (1962) reported autopsy findings in a 45-year-old man with approximately 300 professional fights, among a total of five cases with heterogeneous clinical presentations [31]. Neurological signs included slurred speech beginning about the time he retired from boxing at age 32. Later neurological signs included dragging of the left lower extremity and left arm posturing, ataxia, and continued dysarthria. Autopsy was performed after he suffered an acute myocardial infarct. Areas of "softening" in multiple brain regions were noted, suggestive of cerebrovascular disease. Spillane made no mention of neurofibrillary degeneration and ultimately concluded that "Taken together,

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these cases illustrate the difficulties of aetiological diagnosis and emphasize the extent of our ignorance of this subject."

Case 10 by Mawdsley and Ferguson (1963) depicted the autopsy findings in a 51-year-old ex-boxer who retired at age 35 with 300 professional fights [20]. He was said to be "punchy" with slurred speech, an expressionless face, memory loss, apathy, and brisk reflexes, expiring secondary to squamous cell carcinoma of the floor of mouth. Autopsy showed gross atrophy, cavum septum pellucidum, and septal fenestration. No microscopic examination was provided. Payne (1968) reported neuropathological findings in six professional boxers with extensive boxing histories in addition to significant vascular, psychiatric, and alcohol abuse co-morbidities [32]. In particular, he noted septal abnormalities, miniscars in the cerebral cortex, and foci of white matter degeneration. Senile plaques and early neurofibrillary changes were noted in two cases, which Payne concluded were "nonspecific degenerative phenomena." Burger and Minarovjech (1966) reported nonspecific neuropathology in a 44-year-old former boxer who expired from intracerebral hemorrhage [33]. Betti and Ottino (1969) reported brain biopsy findings in a 38-year-old former boxer with an extensive boxing exposure as well as head trauma from a stairway-related fall [34]. Asymmetrical tremor, abnormal gait, and dysarthria were noted clinically. Brain biopsy, performed for unclear reasons, showed nonspecific loss of neurons.

The landmark study of chronic neuropathology in former boxers was published by Corsellis et al. in 1973 [35] and established the neuropathology of dementia pugilistica until about 1990. This was a retrospective analysis of 15 cases examined in the Department of Neuropathology at the Runwell Hospital Institute of Psychiatry, near London, UK. Since the limiting factor for case acquisition in the Corsellis series was examination at the Institute of Psychiatry, selection bias precludes any discussion of prevalence. The spectrum of pathology is nonetheless noteworthy and perhaps not fully appreciated in modern discussions of long-term effects of mild neurotrauma. Subjects in the Corsellis series boxed between 1900 and 1940, with ages at death ranging from 57 to 91. As noted above, boxers in this era had extensive neurotrauma exposure, vastly exceeding levels of exposure encountered today. Many of the subjects participated in hundreds of promoted fights. Some fought in booths. 7 of the 15 endorsed a history of heavy alcohol use, whereas heightened sensitivity to the effects of alcohol was reported in 6 cases. In only four cases was alcohol use specifically denied. The duration of neurological signs potentially attributed to boxing was often unclear, but ranged from 8 years to 41 years in cases providing such data. The precise onset of symptoms relative to boxing was often unclear.

Neuropathological examination emphasized neurofibrillary degeneration on von Braunmuhl silver impregnation, especially in the substantia nigra and medial temporal lobe. These were often present in large numbers in the face of limited plaque pathology, thus separating the neurofibrillary degeneration of dementia pugilistica from that of typical AD. Substantia nigra neuron loss, cerebellar scarring (with extensive measurements and neuronal counts) particularly involving the cerebellar tonsils, and septal abnormalities with an enlarged cavum and septal fenestrations were also considered among the cardinal manifestations of boxing-relating neuropathology. There was significant heterogeneity in the sample, however. Although not specifically highlighted as boxing-specific, atrophic or flattened fornices were present in several cases. Shrunken and atrophic mammillary bodies were also noted in about half of the cases. Hippocampal atrophy was present in some cases, and hippocampal sclerosis *per se* was depicted in case 1. Cerebrovascular disease with infarcts was present in several cases. Given the important role of cerebrovascular disease in producing focal neurological signs as well as cognitive deficits [36], and the relative lack of effective anti-hypertensive therapy in the early 20th century, the role of co-morbid cerebrovascular disease among classic cases of boxers' encephalopathy may be underappreciated. Structural traumatic brain injury was also evident, with remote contusions and hemosiderin staining of the olfactory bulbs in some cases. A cavernous malformation was present in one case. Tabes dorsalis was present in still another case. Three cases showed no specific pathology, and one was reported to lack a boxing history on further analysis [37]. All told, the Corsellis series is a complex and heterogeneous sample with numerous co-morbidities and structural injuries involving a multitude of brain regions. Despite the heterogeneity of neuropathology in the Corsellis et al. sample, however, the major impact seems to have been the renewal of focus on neurodegenerative pathology and in particular neurofibrillary degeneration.

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As an indicator of the rarity of dementia pugilistica, studies in the 1980 s and early 1990 s often looked to the Corsellis series for application of lately available immunohistochemistry, and offered examination of only a small number of new cases. For example, Roberts [38] in 1988 examined 8 of 15 cases from the Corsellis series with anti-sera to p-tau with no additional cases. Roberts et al. [39] in 1990 examined 14 of 15 Corsellis et al. cases with anti-sera to amyloid-β (Aβ) (in addition to 5 amateur boxer cases that lacked features of classical dementia pugilistica). The 1988 study confirmed the linkage of p-tau to neurofibrillary degeneration in dementia pugilistica, with cases resembling AD, while the 1990 study highlighted variably extensive Aβ-positive diffuse plaques not previously noted with silver impregnation techniques, raising the issue of diffuse plaque pathology as a component of repetitive trauma-induced changes. Tokuda et al. [40] re-examined 7 cases from the Corsellis et al. series, and added one new case, noting extensive *morphologically diverse* plaque pathology with significant overlap with AD. This suggested to Tokuda et al. a role of trauma in AD pathogenesis, but on balance also suggests comorbid aging and/or AD pathology in the original Corsellis case series. Allsop et al. [41] re-examined 6 cases from the original Corsellis series and 2 new cases, also noting more abundant Aβ pathology than previously appreciated from silver impregnation, in addition to Aβ immunoreactive NFT with advanced pathology.

Hof et al. [42] extended the specificity of neurofibrillary degeneration in dementia pugilistica by examining three boxers with extensive boxing exposure. In this study, findings consistent with Corsellis et al. were noted, including abundant neurofibrillary pathology, cerebellar cortical pathology, substantia nigra pathology, and septal pathology. However, the authors noted larger numbers of NFT in superficial cortical laminae, and thus provided an additional point of distinction between trauma-related NFT and aging/Alzheimer-related NFT. Geddes et al. [43] examined the brain of a 23-year-old boxer who suffered an acute boxing-related death. P-tau immunohistochemistry, even in this young subject, showed abundant cortical p-tau, predominantly in frontal and temporal lobes. The immunoreactivity was patchy and showed a predilection for perivascular areas. No clinical signs were ascribed to the p-tau pathology although it was noted that the decedent was "somewhat forgetful" during life.

EVOLUTION OF DEMENTIA PUGILISTICA OVER TIME

The evolution of punch drunk/dementia pugilistica since 1928 is noteworthy, particularly alongside AD in which basic understanding of the clinical substrate, cognitive trajectory, and the pathological hallmarks has remained relatively stable since 1906. As noted above, dementia pugilistica was identified initially because of purely neurological signs, such as dysarthria, pyramidal and extra-pyramidal signs, and ataxia. Concussion-related hemorrhages were the initially-offered pathologic substrate [11]. Alzheimer-like pathology was then erroneously linked to dementia pugilistica as noted above [26]. NFT as a component of dementia pugilistica was reaffirmed by Corsellis et al. [35], who then added septal abnormalities, cerebellar sclerosis, and damage to the substantia nigra to the list of changes. Patchy tauopathy with predilection for superficial cortical laminae and perivascular areas of the cortex and Aβ-positive diffuse plaques appeared in the 1990 s [42, 43], while the specificity of nigral and cerebellar pathology seems to have been abandoned. Additional patterns of tauopathy have been ascribed to chronic effects of trauma in more recent studies [44], and further refined to a single required criterion with several supportive criteria according to a consensus recommendation forged in 2015 [13]. It is further stated that as many as 20% of cases with presumably sport-related p-tau deposits may be missed by routine dementia sampling and immunostaining protocols [13].

It is of note that among the few dementia pugilistica cases reported in the *recent* literature, either no clinical signs were apparent, or clinical signs were attributable to other major diseases [43, 45–48]. Thus, as classical dementia pugilistica has receded over the years, dementia pugilistica diagnosed purely by p-tau immunohistochemistry, either in the absence of neurological signs or in the context of other neurological diseases, has taken its place. This leaves open the question of clinical significance of patchy p-tau immunoreactivity, identified by highly sensitive means.

Figure 1 depicts a case in point. This section was obtained from the NIH Neurobiobank at the University of Maryland. In particular, it was obtained as a *control* case for a brain bank dedicated to neurodevelopmental disorders. The decedent was a 51-year-old man who died of suddenly of pneumonia complicated

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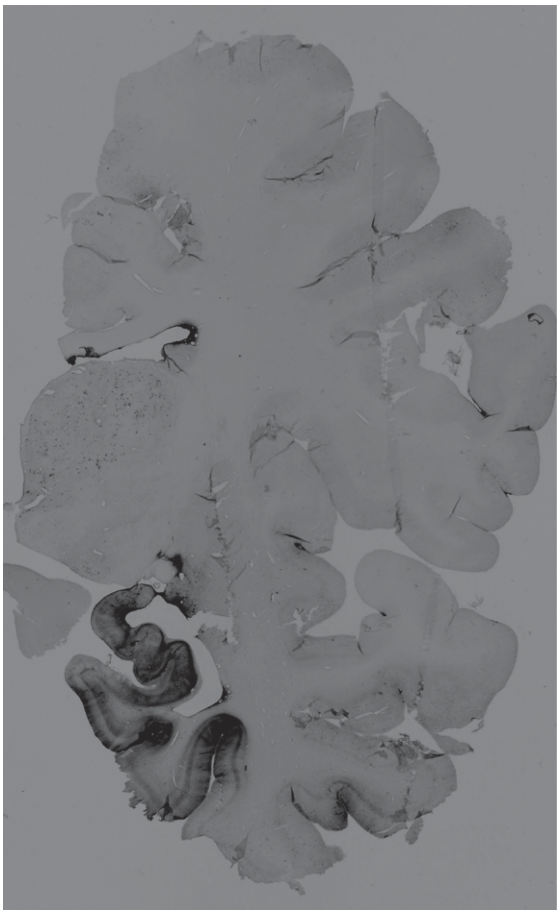

Fig. 1. Whole mount immunohistochemical stain for p-tau (AT8) in a 51-year-old retired professional boxer.

by cardiovascular disease, prior to which he had no health complaints whatsoever. He was neurologically and psychologically healthy. He was on no medications. He did not drink alcohol or use illicit drugs. It was gleaned from the death investigation, however, that the decedent had competed as a professional boxer during his youth. He fought in 14 professional contests and lost 7 of them, two by knock out. At autopsy, his brain was grossly and microscopically normal. Because of his boxing history, and *only* because of his boxing history, extensive immunohistochemical stains for p-tau (AT8) were performed, and indeed there were accumulations of p-tau in neurons and glia, at times in patchy foci at the depths of cortical sulci, meeting modern criteria for chronic traumatic encephalopathy. Given his limited boxing exposure, it seems likely that a similar subclinical stationary tauopathy may be encountered in other asymptomatic athletes with similar or less exposure, and for that matter in non-athletes. This would not be particularly surprising given the ubiquity of more stereotyped p-tau accumulation with age and in the absence of clinical disease [49]. One could speculate about the decedent's neurological or cognitive trajectory had he lived, but the circularity of such speculation, the lack of insight into the kinetics of such changes over time, and the poor correlation between uncomplicated p-tau reactivity (i.e., p-tau reactivity without neuronal loss) and neurological signs, are problematic.

THE ROLE OF TAU PATHOLOGY IN DEMENTIA PUGILISTICA

As stated by Roberts:

> "*It has never been doubted, since it is implicit in the contest, that personal injury occurs in boxing, or that, rarely, accidents happen, as they do in other sports, which result in damage to cerebral functions and even death. Until recently it had not been suggested that the evidently transient incapacity usually sustained might result in permanent, slight but cumulative damage to delicate neural structures.*"

The heavy neurotrauma exposure of the early 20th century boxing thus brought into medical consciousness the intuitively obvious conclusion that brain damage could occur in some participants, and that structural brain injury might be cumulative with time and exposure. A progressive Alzheimer-like neurodegenerative cascade from boxing-induced neurofibrillary degeneration, however, was hypothetical then as it is today. The modern concept of p-tau as a driver of disease likewise sets aside cumulative structural brain injury and instead opens up hypothesis-confirming lines of inquiry, perhaps prematurely, such as 1) tau biomarkers, such as in cerebrospinal fluid or on PET imaging studies [50]; 2) the role of low-n assembly intermediates of p-tau in experimental trauma versus control [51]; 3) the role of any of a number inflammatory mediators in tau phosphorylation [52]; 4) similarities and differences in tau isoform profiles in repetitive trauma versus conventional tauopathy [46]; and 5) templating behavior and prion-like conformers and strains in experimental constructs, among others [53]. The question of whether tau pathobiology is a primary or inherently pathogenic process in the first place seems reasonable to ask, given that p-tau is manifestly downstream in a multitude of conditions [54–57], and that the over-

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whelming majority of heavily exposed boxers are asymptomatic, with most of the rest having stationary neurological deficits.

The amyloid cascade hypothesis may provide some guidance as one attempts to understand the relationship between proteinopathy and clinical disease [58–60]. In terms of a hypothesis for disease etiology or an otherwise rate-limiting factor for disease pathogenesis [61], the role of Aβ in AD is considerably more robust scientifically than p-tau in dementia pugilistica. Pathogenic mutations in amyloid-β protein precursor (AβPP) within and near the Aβ coding region lead to familial autosomal dominant AD with near 100% penetrance. In Down syndrome patients, who possess an extra copy and overproduce AβPP, AD pathology as well as cognitive deterioration from baseline, appear consistently and early compared to sporadic AD and aging. Familial early-onset AD is associated with mutations not only in AβPP, but also in presenilin 1 and 2—components of the γ-secretase complex, required for processing of the AβPP into Aβ. Thus in AD, not only is there strict adherence to central dogma of molecular biology and genotype-phenotype relationships, there is also genetic adherence to genotype-enzyme-substrate phenotype relationships. In addition, animal models in AD are well characterized and consistently recapitulate hallmarks of AD pathology [62]. Therapeutic constructs that reduce Aβ have been shown to reduce behavioral and neurocognitive deficits in AD models [63]. These data have further been extended to clinical trials in humans, in which immunotherapy has been shown to reduce Aβ burden [64]. Yet in the case of AD, despite its robust molecular-genetic underpinnings and detailed extent to which it has been investigated in humans, all major therapeutic trials targeting Aβ have either shown no benefit or have performed worse than placebo [60]. The point here is that lesion- or protein-driven neurodegenerative disease constructs insufficiently address the complexity of neurodegenerative disease in humans, even in the case of AD, a much more homogeneous condition compared to dementia pugilistica, studied in exhaustive detail over three decades with all available technological advancements. By that analogy, the concept of p-tau as a driver of disease or target for therapy in dementia pugilistica seems premature.

CONCLUSIONS

Dementia pugilistica has evolved remarkably over time. Concussion-related hemorrhages, AD pathology, diverse neuropathology encompassing structural brain lesions, neurofibrillary pathology and diffuse Aβ plaques, and, most recently, geographic tauopathy have all been proposed as pathological substrates. The classic clinical presentation includes slurring dysarthia, pyramidal and extrapyramidal signs, ataxia, memory deficits, and personality changes, although the diagnosis is occasionally offered in more recent literature in asymptomatic young athletes and in retired athletes with other major diseases. Assuming that dementia pugilistica is exposure-related, the prevalence of dementia pugilistica or punch drunk syndrome appears to have peaked at 6%, and it makes sense in light of the markedly decreased exposure since World War II that classical dementia pugilistica has reduced to a rarity, notwithstanding subclinical and stationary p-tau deposits in asymptomatic athletes. On the other hand, since cumulative structural brain injury may have been the driver of disease for dementia pugilistica when it was most prevalent, and since single bouts have been shown, on rare occasions, to produce permanent deficits, boxing can never be said to be devoid of risk for permanent brain injury or even death.

Of particular concern is not simply the existence of structural brain injury, including cumulative structural brain injury, which obviously has occurred in some boxers, but the specific concept of repetitive trauma-induced neurodegenerative disease in the manner of AD, propagated by a proteinopathy. Clinicopathological data in boxers with extreme neurotrauma exposure leave considerable doubt that such a process exists today with any frequency, while those anecdotal instances with evidence of progression tend to be confounded either by major co-morbidities, failure to exclude coincidental neurodegeneration disease, or outdated pathomorphological analyses that are not amenable to comparison with current concepts. Also striking is the paucity of new cases of dementia pugilistica in the literature, and in those few cases that have appeared, the lack of the neurological syndrome (dysarthria, ataxia, asymmetric hyperreflexia, etc.) that allowed identification of punch drunk syndrome or dementia pugilistica in the first place. At present, if one is rigorous with the human data, it remains unclear how, and indeed whether, traumatic brain injury from boxing crosses the threshold from structural damage to a progressive neurodegenerative cascade. On the other hand, the finding of p-tau in atypical patterns and distributions, even in asymptomatic athletes, indicates that more research is needed.

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DISCLOSURE STATEMENT

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Assessing the Limitations and Biases in the Current Understanding of Chronic Traumatic Encephalopathy

Nicole Schwab[a,b] and Lili-Naz Hazrati[a,b,*]
[a]*Department of Laboratory Medicine and Pathobiology, University of Toronto, ON, Canada*
[b]*The Hospital for Sick Children, Toronto, ON, Canada*

Abstract. Chronic traumatic encephalopathy (CTE) is considered to be a progressive neurodegenerative disease caused by mild traumatic brain injury (mTBI). Recently there has been a significant amount of media attention surrounding the commonness of CTE in professional athletes, particularly American football, based on several postmortem case series. However, despite the persuasive claims made by the media about CTE, research on the disease and the effects of mTBI in general remain in its infancy. Commonly cited case series studying CTE are limited by methodological biases, pathological inconsistencies, insufficient clinical data, and a reliance on inherently biased postmortem data. These case series do not allow for the collection of any epidemiological data and are not representative of the general population. The exaggerated assumptions and assertions taken from these studies run the risk of creating a self-fulfilling prophecy for individuals who believe they are at risk and have the potential to negatively influence sports-related policymaking. This review outlines the status and limitations of recent CTE case series and calls for future prospective, longitudinal studies to further characterize the pathological and clinical hallmarks of CTE.

Keywords: Athletic injury, chronic traumatic encephalopathy, concussion, dementia, neurodegeneration, tauopathies, traumatic brain injury

INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of death and hospitalization in the United States, with at least 2.5 million TBIs occurring in 2010 alone [1]. Mild TBIs (mTBIs), such as concussions, are most common and are particularly prevalent in contact sports including football, hockey, rugby, and boxing in which players tend to suffer several mTBIs throughout their career. Recurrent brain trauma can lead to broad, long-term symptoms that affect cognition, behavior, mood, and motor skills, and is considered the principal risk factor for developing chronic traumatic encephalopathy (CTE), a progressive neurodegenerative disease [2].

CTE is currently accepted as a distinct neurodegenerative disease characterized by accumulation of hyperphosphorylated tau in an irregular and distinct pattern, TAR DNA-binding protein (TDP-43) positive neurites, ventricular enlargement, septal abnormalities, and atrophy among other supporting features [3]. Clinically, it most commonly presents with irritability, aggression, depression, short-term memory loss, and suicidality among other diverse symptoms [4]. The link between head trauma and long-term symptoms is not novel—this association was first described in the 1920s, when Harrison

*Correspondence to: Lili-Naz Hazrati. E-mail: lili-naz.hazrati@sickkids.ca.

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Martland began to study 'punch drunk syndrome' in boxers [5]. This disease became known as "dementia pugilistica" [6] and the term CTE was later coined in 1940 in a case study of one professional boxer who presented with depression, violence, and poor memory [7]. Throughout the next 50 years a large number of case series [8–10], particularly on retired boxers, were produced in an attempt to causatively link head trauma to CTE and pathologically characterize the disease. In 2005, Omalu et al. [11] published a case study of CTE in a retired football player, renewing scientific interest in the long-term effects of brain trauma and raising questions about the disease's prevalence in professional football. Since 2005, several case series on the presence, pathology, clinical presentation, and diagnostic criteria of CTE in American football have been performed [12–15].

The long-term effects of brain trauma should be considered a significant public health concern, as millions of Americans participate in contact sports [16]. But despite the significance of these concerns, and the compelling media attention surrounding CTE, little is currently known about the pathophysiology of TBI and the clinicopathological correlation in CTE. The current neuropathological and clinical diagnostic criteria for CTE have inherent limitations and biases which must be taken into consideration when drawing conclusions. Since CTE is a neuropathological entity diagnosed only postmortem and not based on clinical features, this paper summarizes the current understanding of CTE and highlights limitations, inconsistencies, and problematic implications, with an emphasis on the neuropathological perspective.

NEUROPATHOLOGICAL PRESENTATION OF CTE

In 2013, Mckee et al. [17] set out to analyze the spectrum of CTE in a study of 85 donated brains with a history of repetitive mTBI and 18 healthy controls. The results of this study lead to the proposal of four progressive stages of CTE differentiated by emergent pathological hallmarks (Table 1) and worsening clinical presentation (Table 4).

Gross pathology

According to the diagnostic criteria initially proposed by Mckee et al. [17] macroscopic changes to the brain are uncommon in early stages (I and II) of CTE. These brains may present normally with no structural changes usually associated with brain trauma, such as hemorrhage or contusions [3]. In more advanced cases, gross pathology may be present (Table 1). Macroscopic pathological changes associated with later stages of CTE include a cavum septum pellucidum, ventricular enlargement of the frontal and temporal horns of the lateral ventricles, widespread atrophy, thinning of the corpus callosum, and depigmentation of the substantia nigra and locus coeruleus [17].

Microscopic pathology

Initially, McKee et al. [17] proposed five preliminary criteria for the microscopic pathological diagnosis of CTE (Table 2). The first stage of CTE was characterized by the deposition of p-tau protein as neurofibrillary tangles (NFTs) or astrocytic tangles (ATs) as focal epicenters in the cerebral cortex, particularly in the perivascular regions at the depths of cortical sulci. Stage II was characterized by the presence of additional NFTs and ATs in the superficial cortical layers adjacent to focal epicenters, as well as in the nucleus basalis of Meynert and locus coeruleus. In stage III, p-tau pathology consisted of dense deposits in the medial temporal lobe structures and widespread throughout the cortex. Stage IV showed dense, widespread p-tau pathology in the cortex and temporal lobe, as well as significant neuronal loss and gliosis of the cerebral cortex and hippocampus [17].

In 2015, after the initial proposal of CTE staging, the National Institutes of Health (NIH) sought out to strictly define the neuropathological criteria of CTE diagnosis with a consensus meeting [3]. The aim of this meeting was to confirm that CTE was a distinct neurodegenerative disease, and to establish diagnostic criteria to distinguish it from other tauopathies. In this meeting, 7 neuropathologists blindly evaluated 25 cases of tauopathies, including 10 cases of CTE. There was strong unanimity among the evaluators and reviewers that CTE represented a distinct tauopathy with both specific diagnostic criteria and supporting diagnostic features. The diagnostic criteria for CTE that came from this meeting was more limited than those initially proposed by McKee et al. [17] and required only p-tau accumulation in neurons and astrocytes in the perivascular regions at the depths of cortical sulci. Additional pathological features including NFTs in superficial cortical layers and macroscopic changes and TDP-43 positive neurites

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Table 1
Detailed summary of the pathological and clinical findings extracted from McKee et al., 2013 [17]

Stage I (very mild)	**Overview** ○ $n = 7$ ○ Age = 17–56 (mean 28.3 years) **Gross pathology** ○ Mild ventricular enlargement ○ No change in brain weight **Microscopic pathology** ○ Foci of perivascular NFTs and ATs in depths of cortical sulci. ○ Isolated NFTs in superficial cortical laminae ○ NFTs in the locus coeruleus (2 cases) ○ NFTs in the hippocampus, entorhinal cortex, and substantia nigra (1 case) ○ NFTs in the medulla (1 case) ○ Scattered axonal varicosities ○ TDP-43 positive neurites in the frontal subcortical matter in 57% of cases. **Clinical Presentation** ○ 1 case asymptomatic ○ 4 cases: Headache, loss of attention and concentration ○ 3 cases: Short-term memory difficulty, aggression, depression ○ 2 cases: Executive dysfunction and explosivity ○ 2 cases diagnosed with PTSD
Stage II (mild)	**Overview** ○ $n = 14$ ○ age = 21–87 (mean 44.3 years) **Gross pathology** ○ No cerebral atrophy, no change in brain weight ○ Mild ventricular enlargement in 6 cases ○ Small cavum septum in 4 cases ○ Pallor of lucus coeruleus and substantia nigra in 3 cases ○ Severe gliosis and atrophy of mammillary bodies in one case **Microscopic pathology** ○ Discrete tau foci in cerebral cortex ○ NFTs in superficial cortical layers ○ NFTs in the locus coeruleus, nucleus basalis of Meynert, and amygdala ○ NFTs and pretangles in the hypothalamus, hippocampus, entorhinal cortex, thalamus, substantia nigra, and midbrain ○ Axonal varicosities and tau in frontal and temporal cortex white matter tracts ○ 79% of cases had TDP-43 positive neurites **Clinical presentation** ○ Common: depression, mood swings, headaches, short-term memory loss ○ Uncommon: executive dysfunction, impulsivity, suicidality, language difficulty
Stage III (moderate)	**Overview** ○ N = 15 ○ Age = 38–82 (mean 56 years) **Gross pathology** ○ Mild cerebral atrophy ○ Ventricular dilation ○ Septal abnormalities in 5 cases ○ Depigmentation of locus coeruleus in 7 cases ○ Depigmentation of substantia nigra in 6 cases ○ Atrophy of mammillary bodies and thalamus **Microscopic pathology** ○ Widespread cortical NFTs ○ Widespread NFTs in the hippocampus, entorhinal cortex, amygdala, nucleus basalis of Meynert, and locus coerulius ○ NFTs in olfactory bulbs, hypothalamus, mammillary bodies, substantia nigra, and midbrain ○ Severe axonal loss and distortion in subcortical white matter of frontal and temporal cortices ○ Widespread TDP-43 positive neurites **Clinical Presentation** ○ Symptoms listed in early stages were common ○ Less common: Impulsivity, apathy, headaches, and suicidality ○ 75% of cases were considered “cognitively impaired”

(*continued*)

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Table 1
(Continued)

Stage IV (severe)	**Overview** ○ N = 15 ○ Age = 51–98 (mean 77.4 years) **Gross pathology** ○ Widespread atrophy ○ Significantly reduced brain weight ○ Ventricular enlargement ○ Cavum septum pellucidum and septal perforations ○ Pallor of locus coerulius and substantia nigra **Microscopic pathology** 1. Neuronal loss in cortex 2. Severe tau pathology throughout the brain and spinal cord, with sparing of the visual cortex 3. Axonal loss and distortion in subcortical white matter 4. Many TDP-43 positive neurites **Clinical Presentation** ○ Most common: Executive dysfunction, memory loss, loss of attention and concentration, language difficulty, visuospatial difficulty, aggressiveness, paranoia, depression, explosivity, gait changes ○ Less common: Impulsivity, dysarthria, parkinsonism ○ 31% of cases were suicidal

ATs, astrocytic tangles; NFTs, neurofibrillary tangles; PTSD, post-traumatic stress disorder.

Table 2
Preliminary criteria for the microscopic pathological diagnosis of CTE as proposed by McKee et al. [17]

(i) Perivascular tau foci in the neocortex in the form of neurofibrillary tangles (NFTs) or astrocytic tangles (ATs);
(ii) Distribution of tau NFTs and ATs in the depths of cerebral sulci;
(iii) NFTs in the superficial layers of the cerebral cortex
(iv) Supportive, but not diagnostic: subpial ATs in the depths of cortical sulci.

were included as supporting features of CTE but are not required for diagnosis. The required diagnostic criteria and supporting features of CTE proposed by the 2015 consensus meeting can be found in Table 3.

CTE in the context of other brain diseases

Several of the pathological entities listed in the diagnostic criteria for CTE are found in other brain diseases, making it difficult to distinguish CTE pathology from either comorbidities or other neurodegenerative diagnoses in some postmortem cases. Some of the neurodegenerative diseases with common pathological features of CTE include Alzheimer's disease (AD), Parkinson's disease, frontotemporal lobar dementia (FTLD), Lewy body disease, and motor neuron disease (MND).

Hyperphosphorylated tau is a common pathological entity in a variety of neurodegenerative disease, collectively referred to as tauopathies. This is the defining neuropathological feature of CTE [3] and can often be distinguished from other diseases. In CTE, tau is distributed perivascularly in the depths of cortical sulci in both neurons and astrocytes, whereas in AD, for comparison, tau is found initially in the temporal lobes and is found only in neurons [17]. CTE can also by distinguished by the relative absence of amyloid-β plaques compared to AD [2].

A common feature of CTE, despite being excluded from the initial proposed diagnostic criteria [17] and the consensus diagnostic criteria [3], is the presence of TDP-43 in neurons of the cortex and brainstem. This is a prominent feature of MND, such as amyotrophic lateral sclerosis (ALS), and FTLD [18]. The pattern and distribution of TDP-43 in CTE is not unique and is similar to the inclusions found in FTLD [19]. It is therefore difficult to distinguish CTE pathology from that of other neurodegenerative diseases by observations of TDP-43 accumulation.

CLINICAL PRESENTATION OF CTE

The initial clinical symptoms of CTE vary significantly between individuals and the disease can present behaviorally or cognitively in early stages. Families of individuals with CTE often describe initial changes such as irritability, aggressiveness, sadness, and apathy [18]. On the other hand, some families describe cognitive changes like difficulty concentrating and loss of short-term memory as occurring first [19]. As the disease progresses into later stages more severe symptoms can emerge such as executive dysfunction, severe memory loss, and visuospatial deficits (Table 4). It has been suggested that suicidality is a common clinical feature of CTE

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Table 3
Summary of the 2015 consensus meeting criteria for the diagnosis of CTE [3]

Required for diagnosis	(i) P-tau aggregates in neurons and astrocytes in the perivascular regions at depths of cortical sulci
Supportive features	(ii) Neurofibrillary tangles (NFTs) and/or pretangles in the superficial layers of the cortex (iii) NFTs and/or pretangles in the hippocampus (iv) P-tau reactivity in neurons and astrocytes of subcortical matter, including the mammillary bodies, hypothalamus, amygdala, nucleus accumbens, thalamus, midbrain tegmentum, nucleus basalis of Meynert, raphe nuclei, substantia nigra, and/or locus coeruleus (v) P-tau in subpial and periventricular astrocytes (vi) P-tau in large grain/dot-like structures (vii) Macroscopic features including ventricular dilation, septal abnormalities, atrophy of the mammillary bodies, and/or contusions (viii) TDP-43 positive neurites in the hippocampus, temporal cortex, and/or amygdala.
Non-diagnostic features	(i) Thorny astrocytes in subcortical white matter (ii) Subependymal periventricular thorny astrocytes (iii) Thorny astrocytes in the amygdala and/or hippocampus

Table 4
Summary of clinical symptoms for each stage of CTE (extracted from McKee et al., 2013 [9])

Stage I (very mild)	• Headaches • Loss of attention • Loss of concentration.
Stage II (mild)	• Depression • Mood swings • Explosivity • Loss of attention • Loss of concentration • Headaches • Short-term memory loss
Stage III (moderate)	• Memory loss • Executive dysfunction • Loss of attention • Loss of concentration • Depression • Explosivity • Visuospatial abnormalities
Stage IV (severe)	• Profound short-term memory loss • Executive dysfunction • Attention loss • Concentration loss • Explosivity • Aggression • Paranoia • Depression • Impulsivity • Visuospatial abnormalities

[19], although it is important to note that not every individual diagnosed with CTE had a history of suicidality. It has also been suggested that individuals with advanced CTE are at a disproportionately higher risk for developing dementia later in life [19]. This diverse array of symptoms indicates that the clinical presentation of CTE greatly differs between individuals and the disease can impact multiple aspects of a person including behavior, cognition, mood, and motor function.

LIMITATIONS OF CTE RESEARCH

Epidemiology

There have been no systematic studies on CTE published to date, and current understanding of the disease is based on a selection of case series. These series have been heavily covered by the media, with headlines such as "CTE found in 99% of studied brains from deceased NFL players" [20] catching the attention of many readers worldwide. However, the studies on which these articles are based have been primarily limited to populations of contact sports players, many of which reported symptoms resembling CTE presentation prior to death. The results of these series primarily reflect contact sports players with a clinical history and is therefore not representative of the general population or even contact sports players as a whole. The media coverage on CTE is therefore misleading.

As CTE can only be diagnosed postmortem, studies on CTE have all been based on autopsy series. These studies are limited in that they cannot inform on new incidences of disease or prevalence of the disease outside of the study population. In other words, true epidemiological data on CTE does not yet exist. A longitudinal population study on CTE could advise on these issues of prevalence and incidence; however, the diagnostic criteria established [3] does not include any clear clinical diagnostic criteria, so a population-based study is not yet possible.

The confounds of brain donation programs must also be considered when evaluating postmortem case studies. Generally, patients and next-of-kin are more likely to participate in brain donation programs if they display symptoms of the disease than if they were not

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[21], commonly referred to as "referral bias". It has also been reported that families of athletes who died by suicide are disproportionately more likely to participate in CTE brain donation programs [21]. The clinical information on these donated brains rely on retrospective interviews with next-of-kin for case history, including playing time, number of concussions, symptoms, and substance abuse among other factors. Clinical analysis is therefore based on information which could be significantly influenced by recall bias. This is insufficient for creating robust clinical diagnostic criteria for CTE in living patients. The design of CTE case series studies is therefore fundamentally flawed by referral and recall biases and cannot answer epidemiological questions.

A lack of proper controls and specificity is also an issue in the study of CTE microscopic pathology. Several large case studies of CTE have failed to acknowledge literature implicating the presence of sporadic tau in aging, but otherwise healthy, brains [22]. In a study of over 2000 postmortem brains aged 1–100 years, Braak et al. [23] found that over 89% of cases under the age of 30 had at least some tau pathology. The authors concluded that advanced age is not a requirement of AD-associated pathology, and that AD-associated pathology can occur in healthy cases with no reported symptoms. It would therefore seem to be helpful to study a group of healthily aged brains in comparison to cases of CTE as controls. However, we do not yet know what the appropriate control group for studying CTE is. Recently a case of CTE was reported in a brain with no history of head trauma [24], indicating that brains without mTBI but with neurodegenerative disease or natural aging may not be the proper control for CTE case series.

Pathology

A majority of the postmortem studies on CTE show diverse neuropathology, with broad gross and microscopic changes not explicitly referenced in the proposed diagnostic criteria. Two of the most influential case series have been those by McKee et al. [17] and Omalu et al. [25], and although the two groups aimed to characterize the same disease with similar methods, they had some contradictory findings. For example, Omalu et al. [25] claimed that atrophy is not a prominent feature of CTE, whereas McKee et al. [17] claims that atrophy of the frontal and temporal lobes is common in advanced stages of CTE. Further, McKee et al. [17] reports ATs as a defining feature of CTE in the first proposed diagnostic criteria, but Omalu et al. [25] did not find these in their cases at all. Both groups note that tau in the depths of cortical sulci is a defining feature of the disease, and both consider the disease unique to other neurodegenerative diseases. The inconsistent findings between these two series highlights the varied neuropathological presentation of CTE and emphasizes the role of individuality between cases. Further, the case series that lead to diagnostic criteria used 50 μm whole-mount sections to study tau pathology [3, 17], which may reduce specificity of tau reactivity while simultaneously increasing sensitivity. The result of this method could be an overestimation of tau pathology in some cases and not reproducible in most diagnostic laboratories.

CTE can supposedly be distinguished from AD based on the abnormal distribution of tau to the depths of cortical sulci and the relative absence of amyloid-β plaques [3, 17]. However, of 68 individuals diagnosed with CTE in the proposed diagnostic criteria case series, amyloid-β plaques were reported in 44.1% [17]. This finding is contradictory and accentuates both the overlap between CTE and other neurodegenerative diseases and the pathological diversity within a cohort of CTE cases. In theory, CTE should be relatively easy to distinguish from AD due to the differential localization and distribution of tau protein, as well as the relative absence of amyloid-β. In practice, however, this distinction becomes blurred in more severe cases. Often, individuals diagnosed with CTE live into old age, presenting with neurological and/or systemic comorbidities [13]. In particular, it is very common to see individuals with both CTE-consistent and AD-consistent tauopathy. With the current diagnostic criteria for CTE, it is unclear whether these individuals should be diagnosed with CTE, AD, or a complex, mixed-type pathology. Further, it is unclear whether the pathological hallmarks associated with CTE are a product of AD, or vice versa.

Age-related tau astrogliopathy (ARTAG) refers to an accumulation of astrocytic tau in patients generally over the age of 60 [26]. It may be present in addition to other primary tauopathies or diseases and is characterized pathologically by widespread tau accumulation in astroglia of the subpial, subepedymal, and perivascular white and grey matter [27]. The features of ARTAG therefore heavily overlap with those of CTE, with the exception of neuronal involvement. Researchers have noted that cases of ARTAG may have been misdiagnosed as CTE in case series [27],

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and that a clear distinction between the two diseases has yet to be made.

The pathological hallmarks of CTE are at times vague and include changes that could be associated with a variety of neurodegenerative diseases and even normal aging. The diagnosis of CTE becomes even more unclear when one considers diagnosed comorbidities within case studies. For example, McKee et al. [17] claimed that of 85 individuals with a history of repetitive mTBI, 68 (80%) were diagnosed with CTE as per their proposed diagnostic criteria. However, of these cases diagnosed with CTE, 1 case was diagnosed with multiple system atrophy, 8 cases were diagnosed with ALS, and 17 cases were diagnosed with one or more of the following: AD, Parkinson's disease, FTLD, progressive supranuclear palsy, or Pick's disease, and multiple cases had cerebrovascular disease. The total number of "pure" CTE cases in these series is therefore only 39 (45%); however, the broad-encompassing definition of CTE allows for a higher estimate of disease prevalence despite the existence of significant comorbidities. This analysis emphasizes the unclear pathological findings in CTE studies and highlights the inconsistency of CTE diagnosis from these proposed criteria.

In particular, a feature found in most cases of CTE [2, 12, 13, 17], despite being excluded from the initial proposed diagnostic criteria [17] and included only as a supportive feature in the consensus diagnostic criteria [3], is the presence of TDP-43 neuronal inclusions in the cortex and brainstem. In a case series of CTE, mild TDP-43 inclusions were reported in 50% of stage 1 cases and were reported to be widespread and significant in later stages [13]. This finding seems quite significant in terms of neuropathological assessment, yet it has been disregarded in formal diagnostic criteria. Further, the presence of this protein is worth discussion as it is a prominent feature of MND, such as ALS, and FTLD [28]. The pattern and distribution of TDP-43 inclusions in CTE is also not unique, as it presents similarly to the inclusions found in FTLD [28]. It is therefore difficult to distinguish CTE pathology from that of other neurodegenerative diseases by observations of TDP-43 accumulation not only because of its similar distribution to other diseases, but because of its general absence from CTE diagnostic criteria.

Confident diagnosis of CTE is not yet possible with such confounding neuropathological findings, and assertions based on this data should be taken with caution. It is not understood how focal perivascular lesions lead to symptoms associated with early stages of CTE [4], especially when one considers the broader context of symptom manifestation in neurodegenerative disease. In Parkinson's disease, for example, clinical symptoms do not appear until about 80% of striatal dopamine neurons and 30% of substantia nigra dopamine neurons are lost [29]. Furthermore, early stages of AD can be pathologically diagnosed in cases who reported as cognitively intact with no symptoms [23]. The idea that a small focal tau lesion could cause large-scale symptoms in early stages of CTE is therefore conflicting.

Clinical presentation

The clinical presentations of CTE have generally been measured with double-blind next-of-kin retrospective interviews [3, 17, 25]. These interviews are often semi-structured and centered around the donor's clinical presentation after suffering TBI. It is difficult to quantify this clinical assessment because there is no baseline (i.e., before the donor acquired mTBI) to compare to. This does not allow researchers to consider individual differences in mood and behavior, for example, in the donor prior to acquiring any head trauma. Further, the families of individuals who participate in CTE brain donation programs are often seeking an explanation for their loved one's potentially disruptive symptoms, and this could influence their answers to interview questions in a way that favors CTE diagnosis. The clinical assessments used in these studies may therefore not be truly representative of the symptoms associated with CTE, as they are biased. In several cases of CTE, abuse of alcohol and drugs by the individual or post-traumatic stress disorder (primarily in veterans) was reported, and so the degree to which neuropathological entities genuinely influenced clinical presentation can be indistinguishable from these external factors.

In a study using a neurodegenerative brain bank, CTE was found in 32% of cases with a history of playing contact sports [30], and their clinical history did not differ from cases without any CTE pathology. This study highlights the lack of clinicopathological correlation in CTE and raises the question of whether or not CTE can be considered a separate disorder from other neurodegenerative diseases.

Suicidality is often cited as a prominent feature of CTE [3, 17, 25], but this assertion is both unsupported and problematic. Families of suicide victims are disproportionately more likely to participate in brain donation programs [25], and so the

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sample from which clinical assessments are taken is inherently biased. Furthermore, risk factors for suicidality, including depression and substance abuse, are strongly associated with CTE [4]. Based on the current scientific evidence, it is therefore impossible to determine if CTE causes suicidality, or if comorbid issues associated with CTE may lead to suicidality. In addition, the portrayal of CTE in the media may lead to excessive worrying by individuals who are allegedly "at risk" for progressive brain disease and trigger suicidal thoughts.

It is important to note that the clinical presentation reported in CTE is not unique and reflect symptoms of many disorders. For example, depression, anxiety, anger, and short-term memory loss are all reported in CTE [21] but are also common side effects of many drugs [31] and mental illnesses [32], and some can even be attributed to normal human life. Indeed, many individuals reported with CTE were professional athletes, a job that comes with a lot of pressure to perform and attention from media. It is possible that these individuals develop cognitive symptoms attributed to poor mental health and may not have any correlation to neuropathology. In order for a longitudinal study on CTE in living patients to be made possible, a unique set of clinical traits and their specific progression through time must be established. This is difficult, as the symptoms seen in cases of CTE seem to vary between individuals in their time and severity of presentation [17].

Insufficient evidence for clinical tests

Currently, there exist no potential biomarkers associated with CTE for clinical diagnosis in a living person. The most promising research into diagnosing CTE in living patients involves radioactive neuroimaging of tau using positron emission tomography (PET) scans [33]. In 2017, a study was published claiming to have diagnosed CTE in a living patient for the first time [34] using this neuroimaging method and confirmed the diagnosis postmortem. The researchers found evidence of tau, amyloid, and TDP-43 in the brain using PET tracers. However, the findings from this study are not very clear. The detection of tau was extremely widespread, including the frontal lobe, temporal lobe, striatum, hypothalamus, midbrain, and pons. Further, the study failed to acknowledge other explanations of tau deposition (e.g., another tauopathy, normal aging, etc.) and the patient was diagnosed with MND two years postscan. Despite a complete lack of specificity and the existence of a comorbidity, the study concludes that PET neuroimaging can be selective for CTE. This conclusion is dangerous as it could lead to serious misdiagnoses in living patients, causing unnecessary anxiety, stress, or a self-fulfilling prophecy.

CONCLUSION

Currently CTE is presented as a progressive neurodegenerative disease with unique pathology caused by repetitive brain trauma. However, looking through all published cases there are significant limitations, biases, and inconsistencies that question this claim. Of these issues, the most significant are insufficient samples, pathological inconsistencies, unreliable clinical data, and flawed study designs.

CTE research has been limited to case series, making incidence and prevalence of the disease impossible to determine. Additionally, clinical analysis of CTE cases is limited and fragmented making potential risk factors for developing CTE impossible to determine with confidence. Despite these limitations, media coverage of CTE is giving the impression that developing symptomatic CTE is almost inevitable if you are a long-term contact sports player [20, 35, 36]. Many news outlets are citing suicidality, dementia, and executive dysfunction as inevitable in the end stages of CTE [37], resulting in a self-fulfilling prophecy for some who fall into the "at-risk" population of contact sports players. For example, a professional hockey player presented with some common symptoms of CTE and was convinced he had developed the disease [38]. The player committed suicide, but postmortem examination revealed no pathological evidence of CTE [38]. This explosion of CTE media coverage has also began to influence policymaking and parental permissions in youth sports [39, 40], despite a lack of scientific evidence.

Throughout the last century, research has clearly correlated repetitive head trauma with long-term cognitive effects. However, the current scientific literature is limited and the pathophysiology of TBI-induced damage is still largely unknown. The pathological hallmarks and clinical presentations of CTE are widespread and variable both within and between case series, making it difficult to uniquely and definitively characterize CTE as a neurodegenerative disease. Prospective longitudinal studies are necessary in order to identify prevalence, incidence, clinicopathological correlation, and risk factors. Epidemiological studies with an emphasis on individual

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factors should also be considered in order to better understand the long-term effects of head trauma.

DISCLOSURE STATEMENT

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The Need to Separate Chronic Traumatic Encephalopathy Neuropathology from Clinical Features

Grant L. Iverson[a,*], C. Dirk Keene[b], George Perry[c] and Rudolph J. Castellani[d]
[a]*Department of Physical Medicine and Rehabilitation, Harvard Medical School, Spaulding Rehabilitation Hospital, MassGeneral Hospital for Children™ Sports Concussion Program, and Home Base, A Red Sox Foundation and Massachusetts General Hospital Program, Boston, MA, USA*
[b]*Department of Pathology, Division of Neuropathology, University of Washington School of Medicine, Seattle, WA, USA*
[c]*College of Sciences, University of Texas, San Antonio, San Antonio, TX, USA*
[d]*Center for Neuropathology, Western Michigan University Homer Stryker MD School of Medicine, Kalamazoo, MI, USA*

Abstract. There is tremendous recent interest in chronic traumatic encephalopathy (CTE) in former collision sport athletes, civilians, and military veterans. This critical review places important recent research results into a historical context. In 2015, preliminary consensus criteria were developed for defining the neuropathology of CTE, which substantially narrowed the pathology previously reported to be characteristic. There are no agreed upon clinical criteria for diagnosis, although sets of criteria have been proposed for research purposes. A prevailing theory is that CTE is an inexorably progressive neurodegenerative disease within the molecular classification of the tauopathies. However, historical and recent evidence suggests that CTE, as it is presented in the literature, might not be pathologically or clinically progressive in a substantial percentage of people. At present, it is not known whether the emergence, course, or severity of clinical symptoms can be predicted by specific combinations of neuropathologies, thresholds for accumulation of pathology, or regional distributions of pathologies. More research is needed to determine the extent to which the neuropathology ascribed to long-term effects of neurotrauma is static, progressive, or both. Disambiguating the pathology from the broad array of clinical features that have been reported in recent studies might facilitate and accelerate research—and improve understanding of CTE.

Keywords: Concussion, neurodegenerative, neuropathology, tau

INTRODUCTION

Chronic traumatic encephalopathy (CTE) has been recognized, but not well studied, for more than 80 years, and the large majority of publications in the past century are case studies [1–6]. CTE was initially conceptualized as a neurological disorder affecting boxers who had tremendous exposure to neurotrauma [1, 2]. Martland [1] described the clinical features as mostly including gait disturbance, dysarthria, tremor, and cognitive impairment. In the 1930s, the syndrome was referred to as traumatic encephalopathy [7] and dementia pugilistica [8]. In the late 1940s,

*Correspondence to: Grant L. Iverson, Ph.D., Center for Health and Rehabilitation Research, Department of Physical Medicine and Rehabilitation, 79/96 Thirteenth Street, Charlestown Navy Yard, Charlestown 02129, MA, USA. E-mail: giverson@mgh.harvard.edu.

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Critchley referred to it as CTE [9] and in 1957 he referred to it as chronic *progressive* (italics added) traumatic encephalopathy [10]. In 1969, the first and only large clinical study of chronic neurotrauma in boxers was published by Roberts as a book. He selected an age-stratified random sample of 250 retired boxers from a cohort of 16,781, located and clinically examined 224, and identified 17% as having the syndrome (i.e., 11% as having a mild form of the syndrome and 6% as having severe traumatic encephalopathy). Omalu and colleagues published the first description of CTE in a retired National Football League (NFL) player in 2005 [11], with additional case studies published in 2006 [12] and 2010 [13]. In 2009, McKee and colleagues described three additional cases, one retired football player and two boxers, within their review of 48 known cases in the world literature [14].

Omalu and colleagues introduced four neuropathological "phenotypes" in 2011 [15], and McKee and colleagues introduced four neuropathological "stages" of CTE in 2013 [16]. These descriptions of phenotypes and stages vary in the diversity and severity of neuropathology. These phenotypes and stages largely underlie the theory and assumption that the pathology and clinical features are progressive. Moreover, the stages vary as a function of age—and Stage III and IV cases often have co-morbid neurodegenerative diseases [16].

In Roberts' large study of boxers in the 1960s, he reported that most with the syndrome had a static course, there were anecdotal cases of improvement after retirement from boxing, and a small subgroup appeared to have a progressive course greater than expected from aging [2]. Decades later, Jordan echoed the conclusions of Roberts and noted that it was unclear whether worsening of chronic brain injury in boxers reflected a progressive neurodegenerative disease, the aging process superimposed on a fixed neurological injury, or both [17]. In contrast, in recent years CTE has been described definitively as a *delayed-onset* and *progressive* neurodegenerative disease, with symptoms appearing "in midlife" [18, 19] or decades after exposure [14, 15, 18–24].

To date, however, there are no prospective, longitudinal, or epidemiological studies that support the theory that CTE pathology is progressive in a manner similar to canonical neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), or amyotrophic lateral sclerosis (ALS). Adding further complexity to theories relating to the natural history of CTE, Omalu [22] has asserted that CTE can begin immediately, days, weeks, months, years, or decades after exposure to neurotrauma, and he states that a *single* injury of any severity [23] or repetitive subconcussive blows can cause CTE [13]. The confident assertions of causation by some researchers stand in juxtaposition with the fact that the etiologies of much better characterized sporadic neurodegenerative diseases, each of which is inexorably progressive, are mostly unknown. This article discusses the complexity of the current conceptualization of CTE pathology and clinical features attributed to that pathology.

NEUROPATHOLOGY

In publications from 2005–2015, CTE has been described as a progressive neurodegenerative disease characterized by a broad and diverse range of both macroscopic and microscopic neuropathology. The gross neuropathology, described as "characteristic" of CTE, includes 1) frontal and temporal atrophy, thinning of the hypothalamic floor, shrinkage of the mammillary bodies, pallor of the substantia nigra, hippocampal sclerosis, and reduction in brain mass; 2) enlarged ventricles; and 3) cavum septum pellucidum with or without septal fenestrations [14, 24, 25]. Microscopic features described as characteristic have included 1) localized neuronal and glial accumulations of phosphorylated tau (p-tau) with varying microscopic morphologies, involving perivascular areas of the cerebral cortex and sulcal depths, and with a preference for neurons within superficial cortical laminae; 2) multifocal axonal varicosities involving deep cortex and subcortical white matter; 3) variable and often absent amyloid-β (Aβ) deposits; and 4) TDP-43-positive inclusions and neurites [14, 24, 25]. A variable distribution and quantity of tau pathology, and the accumulation of other altered proteins such as Aβ, α-synuclein (αS), and transactive response DNA (TDP) binding protein 43-immunoreactivity, occurs with human aging and other distinct diseases, and can also be found in people who are cognitively normal [26]. Most of the above-mentioned gross and microscopic features have not, as yet, been independently verified as specific to CTE [27–30], and virtually all are associated with aging, other neurological diseases, or both.

Prior to 2015, there were no agreed upon neuropathological criteria for CTE, and the criteria put forward by the two research groups in the US differed

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[15, 16]. To address this problem, a panel of seven neuropathologists, convened by the National Institutes of Health (NIH), were provided 10 cases of advanced CTE (two with Stage III and eight with Stage IV pathology [16]) and 15 cases with primary tau-related neurodegenerative diseases to examine blindly [30]. The 10 cases of presumptive CTE were former professional athletes, between the ages of 60 and 85, selected by researchers from Boston University. The pathologists were provided a presumptive *a priori* definition of the neuropathology of CTE from Boston University (see online supplementary material 1 in the original article). They then examined the cases without access to demographic information, clinical history, or gross neuropathologic data, and listed their diagnoses. They then met in person to further refine their interpretations and discuss the overall findings.

Through their work, preliminary consensus criteria for the neuropathology of CTE were developed [30]. It should be noted that the panel did not fully adopt the *a priori* neuropathological criteria (i.e., online supplementary material 1), but they did produce a set of criteria that were similar. They defined a single "pathognomonic" criterion for CTE as an accumulation of abnormal p-tau in neurons, astrocytes, and cell processes around small vessels in an irregular pattern at the depths of the cortical sulci [30]. This finding represents a distribution of p-tau that is believed to differ from that seen in aging and neurodegenerative diseases. The consensus report did not indicate that p-tau increases quantitatively on a whole brain level, greater than what would be expected from aging, pre-clinical neurodegeneration, or comorbid neurodegenerative disease. Rather, the pathognomonic feature relates to specific distributions, not overall burden, of pathology.

They also identified *supportive* criteria as follows: "(1) abnormal p-tau immunoreactive pretangles and NFTs preferentially affecting superficial layers (layers II–III), in contrast to layers III and V as in AD; (2) in the hippocampus, pretangles, NFTs or extracellular tangles preferentially affecting CA2, and pretangles and prominent proximal dendritic swellings in CA4. These regional p-tau pathologies differ from the preferential involvement of CA1 and subiculum found in AD; (3) abnormal p-tau immunoreactive neuronal and astrocytic aggregates in subcortical nuclei, including the mammillary bodies and other hypothalamic nuclei, amygdala, nucleus accumbens, thalamus, midbrain tegmentum, and isodendritic core (nucleus basalis of Meynert, raphe nuclei, substantia nigra and locus coeruleus); (4) p-Tau immunoreactive thorny astrocytes at the glial limitans most commonly found in the subpial and periventricular regions; and (5) p-Tau immunoreactive large grain-like and dot-like structures (in addition to some threadlike neurites)" [30]. Additional pathologies that were considered supportive of the diagnosis were as follows: "(1) macroscopic features: disproportionate dilatation of the third ventricle, septal abnormalities, mammillary body atrophy, and contusions or other signs of previous traumatic injury; and (2) TDP-43 immunoreactive neuronal cytoplasmic inclusions and dot-like structures in the hippocampus, anteromedial temporal cortex and amygdala" [30]. The consensus panel acknowledged the limitation of using only "moderate to late stage" CTE to develop the consensus criteria. Interestingly, the frequent comorbidities in advanced stage CTE might actually hamper the interpretation compared to early stage CTE, which should be more easily separated from both age-related changes and other neurodegenerative diseases. The consensus panel did not adopt the four neuropathological phenotypes [15] or stages of CTE [16], and indicated that the preliminary criteria were a "first step along the path to standardizing the neuropathology of CTE" [30]. Moreover, they did not address whether CTE was a progressive neurodegenerative disease, or whether there were known or predictable clinicopathologic correlations. Instead, they examined whether CTE could be separated neuropathologically from other pathologies.

CTE-LIKE NEUROPATHOLOGY IN AGING AND OTHER DISEASES

The consensus criteria for CTE [30] are mostly similar to, but have some important differences from, the neuropathological criteria described in papers between 2005 and 2015. Many of the gross and microscopic neuropathological features described in past studies were not included in the definition. Moreover, the presumptive diagnostic criteria provided to the panelists prior to the consensus conference were not adopted in whole. For example, the first presumptive criterion, "perivascular foci of p-tau immunoreactive neurofibrillary tangles (NFTs) and astrocytic tangles (ATs) in the neocortex" (see online supplementary material 1) was not adopted—presumably because this criterion is not unique to CTE. P-tau in these regions has recently been described in the postmortem brains of patients who had ALS [31],

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temporal lobe epilepsy [32], and multiple system atrophy [33] but no known history of participation in contact sports or neurotrauma. Gao and colleagues recently described a case of a man with ALS and no known history of neurotrauma who had CTE-like pathology in both depths of sulci and perivascular regions [34].

Perivascular, subpial, and periventricular p-tau immunoreactive NFTs and astrocytic tangles in the neocortex have been reported to be characteristic of both primary age-related tauopathy (PART) [35] and age-related tau astrogliopathy (ARTAG) [36]. PART is characterized by neurofibrillary degeneration most notable in medial temporal lobe, basal forebrain, brainstem, olfactory bulb, and cortex, in association with little or no amyloid-β accumulation—and PART may or may not be associated with cognitive impairment. PART refers to neuronal tau pathology and ARTAG is characterized by astrocytic tau pathology, although they are not mutually exclusive, and appear to be more a manifestation of age than a subtype of neurodegenerative disease. The authors describing ARTAG appear to emphasize p-tau in the sulcal depths as the most specific feature distinguishing CTE and ARTAG, stating "ARTAG has features that overlap those of CTE, including the accentuation of tau pathology around small cerebral vessels and in subpial and periventricular areas. On the other hand, tau pathology, including neuronal and astroglial, in CTE is more abundant in the depths of the cerebral sulci, especially in early stages, an aspect that has not been reported in tau astrogliopathy in the aging brain." This distinction was not made prior to 2015, so in some past published cases PART and ARTAG neuropathology was likely interpreted as CTE-specific neuropathology. Historical cases of dementia pugilistica are interesting in this regard, in that the pathological descriptions in some cases appear indistinguishable from PART [6], consistent with Jordan's hypothesis that dementia pugilistica may in some cases represent the aging process superimposed on fixed structural pathology [17].

In a large-scale post-mortem study of CTE in the United Kingdom, Ling and colleagues screened 268 cases and identified pathology consistent with recent descriptions of CTE in 32 (11.9%) [37]. History of traumatic brain injury, with or without loss of consciousness, was present in nearly all cases (i.e., 93.8%). Only a minority, however, had known participation in sports (34%), and 18.8% were military veterans. Remarkably, of those 32 cases, 13 were women (40.6%). The majority of the screened cases met neuropathological criteria for other neurodegenerative diseases, although some were control subjects. The rates of CTE pathology stratified by neurodegenerative diseases were as follows: progressive supranuclear palsy = 24%, PD = 16%, AD = 10%, corticobasal degeneration = 7.4%, frontotemporal lobar degeneration = 4.2%, multiple system atrophy = 2%, and control subjects over the age of 60 = 12.8%. This study was completed before the NIH consensus criteria were published, so it is possible that the rates would be different (i.e., lower) if those criteria had been applied. It is not known whether, or the extent to which, ARTAG and PART pathology was conceptualized as CTE pathology in that study. In another large-scale neuropathology study [38], 21 of 66 former athletes (31.8%) had tau pathology suggestive of CTE. In contrast, none of the 198 cases who were not former athletes showed CTE pathology, including a subgroup of 33 cases who had a history of a single traumatic brain injury. Nearly all (95%; 20/21) of those with CTE pathology had primary neuropathological diagnoses of a neurodegenerative disease such as AD, frontotemporal dementia, Lewy body disease, or ALS. Of the former boxers, 2 of 9 (22.2%) had CTE pathology and 16 of 43 former football players (37.2%) had CTE pathology.

Koga and colleagues [33] examined 139 autopsy-confirmed cases of multiple system atrophy for pathological evidence of CTE. Using the consensus criteria, they identified CTE pathology in 8 cases (6%). All were men, but only four had a history of participation in sports (three in football and one in basketball). The authors were careful to differentiate cases of ARTAG (10 cases) from CTE in this study, and noted that ARTAG pathology in past studies might have been mistaken for CTE pathology. The authors speculated that the CTE pathology could be related to falls associated with having multiple system atrophy—but there was no reported statistical association between falls and CTE pathology in that study. In addition, the authors did not report other evidence that might be associated with falls and neurotrauma, such as structural pathology (e.g., contusions).

Noy and colleagues [39] examined 111 brains in a routine neuropathology service in Canada for the presence of CTE pathology. The subjects were between the ages of 18 and 60, and they were from a non-selected community-based neuropathology referral base. They set their cutoff age at 60 to reduce the confounding effects of aging and preclinical neurodegenerative diseases. They identified CTE pathology, based on the staging system of

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McKee et al. [16], in 4.5% (three cases of Stage I and two cases of Stage II). However, they made the important observation that there is no lower bound for classifying Stage I CTE pathology, so if they included tiny amounts of pathology characteristic of Stage I, an additional 34 cases were identified (30.6% of the sample). Therefore, of the total sample, 35.1% had some degree of mild CTE pathology. Only one subject had a history of sports participation, and there were three women in the sample. Factors that were associated with the presence of CTE pathology were age, history of traumatic brain injury, and substance abuse. Some of the cases had no known history of traumatic brain injury. There was no association between CTE pathology and psychiatric illness in this sample.

NEURODEGENERATION SEPARATE FROM CTE OR CO-OCCURRING WITH CTE

If one examines the online supplementary material that accompanied the study by McKee and colleagues that describes the stages of CTE [16], it is apparent that some cases conceptualized as having pure CTE also have other proteinopathies (e.g., non-specific p-tau, amyloid-β, α-synuclein, and TDP-43), which raises the possibility of early or preclinical neurodegenerative disease distinct from the pathology attributed to CTE. Therefore, "pure CTE" might be a misnomer in some cases, especially for cases with lesser amounts of region-specific p-tau who might have some degree of PART or ARTAG pathology that is separate from CTE. It is unknown if CTE pathologic lesions begin as purely glial and then progress to involving neurons. If so, ARTAG could be a precursor lesion to CTE pathology in some cases, or CTE pathology could mimic ARTAG in the mildest examples. Without good experimental models, it is virtually impossible to know how pathology progresses.

Neurodegenerative diseases exist in "pure" forms, but it is well understood that polypathology and disease comorbidities are common in those with AD, Lewy body disease, cerebrovascular disease, and hippocampal sclerosis. It can be difficult to determine the extent to which each pathological change contributed to a given person's clinical symptoms and course without rigorous clinicopathologic correlation. Even then, correlation between proteinopathy and clinical signs can be limited [40]. It is now well established that a large percentage of former athletes identified as having CTE neuropathology also have neuropathology associated with age related neurodegenerative diseases such as AD, PD, Lewy body disease, hippocampal sclerosis, and ALS [14, 41–44] to the extent that they meet full neuropathological criteria for an alternative, well-characterized neurodegenerative disease [16]. Often, however, whenever the pathology of CTE is identified, regardless of the amount, the case is conceptualized as CTE, when in fact it might be more appropriate to conceptualize the person as having another neurodegenerative disease with small amounts of CTE neuropathology. A recent case report emphasizes this problem [45]. In fact, in studies involving traditional brain banks, the majority of people identified as having CTE pathology have only mild forms of the pathology (e.g., Stage I or Stage II), in association with a separate and primary relentlessly progressive and fatal neurodegenerative disease [33, 37, 38].

IS THE NEUROPATHOLOGY OF CTE INEXORABLY PROGRESSIVE?

Researchers have not established that the neuropathology defining CTE is inexorably progressive. In fact, some accumulating evidence is inconsistent with the theory that CTE is a progressive neurodegenerative disease. It is well established that some people who have large exposures to repetitive neurotrauma, such as retired professional football players or boxers, had only Stage I or Stage II CTE at the time of their deaths [16], and some of these individuals died in their 80s. This suggests that the kinetics of p-tau accumulation in some people is either non-existent or limited. In the previously discussed large-scale postmortem study of CTE in the United Kingdom, Ling and colleagues screened 268 cases and identified pathology consistent with CTE in 11.9% [37]. All cases were considered Stage I or Stage II based on the staging system of McKee and colleagues [16], and their mean age of death was 81.0 years. The authors assumed that most of the cases with CTE pathology were likely to be clinically asymptomatic. Given the limited pathology (Stage I or Stage II) late in life, a progressive tauopathy, and associated psychiatric or neurologic disease, appears unlikely in these cases.

In another study [38], 21 of 66 former athletes (31.8%) had tau pathology suggestive of CTE. All cases had a separate neurodegenerative disease. Of the 21 cases of CTE pathology, 14 (66.7%) had Stage I or II pathology based on the staging system of McKee

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and colleagues [16], suggesting that if the pathology is progressive it had not progressed to a later stage in most people, in the setting of a primary neurodegenerative disease with extensive proteinopathy that began many years after athletic exposure (and possibly after the deposition of p-tau in a CTE-like distribution). Of the 7 with Stage I pathology, two died with ALS. Of the 5 who did not have ALS, 4 were over the age of 70 at the time of their death, suggesting that the CTE pathology had not or had minimally progressed over their adult lives. Moreover, if the CTE pathology represented a separate progressive neurodegenerative disease, it would be expected that former athletes with CTE, compared to those without CTE, would have earlier onset clinical features of disease and earlier death. However, there were no differences between groups in disease onset, disease duration, or age at death [38].

As previously discussed, Koga and colleagues [33] examined 139 cases of multiple system atrophy and identified CTE pathology in 6%. They classified two cases as Stage I, five as Stage II, and one as Stage III. In the Canadian series of 111 brains from a community autopsy service [39], only 4.5% had Stage I or Stage II CTE pathology, and there were no cases of Stage III or IV pathology. Nearly one in three subjects (30.6%), however, had a very small amount of p-tau which was conceptualized as less than Stage I pathology. In summary, there is emerging evidence from several research groups that CTE pathology might not be inexorably progressive.

ARE THE CLINICAL FEATURES KNOWN?

There are no agreed upon or validated clinical criteria for CTE, although proposed criteria have been published by Jordan [46], Victoroff [47], and Montenigro and colleagues [21]. In the past, based on studies in boxers with extensive neurotrauma exposure, slurred and dysarthric speech, gait problems, Parkinsonism, cognitive impairment, and dementia were considered clinical features [2, 48, 49]. Some descriptions of historical cases also included psychiatric illnesses, severe substance abuse, and other medical or neurological problems. Over the past decade, the clinical features attributed to CTE have been expanded greatly and include virtually any mental health or neurological symptom or problem present prior to death, such as 1) depression and anxiety [15, 16, 24]; 2) suicidality [16, 18–20, 22–24]; 3) personality changes, anger control problems, and violence [15, 16, 24]; 4) poor financial decisions, financial problems, and bankruptcy [15]; 5) marital problems, separation, and divorce [22]; 6) headaches [14–16]; 7) generalized body aches and pain [15]; 8) insomnia [22]; 9) Parkinsonism [11, 16, 24]; 10) mild cognitive impairment (MCI) [15, 16, 24]; 11) dementia [15, 16, 24]; and 12) motor neuron disease resembling ALS [43]. Although suicide is often reported to be a clinical feature of CTE in case studies and general review papers [13, 16, 18–20, 22–24], several reviews focused specifically on suicide [50–53],one retrospective historical case review study [54], and one epidemiological study [55] have concluded that there is minimal or no scientific evidence to support this assertion.

Many of the clinical symptoms that have been attributed to CTE pathology are common in the general population (e.g., depression, anxiety, anger, financial problems, marital problems, headaches, bodily pain, and insomnia). Of these, depression is often ascribed to CTE pathology, or that CTE pathology *causes* depression. Such an assertion should be considered a hypothesis to be tested, not an established clinicopathological correlation or causal association. The theoretical mechanisms by which heterogeneous psychological, functional, and biochemical disturbances underlying depression relate directly to the accumulation of insoluble post-translationally modified proteins have not been discussed or resolved in prior studies. Further, a correlation between CTE pathology and depression has not been established. Moreover, in psychiatry depression is conceptualized as heterogeneous, multifactorial in causation, and it is believed to arise from the cumulative effects [56–58] of genetics [59–62], adverse events in childhood [63–66], and ongoing life stressors [67–70]. In the general population, depression is associated with a wide range of health, mental health, and neurological conditions, such as 1) chronic pain [71–75]; 2) headaches and migraines [76–79]; 3) diabetes [80–82]; 4) low testosterone [83–85]; 5) cardiovascular, cerebrovascular, and small vessel ischemic disease [86–92]; 6) PD [93]; 7) MCI [94–96]; and 8) AD [97]. Therefore, former athletes, military veterans, and civilians who have neuropathology characteristic of CTE might experience depression for a broad range of reasons, similar to people in the general population who have no history of athletic participation or military service.

Clearly, people with psychiatric, neurological, and neurodegenerative diseases have the same symptoms

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and problems as those described in the CTE case studies. Moreover, if one assumes that CTE pathology clinically manifests decades after exposure (something that has not been demonstrated by prospective studies) the likelihood is maximized that any health, psychiatric, or neurological condition that affects a person's brain and behavior could be erroneously attributed in whole or part to the CTE pathology. Simply put, if a former athlete developed depression in association with life stress, marital problems, and chronic pain, that person could be incorrectly assumed to be showing the clinical features of a "progressive neurodegenerative disease," the diagnosis of which could further exacerbate this psychiatric condition. The neuropathology consensus group convened by the NIH noted that it is especially important to understand that it is not yet possible to correlate clinical symptoms or future brain health with the signature pathologic feature of CTE [30]. Research is needed to determine whether neurotrauma associated with athletic participation intrinsically causes localized and variable deposits of post-translationally modified and insoluble p-tau, and whether those protein deposits cause specific symptoms or syndromes, such as depression.

ARE THE CLINICAL FEATURES PROGRESSIVE?

In 1928, Martland reported that some cases remain mild and do not progress, and other cases progress to advanced Parkinsonism and dementia. Carroll, in 1936, described the punch-drunk syndrome as evolving during one's boxing career, progressing for a year or so after, and then becoming stationary. He noted that some boxers, however, deteriorated and needed to be institutionalized [98]. In contrast, in 1957 Critchley emphasized that the condition is gradually progressive [10]. Similarly, in 1963, Mawdsley and Ferguson [4] reported 10 cases of boxers, some of whom noticed the onset of neurological problems, such as slowing down and have slurred speech, while still actively fighting. Others noticed neurological deterioration years after they retired. These cases had a progressive course. In Roberts' large-scale study of more than 200 boxers in the 1960s, he reported that most with the syndrome had a static course, there were anecdotal cases of improvement after retirement from boxing, and a small subgroup appeared to have a progressive course greater than expected from aging [2]. Decades later, in 2000, Jordan reviewed the literature and reported that it was unclear whether clinical worsening in boxers reflected a progressive neurodegenerative disease, the aging process superimposed on a fixed neurological injury, or both [17]. At present, it remains inconclusive as to whether the neuropathology or the clinical features of CTE are inexorably progressive. It also remains inconclusive as to whether p-tau causes specific static or progressive clinical features.

LESSONS LEARNED FROM ALZHEIMER'S DISEASE

Studies over the past 20 years have supported the existence of pre-clinical burden of pathology in AD [99–101], and in 2011 a working group published a conceptual framework and operational research criteria for pre-clinical AD [102]. Nevertheless, after decades of research, both the etiology and the pathophysiologic sequence of AD remain uncertain [102]—and it is well established that the pathological burden of diverse lesions and proteinopathies can be substantial in people who are living independently and are clinically asymptomatic [103–107]. The inflection point or biochemical trigger that separates healthy aging from inexorable progressive neurodegeneration is unknown. Indeed, the conversion from MCI to AD is difficult to predict, and some patients with MCI improve [108]. Because there is evidence that the pathophysiology of AD likely begins many years before the emergence of clinical symptoms or a syndrome such as MCI or dementia, and because the correlation between hallmark lesions and the presence and severity of disease is modest, some in the research community have determined that it is important to disambiguate AD into AD-pathological processes and the clinical phases of the disease, which can be termed AD-clinical [102]. Similarly, it seems prudent to disambiguate the neuropathology ascribed to CTE from the diverse purported clinical features.

CONCLUSION

Between the late 1920s and 2009, the clinical features of CTE were described as mostly including gait disturbance, dysarthria, tremor, and cognitive impairment—including dementia. However, mild forms of neuropsychiatric clinical presentations have also been described. It has not been clear in the literature whether CTE is inexorably progressive. Many

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cases have been described as being static, not progressive. Moreover, it is now well-established that the pathology of CTE is present in people with other neurodegenerative diseases, such as AD, PD, and multiple system atrophy, so the progression of clinical features in those cases is expected as a direct result of those primary neurodegenerative diseases. There are now several studies illustrating that CTE neuropathology, at the time of death, is limited to Stage I or less in many people [33, 37, 39], suggesting that the kinetics of p-tau accumulation are limited—at least in some people. Further research is needed to determine whether some individuals exposed to repetitive neurotrauma from athletic participation suffer a unique progressive neurodegenerative process, rendered susceptible by the extent of exposure, genetic predisposition, or other factors. At present, there is an absence of scientific evidence to conclude that all or nearly all people with CTE pathology have a progressive neurodegenerative disease, and there is some evidence to support the theory that CTE pathology might not be inexorably progressive. It is not known whether the emergence, course, or severity of clinical symptoms can be predicted by 1) specific combinations of neuropathologies (e.g., p-tau, synaptic dysfunction, amyloid-β, and neuronal loss), 2) thresholds for accumulation of pathology, or 3) regional distributions of pathologies. In addition, factors relating to the resistance and resilience of the human brain to damaging effects of repetitive mild neurotrauma are not understood. When one considers, for example, the enormous neurotrauma exposure of boxers in the early part of the 20th century, and the fact that only 17% of those studied developed a diagnosed neurological syndrome, the resilience and plasticity of the human brain is likely remarkable. Therefore, researchers and clinicians are encouraged to be cautious when considering the clinical symptoms and psychosocial problems of former athletes, civilians, and military veterans, and to be mindful of potential iatrogenic effects of diagnosing a progressive neurodegenerative disease in someone with a psychiatric illness due mostly or entirely to other factors.

In conclusion, preliminary consensus-based neuropathological criteria for CTE were published in 2015 and research criteria for the clinical diagnosis of CTE have been proposed. It has been stated definitively in recent years that CTE is a delayed-onset progressive neurodegenerative disease, although minimal scientific evidence to support this theory is currently available. Some people with the neuropathology of CTE do not appear to have clinical signs or symptoms that are attributable to that pathology, and many do not appear to have a progressive tauopathy. To advance science in this area, it seems prudent to disambiguate the neuropathology of CTE from the possible clinical features.

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Tau Biology, Tauopathy, Traumatic Brain Injury, and Diagnostic Challenges

Rudy J. Castellani[a,*] and George Perry[b]
[a]*Departments of Pathology and Neuroscience, West Virginia University School of Medicine, Morgantown, WV, USA*
[b]*College of Sciences, University of Texas, San Antonio, San Antonio, TX, USA*

Abstract. There is considerable interest in the pathobiology of tau protein, given its potential role in neurodegenerative diseases and aging. Tau is an important microtubule associated protein, required for the assembly of tubulin into microtubules and maintaining structural integrity of axons. Tau has other diverse cellular functions involving signal transduction, cellular proliferation, developmental neurobiology, neuroplasticity, and synaptic activity. Alternative splicing results in tau isoforms with differing microtubule binding affinity, differing representation in pathological inclusions in certain disease states, and differing roles in developmental biology and homeostasis. Tau haplotypes confer differing susceptibility to neurodegeneration. Tau phosphorylation is a normal metabolic process, critical in controlling tau's binding to microtubules, and is ongoing within the brain at all times. Tau may be hyperphosphorylated, and may aggregate as detectable fibrillar deposits in tissues, in both aging and neurodegenerative disease. The hypothesis that p-tau is neurotoxic has prompted constructs related to isomers, low-n assembly intermediates or oligomers, and the "tau prion". Human postmortem studies have elucidated broad patterns of tauopathy, with tendencies for those patterns to differ as a function of disease phenotype. However, there is extensive overlap, not only between genuine neurodegenerative diseases, but also between aging and disease. Recent studies highlight uniqueness to pathological patterns, including a pattern attributed to repetitive head trauma, although clinical correlations have been elusive. The diagnostic process for tauopathies and neurodegenerative diseases in general is challenging in many respects, and may be particularly problematic for postmortem evaluation of former athletes and military service members.

Keywords: Dementia pugilistica, phosphorylated tau, repetitive head trauma, tau, tauopathy

SUMMARY OF PURPOSE

The primary purpose of this review is to highlight the complexity of tau in health and disease, and to point out the many uncertainties concerning its role in pathogenesis as well as diagnostic interpretation. It is intended to foster a more circumspect approach to molecular and clinical neuroscience with respect to tau biology, and the avoidance of premature conclusions with respect to: 1) the role of tau phosphorylation as a primary neurotoxic process; and 2) the relationship between tau pathology at autopsy and clinical problems that may have been present during life.

*Correspondence to: Rudy J. Castellani, MD, WVU Department of Pathology, PO Box 9203, Morgantown, WV 26506, USA. Tel.: +1 304 293 3212; Fax: +1 304 293 1627; E-mail: Rudolph.castellani@hsc.wvu.edu.

IDENTIFICATION OF TAU AND THE TAU GENE

Tau was initially identified by Weingarten et al. as a heat stable protein factor that would convert

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6S dimers of tubulin into 36S rings necessary for microtubule polymerization [1]. They named this factor tau (τ) for its ability to induce tubule formation. Phosphorylation of tau was found to promote a conformational change favoring depolymerization of the tubulin assembly [2–4]. Brion et al. first reported immunohistochemical evidence of tau in paired helical filaments (PHF) in 1985 [5]. Later the same year, Grundke-Iqbal et al. [6] reported that bovine tau preparations reacted with antibodies to Alzheimer PHF, and that affinity purified antibodies labeled neurofibrillary tangles (NFT) and dystrophic neurites, but not amyloid-β (Aβ) plaques. Neve et al. [7] subsequently used cDNA clones for tau and mapped the tau gene to 17q21.

The tau gene on chromosome 17q21.31 spans 16 exons of approximately 150 kilobases of genomic DNA. In human brain, alternative splicing of exons 2 and 3 results in three isoforms with either 0, 1, or 2 inserts of 29 amino acids (0N, 1N, 2N) [7–11] (Table 1). Each of the three isoforms may contain 3 repeats (3R) or 4 repeats (4R) of the microtubule binding domain encoded on exon 10, resulting is six isoforms. 1N, 0N, and 2N isoforms comprise 54%, 37%, and 9% of tau in human brain, while 3R and 4R tau species are expressed in roughly equal amounts among 0N, 1N, and 2N tau [12–14]. Expression of tau isoforms is developmentally regulated and tissue specific [9, 15, 16]. In the human fetus, only the shortest isoform (3R, 0N) is expressed, while the same isoform is downregulated in the adult brain. Tau phosphorylation is also developmentally regulated, being high until the end of synaptogenesis, compared to the adult human brain in which only 2–4 mole phosphate are attached per molecule of tau protein [17].

The developmental shift in isoforms roughly coincides with the formation of synapses [18]. The 3R, 0N isoform that predominates during development shows the least microtubule binding affinity, and switches to a relative increase in 4R tau species over time, suggesting pressure for greater microtubule binding affinity in the developed brain, and perhaps a role for the 3R tau species in neuroplasticity or in response to injury. Increased 3R tau during cellular stress, as well as the persistence of fetal tau in the adult brain [19], support this concept.

Table 1
Genetic heterogeneity of tau

Differential regulation of exons 2, 3, and 10 in development and disease (6 isoforms)
Regulated 3R and 4R tau with different microtubule binding affinities
Two haplotypes (H1 and H2) that confer disease susceptibility
Pathogenic mutation causes frontotemporal dementia phenotype

The regulation of tau binding appears to occur by alternative splicing and post-translational modifications. Tau also has a short reaction time with microtubules [20], which might explain why a protein in such abundance within the axon does not interfere with axonal transport. There is evidence that tau has two binding sites for microtubules. Microtubule binding repeats bind protofilaments at the taxol-binding site of beta-tubulin. The proline-rich region binds a protofilament anchoring the projection domain on the surface of the microtubule [21].

It is interesting that exon 10 is constitutively expressed in rodents [22], but is regulated in humans [8, 9]. This may in part underlie human susceptibility to tauopathy compared to rodents. The relative microtubule instability conferred by human 3R tau in response to cellular stress favors a depolymerized phosphorylated species, compared to rodents in which microtubule binding is maintained in a steady state because of constitutive expression of exon 10.

Faulty regulation of exon 10 splicing in humans, and the resulting imbalance of 3R and 4R tau expression, is suggested as a pathogenic basis for human tauopathy [12]. Excessive inclusion of exon 2 and exon 3 has also been reported in gliopathy and spinal cord degeneration [23], although it remains to be determined whether cell specific expression of exon 2 and exon 3 is the basis for this finding.

Two tau haplotypes, referred to as H1 and H2, occur because of a 900 kb inversion polymorphism [24–26]. The H1 haplotype and the H1/H1 genotype is suggested to be a risk factor for progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), argyrophilic grain disease, and idiopathic Parkinson's disease [24, 27–32]. The H2 haplotype is associated with increased expression of exon 3 in grey matter, suggesting that the inclusion of exon 3 might be protective against neurodegeneration [33]. The H1/H2 genotype confers a greater risk of developing dementia before the age of 45 years in individuals with Down's syndrome [32, 34].

Normal tau function

The primary function of tau within the brain appears to be the binding of tubulin to promote polymerization and stabilization of microtubules [1]

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(Table 2). Tau stabilizes and stiffens microtubules such that it supports the lengthy axon. Interactions with tubulin are dynamic processes with equal binding properties to both polymerized and non-polymerized tubulin, which regulates neurite polarity, axonal sprouting, and neuroplasticity, i.e., morphogenesis, and regulates axonal transport through interactions with motor proteins [35–41]. Microtubule binding confers a conformational change [3, 4], influences other diverse cellular processes [42–45], and interacts with other natively unfolded protein such as TDP-43, FUS, and alpha-synuclein [46–48]. A number of studies suggest alternative functions, including cell cycle regulation via tyrosine kinase, plasma membrane interaction, and synaptic function [42, 43, 46, 49].

Physiologic tau phosphorylation is therefore integral to life across species as a productive response to a variety of stressors including insulin dysfunction, glucose deprivation, starvation, hypothermia, hibernation, anesthesia, and glucocorticoids, among other conditions [50–57] (Table 3). Physiologic tau phosphorylation may also regulate subcellular localization of tau, which in turn may influence signaling cascades or synaptic function [58, 59]. A number of post-translational modifications apart from phosphorylation also occur which may have functional implications [58]. Among these are O-glycosylation, advanced glycation and the Maillard reaction, ubiquitination, nitration, SUMOylation, prolyl-isomerization, acetylation, and truncation [60–67].

Table 2
Some physiologic functions of tau

Stabilization of microtubules
Actin binding and cytoskeletal integrity
Regulating neurite polarity
Axonal sprouting
Neuroplasticity
Axonal transport
Cell cycle regulation
Plasma membrane interaction
Synaptic transmission (“synaptic brake”)

Table 3
Some stimuli for tau phosphorylation

Insulin dysfunction
Glucose deprivation
Starvation
Hibernation
Hypothermia
Anesthesia
Glucocorticoids
Opiates
Alcohol

Studies increasingly suggest a role for physiologic tau phosphorylation in synaptic function [59]. Tau is normally present at both pre- and post-synaptic sites [68], and accumulates as hyperphosphorylated tau at these sites in AD [69]. Whether tau diffuses across the synapse under normal conditions is an open question. Synaptic stimulation nevertheless induces site-specific, subsynaptic tau phosphorylation [70–72]. Tau mRNA has also been identified in axons and at subsynaptic sites, suggesting a role for local translation of tau in maintaining axonal integrity and synaptic function [73, 74]. Tau may also modulate signaling of synaptic neurotransmitter receptors, with post-synaptic tau phosphorylation acting as a “synaptic brake” via a complex and incompletely resolved mechanism. Glycogen synthase kinase 3 beta (GSK3β)-mediated tau phosphorylation, for example, may regulate neurotransmitter receptor endocytosis and negatively influence long term depression [59, 72].

The biology of hibernation is interesting in that tau protein transitions to a PHF-like phosphorylated state, involving epitopes typically related to tau phosphorylation in AD. Yet the phosphorylation state is completely reversible upon arousal from torpor and return to euthermic conditions [75]. This tends to suggest that tau phosphorylation in AD is a reactive phenomenon rather than a primary toxic process, and raises the issue of whether controlled hyperphosphorylation of tau confers cellular protection.

Tau has been shown to bind filamentous actin of dendritic spines as further evidence of its role in cytoskeletal integrity [76, 77]. Other studies have localized tau to the nucleus and the centrosome, in addition to the mitotic spindle microtubules of dividing cells [78–80], suggesting that tau phosphorylation might be involved in nucleus-cytoplasm translocation and cell cycle transition. Tau can also bind DNA, whereas tau phosphorylation may prevent DNA binding [81, 82]. Nucleolar organization and protection of genomic DNA is still another potential function [83, 84]. Tau is found in association with RNA as part of a ribonucleoproteome, complexing with RNA and a variety of proteins [48, 85, 86]. Finally, tau is also expressed in astrocytes and oligodendrocytes, the latter with all six isoforms, although with a lesser degree of microtubule binding [87–89]. Oligodendrocyte tau appears to be involved in microtubule stability during morphogenesis and myelination [90].

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Tau protein phosphorylation and hyperphosphorylation

Normal tau is a highly soluble natively unfolded protein [91–93], which contrasts with hyperphosphorylated p-tau in NFT which is highly insoluble [94]. The latter should be distinguished from physiologic tau phosphorylation, which is an ongoing dynamic process in the brain, and a necessary, tightly regulated process [75]. Phosphorylation regulates interactions involved in subcellular distribution and axonal transport [95, 96], organelle delivery to the somatodendritic compartment [97], neurotransmitter receptors [98], apolipoprotein E [99], Src kinases [49, 100, 101], and Pin1 [102]. Because of its high number of serine and threonine residues, tau protein is an excellent substrate for protein kinases, especially proline-directed kinases such as GSK3β [75]. Tau phosphorylation by cyclin dependent kinases and mitogen activated protein kinases [103–105], emphasize the role of tau metabolism in cellular division and proliferation. Non-proline directed kinases are also involved [106]. GSK3β and cdk5 may play a relatively more prominent role in tau phosphorylation in the human brain [75]. Interconnection of the kinase network, promiscuity of protein kinases, and the tendency of phosphorylation sites to cluster present technical challenges to the study of tau phosphorylation *in vivo*. The phosphorylation yield at any given site is low and can be difficult to assess. Site directed mutagenesis results in complex alterations in ionic properties, which limits the significance of experimental findings [75].

Numerous phosphatases dephosphorylate tau *in vitro* [43, 107], especially PP2A which is thought to also play a role *in vivo* [108]. Activity of tau protein phosphatases is further regulated by endogenous inhibitors, which themselves are subject to regulatory phosphorylation [75], emphasizing the complexity of tau phosphorylation.

The broad property of "hyperphosphorylation" is a hallmark of tau aggregates in AD, numerous other tauopathies, and aging [17, 109, 110]. Many phosphorylation sites occupied in PHF tau may be occupied in the normal brain [75]. In advanced disease, most of the approximately 39 potential AD phosphorylation sites [111, 112] are phosphorylated, with total phosphate content in p-tau pathological aggregates three times that of physiologic tau [17, 113]. One study in transgenic mice reports that pathological hyperphosphorylation is characterized by an increase in the proportion of phosphorylation at given residues, rather than an increase in the total number of phosphorylated residues [58], suggesting that tau "hyperphosphorylation" reflects an exaggerated physiologic phosphorylation, rather than disorganized phosphorylation at random sites receptive to phosphate groups. Still other studies suggest a role for molecular isomerism catalyzed by proline isomerase, with cis isomers of the Thr231 proline motif of p-tau variously labeling lesional brain tissue in AD and former professional athletes, as well as acutely traumatized murine neurons and axons in acute or recent trauma in humans [114, 115]. Trans isomers of p-tau are said to be "physiological" [114], although their specific role in the diversity of cellular tau functions is unclear.

It is noteworthy that antibodies used in p-tau analyses *in vitro* and *in vivo* react to highly selective epitopes, each with functional and pathological implications. The widely used monoclonal antibody AT8, for example, is used to identify tau phosphorylation at Ser 202, Thr 205, and Ser 208, which in turn identifies a wide spectrum of tau aggregates including the "pre-tangle" in autopsy brain [116]. Pretangle aggregates are not otherwise apparent using histologic dyes such as hematoxylin and eosin, or silver impregnation techniques such as Bielschowsky silver. For this reason, p-tau as identified by AT8 immunohistochemistry may lack any associated pathological alteration (such as a morphologically identifiable NFT). Pathology with a hypothesized link to repetitive traumatic brain injury (TBI) for example is often entirely immunohistochemical, with no tissue reaction that would otherwise suggest that an injury has taken place. This tends to raise questions about p-tau immunoreactivity as an indicator of cell death with repetitive TBI exposure. This may also explain the lack of eloquence regarding p-tau and clinical signs [117–120]. Phosphorylation at Thr 212 and Ser 214, identified in tissues by monoclonal antibody AT100, may be a better indicator of more advanced pathology [121], less sensitive than AT8 but more specific for pathological aggregates.

Decomposition and associated artifacts are synonymous with postmortem human brain analyses, and may be underappreciated. It is known, for example, that postmortem changes in the phosphorylation state is a dynamic process, with dephosphorylation of p-tau occurring rapidly postmortem, in a site-specific manner [122–128]. P-tau autopsy tissues may preferentially label buried epitopes, i.e., resistant to degradation. The patterns of immunoreactivity

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in the human brain may therefore be skewed toward postmortem artifact and away from solubility or *in vivo* biological relevance.

Hyperphosphorylation of tau may result from an imbalance in the activity of tau protein kinases and tau phosphatases, which in turn may be necessary for the formation of pathological fibrils. The conversion of physiologic tau to filamentous tau is believed to be a multi-step processes, with microtubule detachment as the initial step [129–131]. A number of biological mechanisms have been suggested [132–136]. Higher concentrations of tau may also influence conformational changes necessary for fibril formation [129]. Interestingly, 3R tau is said to facilitate twisted paired helical filaments such as those seen in classical AD NFT, while 4R tau has a tendency to assemble into straight filaments such as described in PSP [137]. Whether fibrillar or PHF tau signifies cytotoxicity, versus a productive response to the aging process or cellular stress, remains an open question [138]. Direct experiments verifying a feed-forward pathological cascade are sparse, with some studies showing no correlation between NFT accumulation and length of microtubules [139]. Still other studies demonstrating adduct formation (e.g., advanced glycation, advanced lipid peroxidation), and sequestration of redox active transition metals, may indicate that p-tau aggregation, up to and including PHF tau, is a productive response to cellular stress [140, 141].

Studies in recent years have increasingly implicated soluble, low-n tau assembly intermediates as the toxic or biologically active species [142–149]. The same concept is invoked for Aβ in AD [150]. This again suggests that the most readily identifiable postmortem lesions detected by immunohistochemistry may be the least biologically relevant. In one inducible transgenic model, progression of insoluble tau pathology was noted after suppression of mutated tau gene expression, during the process of functional recovery [144].

"Tauopathy"

The broad term "tauopathy" was first suggested in 1997 for a familial degenerative tauopathy [151] and is often used to connote diverse neurodegenerative diseases characterized by p-tau accumulations with various morphologies and clinical correlates (Table 4). To the extent that tauopathy implies the accumulation of p-tau as a rate-limiting factor for disease pathogenesis, the terminology may be unfortunate. A convincing case could be made that p-tau is a disease response, perhaps even a productive disease response [117, 138]. The term "tauopathy" may be subclassified into "primary" tauopathy, in which p-tau accumulation is the major pathological finding, or "secondary" tauopathy, in which some other protein deposit occurs (e.g., Aβ, prion protein) [75]. P-tau in sporadic primary tauopathies may not correlate with neuronal loss in some diseases [120]. Rigorously defined, true primary tauopathies may be limited to frontotemporal lobar degenerations associated with pathogenic mutations of the tau gene (MAPT) on chromosome 17 (FTDP-17) [152]. Like familial AD with APP mutations, the role of tau mutation in the molecular pathology is unclear. Some studies suggest that MAPT mutation causes chromosomal instability and aneuploidy [153, 154], rather than the elaboration of a toxic tau species *per se*.

Sporadic tauopathies are currently classified as frontotemporal lobar degeneration-tau (FTLD-tau), which encompasses Pick disease, PSP, and CBD [120]. Interestingly, MAPT remains the most substantial association by genome wide association analysis [155], and patients with MAPT tau mutation have clinical and pathological features that overlap with PSP and CBD [156, 157]. CBD and PSP clinical

Table 4
Diseases with tau neuropathology

Frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17)
Alzheimer's disease
Aging
Primary age-related tauopathy
Aging-related tau astrogliopathy
Progressive supranuclear palsy
Pick's disease
Argyrophilic grain disease
Corticobasal degeneration
Progressive subcortical gliosis
Amyotrophic lateral sclerosis/parkinsonism-dementia complex
Diffuse neurofibrillary tangles with calcification
Dementia pugilistica
Tangle-only dementia
Down syndrome
Gerstmann-Straussler-Scheinker disease
Hallervorden-Spatz disease
Creutzfeldt-Jakob disease
Globular glial tauopathy
Niemann-Pick disease type C
Prion protein cerebral amyloid angiopathy
Subacute sclerosing panencephalitis
Myotonic dystrophy
Non-guanamian motor neuron disease with neurofibrillary tangles
Postencephalitic parkinsonism
Meningioangiomatosis
Tuberous Sclerosis

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Table 5
Experimental and other limitations of the tau prion concept

Inefficiency of templating in culture
Methods for generating neurotoxic species not standardized
Fibril characteristics necessary for seeding are poorly defined
No consensus standards for tau seeding in culture
Relevance of mutant tau
Selective isoform expression experimentally versus nonselective expression *in vivo*
Tau expression promiscuity in transgenic animals
Tau leakiness
Axonal (as opposed to perikaryal) expression of tau
No natural tauopathy in rodents
Phenotypic propagation of neurodegeneration as a function of strain is not demonstrated
Does not explain selective vulnerability
Contradicted by early appearance of tau in structures with diffuse projections

phenotypes tend to contain lesions composed mainly of 4R tau, which supports dysfunctional microtubule binding as a factor in neurodegeneration. Pick disease phenotype (the least common of the sporadic FTLD-tau phenotypes), on the other hand, contains lesions comprised of 3R tau. Given the tendencies toward tau isoform specificity in FTLD-tau, it is tempting to suggest specific isoforms as therapeutic targets [158]. Such a construct would require p-tau as inherently toxic, however, which is not established.

The tau prion

Clavaguera et al. first demonstrated the elaboration of tau filaments following injection of wild-type mice with tau derived from P301S transgenes, raising the issue of protein-only transmission of phenotypic characteristics [159]. Relevance to human disease nevertheless requires the presumption that p-tau is neurotoxic in the human brain *in vivo*, which remains an open question. Conceptualizing the tau prion is challenging, and involves putative processes such as seeding, templating, spread, strain variation, transcellular propagation, trans-synaptic propagation, functional connectivity, selective vulnerability, and prion-like, each with a level of imprecision [160]. Pliability of definition is evident with terms such as "infectious prions", "non-infectious prions", "quasi-prions", and "transcellular prionoids" [161]. The tau prion nevertheless provides a framework for neurodegeneration based on non-mendelian, horizontal transmission of deleterious information, which is at issue in genuine prion disease. Protein-only transmission of phenotypic information in yeast is well-characterized [162]. By the prion analogy, p-tau would template or seed brain tissue, confer adverse biological properties on naïve tau molecules, and perpetuate an autocatalytic neurodegenerative cascade. Kaufman et al. provide evidence for seeding phenomena and strain variation in tauopathy [163, 164], although their relationship with neurodegeneration in progressive tauopathies is unclear.

There are some limitations of the tau prion concept (Table 5). In an early seminal study by Frost et al., extracellular tau at supraphysiological levels templated intracellular tau in less than 2% of the cells, while the conformationally templated p-tau in cell culture showed little, if any, resemblance to NFT [165]. Guo and Lee used recombinant 4-R tau [166], which has weak amyloid-like properties in human tauopathies compared to mixed 3R and 4R tau in AD. Sonication is often used to generate neurotoxic species for *in vitro* analyses, which is not standardized across laboratories. Characteristics of tau fibrils necessary for seeding experiments are poorly defined. Consensus standards for tau seeding in cell culture studies do not exist [160]. Many studies employ mutant tau, which is of doubtful relevance to sporadic disease since MAPT mutations do not occur in the overwhelming majority of human tauopathies. Most studies on tau propagation also utilize truncated tau [160], mutated or not, rather than full length tau. While reasonable in theory given C-terminally truncated tau in the synapse, it generally ignores the multiple isoforms in humans with variable splicing of the C-terminus and N-terminus. Many studies use recombinant tau rather than tau filaments derived from human disease, raising an issue of biological relevance. Propagation studies that rely on selective expression of specific isoforms do not take into account the fact that expression of 3R and 4R isoforms occurs in all human tauopathies, regardless of whether the predominant form in pathological lesions is 3R, 4R, or a mixture of both [160]. Transgenic constructs relying on conditional expression of tau [167] may have unaccounted for promiscuity

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(e.g., expression outside the entorhinal cortex), tau "leakiness," [160] or axonal tau mRNA and expression outside the cell body [73, 74].

Transgenic mice expressing, or overexpressing, a single isoform of wild-type human tau, do not develop tauopathy or neurodegeneration [160], while various other experimental constructs show tau expression without neurodegeneration [168, 169]. Tau prions have yet to be re-derived and re-injected with phenotypic changes in subsequent passages, separating tau prions from conventional prion disease. It may finally be pointed out that p-tau accumulates initially within neurons of the locus ceruleus, appearing as early as childhood [170]. Since the locus ceruleus is said to be "unsurpassed" among brain regions in the diffuseness of its connections [171, 172], trans-synaptic or transcellular neurodegeneration appears to be limited in the aging process *in vivo*.

Age-related p-tau

Recent analyses of primary age-related tauopathy (PART) [118] and aging-related tau astrogliopathy (ARTAG) [173] have expanded the spectrum age-related p-tau accumulation patterns. PART is, in essence, an accumulation of p-tau within medial temporal lobe and subcortical structures, with little or no Aβ deposition. Clinical symptoms range from no symptoms to mild symptoms involving the memory domain. Most cases previously referred to as "tangle only dementia" or "tangle-predominant senile dementia" are likely within the PART spectrum. It is noteworthy that classic studies of dementia pugilistica (DP) describe neurofibrillary degeneration in a similar distribution [174], which raises the possibility of coincidental p-tau pathology.

The related condition ARTAG, refers to p-tau accumulation within astrocytes, with a tendency for subpial, subependymal, and perivascular areas, and in subcortical white matter. Like PART, ARTAG has no predictable clinical substrate and overlaps substantially with pathology hypothesized as a substrate for repetitive neurotrauma [173]. PART is said to be primarily neuronal although tau astrogliopathy may co-exist with PART. Interestingly, PART is a mixed 3R/4R tauopathy whereas ARTAG is a 4R tauopathy, suggesting some degree of cell type specificity. This may in part explain why AD is a mixed 3R/4R tauopathy, while PSP and CBD, with an abundance of 4R tau, have prominent glial p-tau accumulation. A recent study of 687 postmortem brains from a spectrum tauopathies suggested variable distribution patterns of ARTAG, and differing pathogenesis possibly related to cerebrospinal fluid circulation or mechanical forces. The clinical significance of these patterns was not studied. The issue of spread among astrocytes was raised but remained speculative [175].

TBI and tauopathy in athletes and military service members

Athletes

The relationship between repetitive TBI and neurodegenerative tauopathy has been poorly understood for decades. It remains theoretical and is problematic for a number of reasons [176] (Table 6). The TBI component of the equation itself presents a significant challenge for study. Mathematical thresholds for parenchymal and vascular injury are impossible to quantify (reviewed in [177]). TBI repetition is largely undefined, and the role of TBI repetition of whatever extent on injury thresholds or putative tauopathy is unknown. Given myriad conditions associated with p-tau accumulation, as well as the numerous biological processes associated tau phosphorylation, it is

Table 6
Limitations of the TBI-progressive neurodegenerative tauopathy concept

Neurological signs attributed to early 20th century boxing were not progressive in most cases
Index case of DP at autopsy was most likely familial AD in a former boxer
Index case of putative DP-like disease in a football player depicted age-related changes [184]
Putative disease process is currently defined solely by immunohistochemistry (no clinical correlate required; no neurodegeneration (neuron or axon loss) required)
TBI in athletes is inferred from participation; otherwise undefined and impossible to quantitate
Athletes in modern case series were neurologically asymptomatic or had known neurodegenerative diseases in most cases
National Football League cohort has less cancer, fewer suicides, lower mortality, and better cardiovascular health compared to controls (no evidence of a pervasive, fatal disease related to occupational exposure)
Studies suggesting AD risk with mild TBI are inconsistent (no risk or modest risk)
AD is not confirmed pathologically in studies showing AD risk with moderate or severe TBI (dementia from structural brain injury in some cases not excluded)
No longitudinal data exists demonstrating TBI, latency, clinical neurodegeneration, and neurodegenerative pathology

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nevertheless expected that TBI at some level of severity may stimulate physiologic tau phosphorylation, and even that p-tau inclusions may appear over time following TBI in some instances, for reasons not fully elucidated.

TBI-neurodegenerative disease theory began with the investigation of boxers in the early part of the 20th century, some of whom demonstrated neurological signs and a putative condition known in boxing circles as "punch drunk." [178] Signs included dysarthria, gait disturbance, tremor, and cognitive impairment, as well as dementia in some cases (later termed 'dementia pugilistica' by Millspaugh [179]). It is important to note that neurotrauma exposure in boxers of this era was extreme [180, 181]. Many hundreds of fights over a lengthy career, with additional exposure in boxer booths and as sparring partners for elite fighters, were commonplace. Medical oversight was limited, with no mandatory exclusion times. Boxers often used light gloves. Little care was taken to match evenly weighted or evenly skilled boxers, and there was little inclination to stop fights short of incapacitating and often multiple TBI of a range of severities. The emergence of neurological manifestations of TBI in this setting is therefore not surprising.

Theoretical constructs suggesting a relationship between TBI and chronic disease have evolved considerably. Concussion-related hemorrhage was suggested by Martland as a pathological substrate for Punch Drunk, but was discarded by the 1940s. Martland included autopsy data of acute TBI in a non-boxer to support his theory, although there was no mention of NFT or other neurodegenerative inclusions. The case itself depicted gross features of diffuse axonal injury, suggesting conflation with severe, acute TBI. Later pathogenic theory by Millspaugh also emphasized acute traumatic injury with no mention of NFT [179], suggesting some difficulty with an explanation for chronic disease and disease progression. Interestingly, the first report of microscopic neuropathology by Brandenburg and Hallervorden in 1954 [182] indicated in retrospect a case of early-onset, and likely familial autosomal dominant AD, having nothing to do with boxing. The depiction of lesions that could aptly be regarded as "cotton wool" plaques, early-onset dementia, and extensive cerebral amyloid angiopathy suggest presenilin 1 mutation [183].

In 1973, Corsellis and colleagues [174] reported neuropathological features in 15 boxers from the early 20th century, most of whom had severe neurologic impairment. The findings included NFT, prominent in the medial temporal lobe and out of proportion to plaque pathology (distinguishing DP from AD), loss of pars compacta neurons of the substantia nigra, scarring of the cerebellar tonsils, and fenestrated cavum septum pellucidi. Thin fornices and atrophy of the mammillary bodies were common in this series. Vascular disease with infarcts and other co-morbidities such as contusions, neurosyphilis, and cavernous malformation were also present. The NFT assumed primary importance, however, eventually placing DP in the lexicon of tauopathies, and solidifying the notion of TBI-induced progressive degenerative tauopathy, perhaps prematurely. Careful examination of the clinical data and neuropathology from historical cases casts doubt on the concept of a progressive AD-like or PD-like neurodegenerative disease, or otherwise progressive degenerative tauopathy following a period of latency, even among early 20th century boxer with extreme levels of neurotrauma exposure [176].

The Corsellis et al. series was reported prior to the advent of immunohistochemistry (i.e., the concept of tauopathy), although case material from that series was relied upon for later immunohistochemical studies [185–188], likely because of reduced neurotrauma exposure in boxers and few new DP cases. Studies in recent years more often include asymptomatic boxers [189] (notwithstanding one death from acute TBI), or boxers who became symptomatic from other neurodegenerative diseases [190–193]. Boxing-related neuropathology has also become progressively more subtle, limited to immunohistochemical reactivity in some cases [194]. In essence, there has been a shift in case material from men with unambiguous neurological signs due to head trauma from boxing, to deceased men who happened to have boxed.

Attempts to link TBI to neurodegenerative tauopathy in non-boxers from 2005 forward follow a similar pattern. Subjects either lacked neurological signs attributable to TBI or had other neurodegenerative diseases. Diagnosis instead relies only on brain p-tau interpretation [195, 196]. Identification of p-tau in some cases requires whole brain screening with free-floating immunohistochemistry of 50 μm hemispheric sections obtained from a sledge microtome [198]. Given the ubiquity of p-tau with age [170], the high frequency of p-tau deposits in former American football players is not surprising [197], nor is the fact that p-tau patterns attributed to TBI are described in people with no history of neurotrauma [198–202]. Data from other athlete cohorts are sparse, but tend to be similar. Autopsy studies of p-tau in

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former soccer players, for example, describe p-tau in athletes with either no neurological signs [203], or neurological signs attributable to known neurodegenerative diseases [204–208].

In summary, available scientific evidence does not demonstrate a causal link between athletic participation and progressive neurodegenerative tauopathy in athletes, or for that matter a risk for genuine neurodegenerative disease [209], either from TBI inferred from athletic participation or athletic participation in general. Such a link would also be in conflict with epidemiological data demonstrating that NFL athletes in particular have better overall health (lower cancer rates, lower mortality, better cardiovascular health), and lower suicide rates [210], notwithstanding a modest increase in AD and amyotrophic lateral sclerosis (ALS) [211], which may be explained by the lower mortality. Indeed, the superior overall health of the NFL cohort, combined with the reported high frequency of focal p-tau immunoreactivity, indicate axiomatically that the focal p-tau as described has no clinical impact across the group as a whole.

Military service members

Military service members are vulnerable to TBI because of the nature of armed conflict and military training, and also because of the increased use of improvised explosive devices (IEDs) [212]. Most military service-related TBIs since 2006 have been associated with IED blasts [213]. Case reports and small case series have likewise described focal p-tau immunoreactivity patterns, hypothesized to be due to blast-related TBI sustained in the service [214, 215]. Reports have gone so far as to suggest that post-traumatic stress disorder may share common neurobiological underpinnings with neurodegenerative tauopathy [214–216].

Some studies have suggested that military service-related TBI is a risk for AD specifically. A study of World War II veterans suggested that AD risk was increased in subjects with a history of moderate or severe TBI in a dose dependent fashion, with moderate TBI conferring roughly two-fold risk, and severe TBI conferring a roughly four-fold risk [217]. The study did not find an AD risk with mild TBI, which is in line with one systematic review [218]. However, one recent, large-scale case-control study of US veterans concluded that mild TBI without loss of consciousness conferred a modest risk for dementia as well as AD specifically [219]. The risk was higher in mild TBI with loss of consciousness, and higher still with moderate or severe TBI, again suggesting a dose-dependent relationship between TBI and AD. A recent large cohort study of civilians in Denmark concluded that mild TBI conferred a modest risk for both dementia and AD [220].

Causal assertions from epidemiological studies remain hypothetical, however. The risks are overall modest as noted. The dose–response relationship between AD and TBI severity is also problematic in that severe TBI causes dementia and reduced life expectancy, while AD increases exponentially after middle age. Small relative risks in this setting may be due to misclassification of TBI-related dementia as AD in subset of cases. For example, Lewin et al. [221] studied 75 severely head-injured patients and found that patients often had dementia from TBI, with few surviving more than a decade. In another study, of 288,009 hospitalized survivors of TBI, 124,626 developed long-term disability including dementia [222]. Accurately assessing AD risk in this setting may therefore require pathological confirmation (generally not available in large scale epidemiological studies), since moderate and severe TBI often include structural brain damage [219] (e.g., contusion, laceration, diffuse axonal injury), which may in turn cause "dementia." To date, no longitudinal study demonstrating the sequence of TBI, a period of latency, clinical neurological deterioration, and autopsy-confirmed AD has been presented. More research is needed before the null hypothesis—that the reported dose-response relationship with service-related TBI and AD is a statistical artifact—can be rejected.

Blast-related TBI has emerged as a major cause of morbidity and mortality in military service. Blasts have been the most common cause of injury in American soldiers since 2006; of the ∼1 million veterans screened for TBI between 2007 and 2015, 8.4% reported TBI, the majority of which were mild and associated with blast [213]. Injury to the brain associated with blasts is heterogeneous [212]. Primary blast injury due to positive and negative pressure waves, secondary injury due to shrapnel, tertiary injury due to acceleration of the head and body across the war theater, and quaternary injury due any downstream pathology, including burns, lung injury, mass effect from brain swelling, ischemic brain injury, etc., are components of the blast injury complex. Neuropathological sequelae of primary blast injury are unclear, although early data suggest astroglial scarring at sites of differing tissue density (gray-white interface, periventricular tissue, perivascular areas, subpial areas) [223]. P-tau proteinopathy was

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inconsistent in this series, arguing against the hypothesis of blast-induced tauopathy.

Tau immunohistochemistry, TBI, and diagnostic challenges

Given the complexities of tau biology as well as the unproven concept that TBI causes neurodegenerative tauopathy, limitations in the diagnostic process, for which there is a paucity of literature, may not be fully appreciated (Table 8). Pathological assessment and tissue sampling typically involve a multitude of brain regions, the standards for which are variable and evolving. Antibodies in common use for immunohistochemistry react with only one of a large number of candidate epitopes, and may have differing reactivities as a function of epitope, time, and lesion type [224]. Human tissue is limited to postmortem brain, which is by definition partly decomposed and subjected to phosphatase activity as noted above. Epitopes that survive phosphatase activity are amplified by polymers [225], such that the tissue expression overestimates the true amount of p-tau. Cross reactivity is usually controlled for by omitting the primary antibody rather than by absorption with purified protein. The extent to which p-tau antibodies react with tau epitopes *per se* in any given case is, strictly speaking, unclear.

Neuropathological assessment by immunohistochemistry is therefore an entirely empirical exercise, permitting no conclusions about the nuances of tau pathobiology. The focus is instead on microscopic morphology [118, 173], which may be misleading as an indicator of disease or neurotoxicity (reviewed in [226]). P-tau immunohistochemistry also calls attention to selective vulnerability, for which there is no explanation. The questions of why, for example, the neurons of the cerebellar cortex are spared of p-tau even in end-stage neurodegenerative tauopathy, or why the neurons of the locus ceruleus or the basal nucleus of Meynert may accumulate p-tau early in life, separately or together, are unanswered. Factors responsible for AD variants such as limbic predominant AD and hippocampal sparing AD, are similarly elusive [160].

Table 7
Some p-tau microscopic lesions

Neurofibrillary tangle
Flame-shaped neurofibrillary tangle
Globus neurofibrillary tangle
Ghost tangle
Pre-tangle
Dystrophic neurite
Neuropil thread
Grain
Tufted astrocyte
Equivocal tufted astrocyte
Coiled body
Astrocytic plaque
Globular astroglial inclusion
Ramified astrocyte
Thorny astrocyte
Fuzzy astrocyte

Added to the diagnostic challenges are limitless morphological variations and patterns of immunoreactivity [75, 173, 227] (Table 7). The NFT, dating to the early 20th century [228], was the primary lesion of interest until the identification of tau as the major protein component of NFT and the advent of immunohistochemistry. Neuropil threads, dystrophic neurites, and a variety of morphologic p-tau presentations within neurons in AD and FTLD-tau were described subsequently [120, 229]. Various morphological subtypes of glial inclusion are reported in recent literature, including tufted astrocytes, oligodendroglial coiled bodies, astrocytic plaques, globular astroglial inclusions, ramified astrocytes, "equivocal tufted astrocytes," thorn-shaped astrocytes, and granular or fuzzy astrocytes,

Table 8
Challenges in addressing TBI-p-tau theory at autopsy

Poor correlation of p-tau accumulations with clinical signs
Frequent lack of detailed TBI history
Evolving standards for sampling, immunohistochemistry, and diagnosis
Subjectivity in interpreting p-tau accumulations and tissue architecture
Broadening spectrum of benign, age-related p-tau patterns
Lack of guidelines for assessing vascular disease, metabolic derangements, polypharmacy
Unknown error rate between and within neuropathologists
Variable clinical characterization of individual cases during life
Absence of genetic data
Broad public misunderstanding of TBI consequences driven by scientifically naïve media
Absence of patient consequences for misdiagnosis at autopsy
Vulnerability to *ipse dixit* interpretation

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each considered somewhat specific among the tauopathies [120, 227, 230–234]. The error rate in distinguishing among these descriptive morphologies, irrespective of clinical correlation, is entirely unknown.

Clinical correlations in neurodegenerative proteinopathy are also more limited than may be appreciated [235]. For example, AD proteinopathy cannot predict the level of cognitive dysfunction unless the pathology is end-stage [119]. Decedents with a substantial level of p-tau pathology in their medial temporal memory circuitry are often cognitively normal [118]. In the elderly, proteinopathy is virtually meaningless as a predictor of cognitive function by blinded analysis [236]. Proteinopathy cannot distinguish clinical Parkinson's disease from the clinical presentation of Lewy body dementia [237]. PSP p-tau pathology may be associated with CBD clinical manifestations, and vice versa. Both PSP and CBD p-tau pathology may occur in patients with the behavioral variant of frontotemporal dementia or primary progressive aphasia [238]. Patients with frontotemporal lobar degeneration may show signs of ALS, and patients with ALS may develop the spectrum of frontotemporal dementia phenotypes [238], none of which have been shown to correlate with proteinopathy burden with any degree of precision. The presence of neurodegeneration, i.e., neuronal/axonal degeneration, has an inconsistent relationship with p-tau pathology in CBD and PSP [120]. Clinical signs correlate more with neurodegeneration than proteinopathy [120], suggesting that proteinopathy may be epiphenomenal in some cases. This may explain substantial proteinopathy with intact cognition [118, 170, 173, 236], or the lack of a clinical correlate of p-tau pathology described in former athletes or military service members. There are also few guidelines for assessing metabolic derangements, numerous medications, and microvascular disease [239], which influence cognition independent of proteinopathy.

Some guidance is available in terms of consensus recommendations [118, 119, 173, 238, 240–242], although these tend to be provisional and subject to repeated modification. Because of the nature of consensus guidelines, i.e., the formal recognition that the science is unresolved, their application in neuropathology tends toward precision (consistency in pathological assessment), rather than accuracy in identifying clinical disease. This is reflected in consensus recommendations for AD, in which the preferred terminology is "Alzheimer's disease neuropathologic change," irrespective of clinical findings during life [119]. Similarly, consensus recommendations for frontotemporal lobar degeneration are concerned with patterns of neuropathology rather than clinical subtypes [242].

Added to the bewildering array of pathological lesions and clinical correlations, is the human element. The breadth of circumstances associated with prospective case material, and interpretation for the sake of diagnosis for interested families, may be considerable. Any given case may present with little or no clinical information, and variably rigorous clinical disease classification during life. The specialization of treating physicians may vary from general family practitioners to neurologists with specific expertise in dementia and movement disorders. Genetic data is often not available. Imaging studies may cloud the diagnostic process by suggesting some conditions over others based on variable image acquisition sequences and soft anatomical data.

The diagnostic process may be even more challenging in the arena of presumed repetitive neurotrauma. TBI history may be absent or incomplete, or inferred from a history of athletic participation or military service. Surviving next-of-kin may believe that recent onset of psychiatric signs is due to sport participation many decades prior. A family may be struggling with an inexplicable neurodegeneration in a family member. They may believe that concussion causes suicidal ideation or neurodegeneration because of scientifically naïve media reporting [243–245], or they may be unwilling or unable to accept that a family member took his or her own life. Families may demand that certain items appear or not appear on death certificates, or they may be interested in seeking damages from a third party, which may in turn lead to profligate tissue sampling and p-tau immunostaining in an attempt to confirm a desired diagnosis. The diagnostic process further takes place in the autopsy setting, in which misdiagnosis has no impact on the patient. These factors taken together may encourage *ipse dixit* interpretation, and present major challenges to objective and accurate disease classification.

Conclusions

The foundation for tau toxicity theory dates to Alzheimer's description of the NFT in 1906. It began in earnest with the identification of tau, a protein co-factor involved in the polymerization of tubulin, as a major protein component of

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the NFT. Subsequent pathogenic theory, including kinase-phosphatase imbalance, soluble assembly intermediates, and prion-like propagation, is rooted in the concept that pathological lesions represent neurotoxicity. The limiting factor for this neurotoxicity bias may be the light microscope. But for the visible microscopic inclusions, p-tau neurotoxicity theory and the extensive literature that now accompanies it, would not exist. The question nevertheless remains whether neurodegenerative inclusions embody a dynamic, primary etiopathogenesis, or instead downstream epiphenomena that distract from a more fundamental upstream biology.

Investigations from multiple perspectives including molecular, genetic, experimental, pathological, and clinical, indicate complex tau biology that bridges normal metabolic processes, neurodevelopment, healthy aging, and neurodegenerative disease. Normal tau, including tau phosphorylation, is necessary for development, cell cycle activity, synaptic function, and neuroplasticity. P-tau in postmortem brain may tend toward buried epitopes, insolubility, and limited biological meaning. Clinicopathological correlations with inert p-tau inclusions are fraught with imprecision. Neurodegeneration in the true sense, that is degeneration of neurons with an associated tissue reaction, is the better clinical correlate, while p-tau immunoreactivity in the absence of neurodegeneration and clinical signs may be extensive. P-tau neuropathology is ultimately a superficial indicator of tau pathophysiology, and may be misleading in terms of cause and effect.

Uncertainties in the repetitive TBI-tauopathy paradigm are considerable. TBI definitions are widely variable. Thresholds for mechanical tissue injury are impossible to quantify. Human data in athletes are limited to case reports and heterogeneous case series with little to no TBI history other than that inferred from participation. Brain tissue interpretation may require expensive, labor-intensive research methodology that has yet to be validated for diagnostic purposes. The concept of a decades' long period of latency between TBI exposure and neurodegenerative disease is often asserted, but has not been convincingly demonstrated, even in boxers. P-tau, especially p-tau identified in postmortem brain, is debatable as a driver of clinical disease, and is in part an artifact of postmortem decomposition. Importantly, epidemiological data indicate better overall health in the NFL athlete cohort, compared to the control population, casting doubt on the idea of a pervasive neurodegenerative disease from athletic participation. In former military service members, epidemiological studies of dementia or AD (and associated tauopathy) risk with mild TBI show modest risk or no risk, which essentially precludes causality. The dose-dependent risk of AD with TBI severity is based on large-scale epidemiology with no pathological confirmation of tauopathy. To these uncertainties and contrary data are added the tau prion concept, soluble low-n assembly intermediates, and geometric isomers, making for limitless theoretical possibilities and the potential for constructs more metaphysical than biological.

Finally, the diagnostic process with respect to tauopathy in postmortem brain is problematic. Clinical correlation is poor, standard methodology for postmortem brain examination is lacking, diagnostic error rates are unknown, and there may be external influences that degrade diagnostic accuracy. Such challenges, along with the consequence-free environment of postmortem diagnosis, may risk autopsy confirmation of individual preferences rather than genuine neurodegenerative disease.

DISCLOSURE STATEMENT

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Chronic Traumatic Encephalopathy and Neurodegeneration in Contact Sports and American Football

Scott L. Zuckerman[a,b,*], Benjamin L. Brett[a,c,d], Aaron Jeckell[a,e], Aaron M. Yengo-Kahn[a,b] and Gary S. Solomon[a,b]
[a]*Vanderbilt Sports Concussion Center, Vanderbilt University Medical Center, Nashville, TN, USA*
[b]*Department of Neurological Surgery, Vanderbilt University Medical Center, Nashville, TN, USA*
[c]*Department Neurology, Medical College of Wisconsin, Milwaukee, WI, USA*
[d]*Department of Neurosurgery, Medical College of Wisconsin, Milwaukee, WI, USA*
[e]*Department of Psychiatry, Vanderbilt University Medical Center, Nashville, TN, USA*

Abstract. Chronic traumatic encephalopathy (CTE) is a neurodegenerative disease characterized by the presence of abnormally phosphorylated tau protein in the depths of one or more cortical sulci. Controversy over the risk of CTE and neurologic disorders later in life among contact sport athletes has taken hold in the public spotlight, most notably in American football. Players, parents, coaches, and legislators have taken action based on the commonly held notion that contact sports invariably lead to neurodegenerative disorders. However, to fully understand the science behind this assumed association, a critical appraisal of the evidence is warranted. With regards to CTE in sports, the objectives of the current report are to: 1) describe the history of CTE, 2) review current CTE definitions, 3) critically evaluate the empiric data, divided into all contact sports and exclusively American football, and 4) summarize notable themes for future research.

Keywords: Chronic traumatic encephalopathy, concussion, football, neurodegenerative diseases, sports, traumatic brain injury

INTRODUCTION

Significant public attention has surrounded the topic of chronic traumatic encephalopathy (CTE) and neurodegenerative diseases in athletes exposed to concussive and sub-concussive impacts. Based largely on anecdotal case reports from boxers [1] and convenience samples of football players [2], it has quickly become accepted that repetitive neurotrauma suffered in sport has deleterious effects on long-term brain function. Legal actions against prominent sporting organizations have ensued, [3] and sweeping legislation affecting youth sports has been proposed [4]. Furthermore, widely disseminated and publicized accounts of CTE in prominent former NFL players have portrayed an assumed perception of inevitable later-life cognitive dysfunction in former football players [5]. It is possible that this bias is a result of the *availability cascade*, [6, 7]where dramatic, individual examples readily available to the public are believed to be the rule, in place of existing empirical data. The availability cascade [6, 7] may have contributed to the departure from a measured, scientific approach to a complex problem.

*Correspondence to: Scott L. Zuckerman, MD, MPH, Department of Neurological Surgery, Vanderbilt University Medical Center, Medical Center North T-4224, Nashville, TN 37212, USA. Tel.: +1 914 980 3339; Fax: +1 615 343 6948; E-mail: scott.zuckerman@vanderbilt.edu.

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Though knowledge of CTE has existed in some variant since the 1920s, modern accounts of CTE have brought forth more questions than answers. No accepted clinical criteria exist, and diagnosis can only be made postmortem. Whereas some neurodegenerative diseases have had rigorous clinical and pathologic diagnostic criteria for several years, [8] consensus neuropathologic guidelines for CTE have existed only since 2016.

Forty-three years prior to the consensus guidelines, Corsellis et al. [1] first proposed four neuropathologic criteria necessary for a CTE diagnosis, while the modern criterion relies on a single finding. In the last decade, the two different sets of pathologic criteria that preceded the consensus guidelines, provided by two major research groups, varied considerably [9]. Currently, the hallmark neuropathologic finding is abnormally phosphorylated tau protein (p-tau) accumulation in the depths of one or more cortical sulci, the only overlapping feature among prior proposed criteria.

Controversy over a 90-year-old disease has been reignited. Given the widespread public health implications for all those involved in contact sports, a thorough review of the existing evidence is warranted. The objective of the current report is to: 1) discuss the history of CTE in sports, 2) define previous and current guidelines of CTE, 3) critically review the literature on CTE in contact sports (divided into football and other contact sports), and 4) synthesize these findings to summarize notable themes and directions for future work.

METHODS

In the current narrative review of CTE in sports, studies were identified through an electronic search of the English Literature, using PubMED, Google Scholar, and the Cochrane Database. The following search terms were used: *chronic traumatic encephalopathy, CTE, neurodegenerative diseases, professional athletes, concussion, subconcussion, traumatic brain injury, and multiple sports.* Abstracts were screened and articles discussing CTE in athletes, spanning the high school to professional level, were reviewed. Any relevant references from the selected literature were searched to ensure a thorough capture. Only human studies published in English were included. Specific studies were selected for in depth discussion of methodological strengths and flaws.

The literature regarding CTE and neurodegenerative disorders in athletes as they relate to psychiatric diagnoses and repetitive head trauma is expansive. For the purposes of this narrative review, we aim to focus on CTE and similar neurodegenerative disorders. While brief mention will be made to associated diagnoses, exclusive discussion of depression [10, 11], suicide [12, 13], and repetitive brain trauma [14] will be limited.

BRIEF HISTORY

Since as far back as Hippocrates (460-370 BCE), humans have been aware of the potential harm of brain injury during sport [15]. The Muslim physician Rhazes (850–923 AD) was the first to use the term "concussion" in the modern sense, identifying it as a transient psychologic state distinct from other severe brain injuries [16]. Throughout the 17th to 19th centuries, various explanations for concussion prevailed, from "molecular vibration" [17] to "nerve cell shock" [18].

Research on concussion in the early 1900s was fueled in part by the severity of injuries incurred in early college football [19, 20]. After the death of a Harvard football player in 1906, team physicians Nichols and Smith (1906) published *The Physical Aspect of American Football*, [21] a paper that described the types of injuries sustained by members of the Harvard football team, among the most prominent of which was "cerebral concussion." Nichols and Smith noted that concussions were alarmingly prevalent and treated as a trivial injury, yet there remained the "possibility of serious after effects." The authors concluded that significant changes to the game would be required to improve player safety [21]. The American Medical Association (AMA) echoed these sentiments in a 1906 editorial, stating that, "Perhaps the most serious feature of these accidents is the number of concussions of the brain reported... At the present time no one is ready to say whether concussion of the brain may or may not have serious consequences in after life" [22].

In 1905, as a response to the growing worry regarding the casualties in amateur football, President Theodore Roosevelt met with leaders from Ivy League schools to identify ways to improve college football safety [23]. Changes were made that reduced the number of collisions, legitimized the forward pass, and ultimately made it easier for officials to

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intervene in the case of injury [24]. These meetings also established the Intercollegiate Athletic Association of the United States, which in 1910 became the National Collegiate Athletic Association (NCAA) [25]. Concurrent to these changes came efforts in the medical community to advance the understanding of concussion and its sequelae.

In 1924, Cassasa reported on five autopsies demonstrating "multiple traumatic cerebral hemorrhages" [26]. He correlated these injuries with concussion, and postulated that the mechanism was related to laceration of vessels due to changes in pressure of cerebrospinal fluid throughout the perivascular and perineuronal spaces of the brain [26]. In 1927, Osnato and Giliberti presented "Postconcussion Neurosis-Traumatic Encephalitis" and concluded that with advances in anatomic and pathologic understanding, an updated definition of the term "concussion" was overdue. Like Cassasa, they attributed the signs and symptoms seen in "traumatic encephalitis" to hemorrhages in the perivascular regions of the brain. They went on to state that, "Not only is there actual cerebral injury in cases of concussion, but in a few instances complete resolution does not occur, and there is a strong likelihood that secondary degenerative changes develop." They also advocated that individuals with long term effects from these injuries be labeled as having "traumatic encephalitis" [27].

The following year, Harrison Martland published "Punch Drunk" in the Journal of American Medical Association (JAMA), detailing the neurocognitive and neurobehavioral condition of 23 professional boxers. Martland attributed the "punch drunk" fighter to "traumatic multiple hemorrhages", asserting that, "one half of the fighters who have stayed in the game long enough develop this condition, either in a mild form or severe and progressive form which often necessitates commitment to an asylum" [28].

Bowman and Blau first coined the term Chronic Traumatic Encephalopathy (CTE) in 1940 after publishing their examination of a 28-year-old boxer who manifest multiple psychiatric symptoms including paranoia, depression, and "childish behavior" [29]. That individual was initially diagnosed with "Traumatic Encephalopathy" but after his condition did not improve over the course of 18 months the modifier "Chronic" was included, giving rise to the term used today.

Later in the 1940s, Denny-Brown and Russell [30] theorized that the deleterious effects of concussion occurred at the level of the individual neuron. In a 1962 publication of *The Lancet*, Sir Charles Symonds [31], suggested that, "the instantaneous loss of cerebral function . . . is due to sudden, direct damage, by stretching or compression, to the nerve cells or fibres of the brain."

In 1969, Roberts [32] published the first *in vivo*, clinical examination of traumatic encephalopathy in retired boxers. From an age-stratified random sample of 225 British boxers, he concluded that 17% had CTE, 11% with a mild form and 6% severe. He felt that the syndrome appeared stable over time, with a small subset demonstrating worsening of Parkinsonian symptoms.

In 1973, Corsellis et al. [1] published the first neuropathologic criteria for diagnosing CTE. In an article titled, "The Aftermath of Boxing", four hallmark neuropathological features were established by consensus after autopsy of 15 boxers. Roberts et al. [33] later re-examined these brains using improved immuno-histochemistry techniques and found that nearly all boxers had amyloid-β deposition consistent with what might be seen in Alzheimer's disease (AD).

In 1974, neurosurgeons Ommaya and Gennarelli [34] published their work on primates subjected to concussive forces and concluded that concussion was due to diffuse axonal injury. The next 20 years saw an increase in the neuropsychological influence on the field of concussion. Pioneered by Harvey Levin [35] and Jeff Barth [36], and refined by many others including Mark Lovell, neuropsychological testing provided an additional view into the impact and complexity of concussion. In 1984, Casson et al. [37] published a study of brain damage in boxers using EEG, head CT, and neuropsychological testing. Thirteen years later in 1997, Jordan et al. [38] published their findings on the interactive nature of the apolipoprotein Eε4 gene and boxing exposure and the later development of chronic traumatic brain injury.

Omalu and colleagues [39] published reports of CTE in former NFL players in 2005 [39], 2006 [40], and 2010 [41], and one former wrestler in 2010 [42]. In 2007, Boston University partnered with the Sports Legacy Institute (now known as the Concussion Legacy Foundation) to create the Boston University Center for the Study of Traumatic Encephalopathy (BU-CSTE), a group that has been on the frontlines of the current CTE debate [43]. Led by Dr. Ann McKee, the BU-CSTE published their first case series of CTE in 2009, [43] along with multiple subsequent CTE-related publications.

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DEFINITIONS OF CTE AND NEURODEGENERATIVE DISEASES

Since its inception in 1928, many different definitions of CTE have been published. Generally, CTE is characterized by p-tau accumulation in specific brain locations, and is associated with multiple neuropsychiatric signs and symptoms. Several authors have pointed out the complexity of diagnosing CTE, given the "classic" [1] versus "modern" [44] descriptions, lack of antemortem clinical criteria, and coexisting neurodegenerative diseases [45–48]. Prior to understanding the literature on CTE in sports, we must first define this clinical entity and similar neurodegenerative disorders.

What is a tauopathy?

Tau is a protein that fortifies microtubules in neurons and exists in all individuals. Tau is modified after translation in several ways such as glycosylation, nitration, and truncation, but one post-translation moderation important in neurodegeneration is hyperphosphorylation of tau. Hyperphosphorylated tau, known as p-tau, is associated with several disorders known as tauopathies, including AD, frontotemporal dementia, Parkinson's disease (PD), progressive supranuclear palsy (PSP), corticobasal degeneration, multiple system atrophy, and others [49]. While the finding of p-tau can be pathologic, it is also present in normal aging. Braak et al. [50] reported that only 10 of 2,332 consecutively autopsied brains (0.004%) were absent of any abnormal tau, and all were under the age of 30 years. The NINDS CTE Neuropathology Consensus group [44] identified p-tau in a specific region and pattern to diagnose CTE, and stated that the p-tau of CTE was different from the age-related tau astrogliopathy or primary age-related tauopathy, previously noted by Braak et al. [50] Overall, due to their relative rarity, these tauopathies are often grouped together as a single endpoint to predict the occurrence of neurodegenerative diseases. The overlap between these tauopathies is an active area of research, and describing discrete differences between each tauopathy remains elusive.

Classic CTE

A recent systematic review of CTE by Gardner et al. [45] proposed two distinct variants of CTE to be described: "classic" and "modern" CTE. This proposal came after the authors concluded distinct clinicopathological differences between the earlier accounts of CTE compared to CTE mentioned over the last two decades. Compared to "classic" CTE, "modern" CTE includes a wider range of clinical symptoms and only one, but more specific neuropathologic finding, mostly seen in former athletes.

While "classic" CTE encompasses almost 80 years of research, it can be boiled down to the work by Roberts et al. [32] in 1969 and Corsellis et al. [1] in 1973. As mentioned above, Roberts evaluated 250 retired boxers in the United Kingdom and reported that central nervous system disorders were present in 17% of cases. Though 37 cases were reported, clinical and pathologic details were included in only 11, and a limited description of each was provided.

Four years later, Corsellis et al. published the first neuropathologic criteria for diagnosing CTE based on autopsies in 15 boxers. These criteria included cerebral atrophy, enlarged lateral/third ventricles, thinning of the corpus collosum, a cavum septum pellucidum with fenestrations, cerebellar scarring, and agyrophilic neurofibrillary degeneration. The specimens used by Corsellis were later reexamined by Roberts et al. [33] who found that nearly all had amyloid-β deposition, suggesting the possibility of AD. Gardner and colleagues continued to note that "classic" CTE does not represent a progressive disease with worsening stages [45]. Moreover, the early descriptions of "classic" CTE were limited by a lack of control for confounding variables, including psychiatric illness, family history variables, substance abuse, or genetic risk factors, limiting the generalizability of their findings.

Early modern definitions of CTE

In 2009, the first contemporary CTE staging system was proposed by McKee and colleagues based on autopsies of 85 individuals with repetitive mild traumatic brain injury (mTBI), 68 (80%) of whom were found to have CTE [43]. Microscopic examinations were performed by a single, blinded neuropathologist—McKee herself—and confirmed by two additional Boston University (BU) colleagues, Dr. Thor D. Stein and Dr. Victor E. Alvarez. The preliminary CTE definition used before the study began included: 1) perivascular foci of p-tau immunoreactive neurofibrillary tangles (NFTs) and astrocytic tangles (ATs); 2) irregular cortical distribution of p-tau immunoreactive NFTs and ATs at the depth of cerebral sulci; 3) clusters of subpial and periventricular NFTs in the cerebral cortex, diencephalon,

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basal ganglia, and brainstem; and 4) NFTs in the cerebral cortex preferentially in the superficial layers. After the study results, McKee and colleagues proposed four neuropathologic CTE Stages I-IV. An attempt was also made to match clinical symptoms with neuropathologic findings, with corresponding Stages I-IV representing worsening symptoms.

In 2011, Omalu et al. [9] described a distinctly different set of CTE guidelines from McKee's, which included four phenotypes of CTE based on 17 autopsy findings—8 professional football players, 4 wrestlers, 1 mixed martial arts fighter, 3 high school football players, and 1 boxer. Unlike McKee's classification system, each phenotype was not meant to be an advanced form of the prior, and no clinical sign or symptom criteria were mentioned.

Current NINDS CTE neuropathology consensus guidelines

In 2013, the NIH and NFL funded an effort to define the pathological criteria of CTE and long-term outcomes of TBI, which in 2016 produced the first published consensus guidelines of CTE. At the outset, the primary objective was to determine whether CTE was a distinctive tauopathy that could be reliably distinguished from other tauopathies. A total of 25 cases of various tauopathies were selected by four neuropathologists not involved in grading: two from the BU group, one from the Mayo Clinic Jacksonville, and one from Mt. Sinai. A total of 7 neuropathologists experienced in neurodegenerative diseases participated in specimen examinations, including faculty from Washington University School of Medicine, Mayo Clinic Jacksonville, Brigham and Women's Hospital, University of Washington School of Medicine, Uniformed Services University of Health Sciences, Boston University, and Columbia University. The neuropathologists rated each specimen as *1-unsure, 2-possible, 3-probably,* or *4-definite* after gross inspection. After initial evaluations, evaluators were then sent gross findings and clinical summaries for each case, and asked to reevaluate the diagnosis and provide a second level of conviction.

The expert panel produced an agreed upon neuropathologic definition of CTE that included: "pathognomonic lesion consist of p-tau aggregates in neurons, astrocytes, and cell processes around small vessels in an irregular pattern at the depths of the cortical sulci." Several supportive neuropathological features of CTE were also included, along with mention of age-related p-tau astrogliopathy that may be present, which were non-diagnostic and non-supportive of a CTE diagnosis.

When assessing agreement for any tauopathy in the 25 cases presented at the consensus conference, the agreement level was 67%. When assessing agreement for a diagnosis of CTE specifically, the agreement level was 78%, meaning that 22% disagreed on a CTE diagnosis. Among non-CTE cases, more agreement was seen for AD, corticobasal degeneration, and primary age-related tauopathy, yet frequent discrepancies were seen with PSP and argyrophilic grain disease. When a clinical history and pathologic summary were provided, the degree of certainty rose from 3.1 to 3.7, meaning the clinical context made the evaluators more certain of the diagnosis. Comparing these inter-rater agreement values for CTE to AD, a 1997 study by Nagy et al. [51] evaluated a method to diagnose AD based on the sequential accumulation of NFTs in the cortex, without taking into account age, and reported a kappa of 0.90 among 41 brains. A similar study of AD by the same authors assessed the reliability of AD staging in thin paraffin sections and reported kappa values ranging from 0.6–0.8 for both the interrater and the intrarater reliability. Interestingly, a larger study concluded that absolute agreement was 91% when NFTs were substantial, but dropped to 50% in early stages of AD [52].

CTE IN OTHER CONTACT SPORT ATHLETES

Studies investigating CTE in non-football sports have been limited to single case reports or part of a larger series, often predominantly involving football [53]. The description of CTE in soccer, rugby, hockey, and other contact sports has been underrepresented yet is growing. Below, a review of non-football sport participation and long-term neuropathological, structural, and psychosocial outcomes is provided [45].

Soccer

The first identified case of cytoskeletal and pathological changes observed in a former amateur soccer player was presented by Geddes et al. [54], who identified pathological findings of neocortical NFTs and neuropil threads in the absence of amyloid-β protein. This finding occurred in 1999, prior to the consensus criteria established in 2016, thus the degree of accordance with the current pathological criteria (p-tau in sulcal depths) is unknown. Personal or medical histories were not provided for this case. Additionally,

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this athlete suffered a subdural hematoma resulting in death, which also influenced the neuropathologic findings and decreased the generalizability of the findings.

In the 2014 case series entitled "The Neuropathology of Sport," McKee and colleagues [55] reported a single case of CTE identified in a 29-year-old semi-professional soccer player without a history of CTE. The athlete was diagnosed with amyotrophic lateral sclerosis (ALS) three months after initially presenting with fatigue and lower extremity/hand weakness at the age of 27 and died of respiratory insufficiency 21 months after diagnosis. CTE stage II with motor neuron disease was identified postmortem, though many of the pathological findings were consistent with changes seen in ALS (degeneration of the anterior horn cells).

The largest, non-football study of CTE involved 6 retired, male soccer players with dementia who were followed between 1980 and 2010 [56]. Of these six cases selected based on an a-priori criteria of cognitive decline (inclusion criteria of dementia), CTE pathology according to the latest consensus criteria was confirmed in four patients [44]. Across the six cases, clinical presentation varied widely and included mood/anxiety, gait/motor, and behavioral changes, with memory impairment recorded as the most prevalent. Similar to many of the studies highlighted above, limitations were inherent in the means of clinical data collection, as the authors relied solely on retrospectively collected collateral history obtained through relatives and medical records. Furthermore, the criteria used to determine behavioral, mood, and cognitive changes were not well-defined or provided. While repetitive head impacts were highlighted as the basis for the observed neuropathology in the series (all were described as "skilled headers of the ball"), findings regarding history of head injury were mixed. Two athletes independently sustained head-to-head collisions during play that resulted in loss of consciousness, and CTE was found in one of these athletes but not the other. Additionally, no CTE was found in a player who also participated in amateur boxing. Interestingly, all six cases exhibited AD pathology and aspects of age-related tau astrogliopathy. Further parallel pathology observed across the six cases included TDP-43 ($n = 5/6$), cerebral amyloid angiopathy ($n = 5/6$), hippocampal sclerosis (N = 2/6), corticobasal degeneration (N = 1), dementia with Lewy bodies ($n = 1/6$), and vascular pathology ($n = 1/6$). No "pure CTE" was seen. The various concomitant neuropathology combined with less than ideal clinical history collection further cloud interpretation of these findings.

The studies of CTE in soccer are sparse, but more common are studies that assess the impact of heading on long-term neurologic function. Several studies have failed to demonstrate a significant relationship between frequency of heading the ball and adverse cognitive sequelae across a range of time and developmental periods [57–63]. While most systematic or meta-analytic reviews have concluded that no evidence currently exists to suggest a relationship between heading and cognitive impairment, [64–66] the studies that reported a positive relationship were older, taken at a time when the sport used a heavier leather ball prone to water absorption, and included athletes with a history of alcohol abuse [67–70]. A recent systematic review by Tarnutzer and colleagues [71] concluded that the majority of studies reporting persistent impairment from heading were less likely to control for Type I error and select an appropriate control sample when compared with studies showing no impairment. Studies demonstrating an association between heading and cognitive deficits were also more likely to have lower quality methods of assessing and quantifying heading (e.g., retrospective query) than studies reporting no such correlation.

Rugby

Similar to soccer, the association between CTE pathology and rugby has been limited to case series [53]. In the aforementioned study by McKee and colleagues [55], a 77-year-old former rugby player presented with stage IV CTE and dementia. The athlete began playing rugby at the age of 13 years and played for 19 years; level of play was not mentioned. Macroscopic changes associated with severe atrophy and widespread p-tau immunoreactive neurofibrillary pathology and neuronal loss in diffuse cortical and subcortical areas was reported, yet histological staining for other neuropathology was not included. The athlete first exhibited cognitive difficulties in his mid-50s, including memory loss, executive dysfunction, and attention difficulties. This was reportedly followed by depression and anxiety, as well as behavioral changes of explosivity and impulsivity that reportedly progressed to physical/verbal abuse and paranoia by his mid-60s. The method in which these data were collected was not reported, nor was information regarding premorbid mood and behavioral problems, prior head injuries, family history, and substance abuse.

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To date, a single pathologically confirmed *comprehensive* report of CTE in a former professional rugby player was provided by Stewart and colleagues [72]. The individual reportedly possessed a number of vascular risk factors, asthma, alcohol abuse, and "countless head injuries" as reported by family. No psychiatric history was provided. Onset of cognitive difficulties in attention, organization, and memory started at the age of 51 with positive neurological exam findings, including axial rigidity, asymmetrical upper limb rigidity, bradykinesia and ideomotor apraxia. Age-related white matter changes were noted on the MRI scan and a PET-scan revealed reduced accumulation of dopamine isotope in the caudate and putamen nuclei bilaterally, as well as evidence of frontal and posterior cingulate hypometabolism. Following death at the age of 57, widespread p-tau pathologies were recorded throughout the neocortex, hippocampus, and striatum. These findings were greatest in perivascular, subpial, and sulcal depth locations. An antemortem clinical diagnosis of PSP had been made, while postmortem pathological findings included classical Lewy bodies, PSP-like globose tangles, amyloid-related pathologies, and CTE pathology. Though the comorbidity of PSP does obscure the interpretation of clinicopathological interpretation, a strength of this case report is the inclusion of a comprehensive history and findings from clinical examination.

Studies of long-term outcomes in former rugby participation have been generally equivocal. In a retrospective study of 52 retired male Scottish international rugby players between the ages of 26 and 79 years (mean = 53.5) and 29 age-matched controls, McMillan et al. [73] failed to demonstrate significant differences in various health conditions, social or work functioning, and self-reported measures of mental health, which fell within the "normal range." The retired rugby athlete group did exhibit poorer performance on a metric of verbal learning (Rey Auditory Verbal Learning Test) and dominant hand fine motor dexterity; however, there was no correlation between frequency of concussion (mean = 13.9) and cognitive functioning.

A second cross-sectional study examined long-term outcomes in 259 former elite, male rugby players (mean age = 60.1 + 16.1), as compared to population-based standardized morbidity rates (SMR) based on multiple population-based health-related surveys [74]. Former elite rugby athletes who averaged 22 years of sport exhibited similar long-term rates of dementia and depression following retirement compared to population-based SMR. A fair criticism of the study, as acknowledged by the authors, was the significant difference in age of the population-based reference group (older on average). However, these outcomes remained the same when controlling for age. In the same study, former elite rugby players were two times more likely to experience anxiety. Interestingly, 85% of former rugby athletes did not endorse experiencing anxiety and depression on a separate health-related quality of life instrument (EQ-5D-5L). The authors of the study attributed these mixed results to the timeframe of symptoms solicited; the significant findings asked questions of lifetime prevalence, whereas the non-significant findings asked about current symptomatology. Regardless, SMR were significantly higher in former elite rugby players, which raises suspicion. As part of the study, there was no discussion of CTE or neurodegenerative diagnoses other than "dementia."

Regarding structural brain changes, Wojtowicz and colleagues [75] examined cortical and frontal thickness, as well as subcortical brain volumes in 24 relatively young (mean = 33.3 years-old), active, and retired professional rugby athletes, as compared to age- and education-matched controls. No between-group differences in psychiatric symptoms (depression and anxiety), cognitive functioning across a number of domains (with the exception of a single visual memory-related metric), or whole brain cortical and frontal lobe thickness were seen. Although rugby athletes did exhibit smaller bilateral hippocampi and left amygdala, follow-up analysis suggested that differences in subcortical nuclei volumes were likely attributable to alcohol use, especially in the rugby group.

Ice hockey

As part of a larger case series of individuals reportedly exposed to repetitive head mTBI [76], eight former hockey players (5 professional, 2 college/amateur, and 1 high school) were examined for CTE pathology. Of the eight, three were identified as exhibiting no CTE-related pathology and three were classified as possessing early disease stage pathology (Stage II). Of the two exhibiting more "progressed" pathology consistent with later stages of the disease (stage III and IV), both possessed multiple concomitant neuropathologies (e.g., diffuse amyloid-β, alpha synuclein, and neuritic amyloid-β plaques) and parallel neurodegenerative diagnoses (AD and Lewy

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body dementia (LBD)). Similar to the previously discussed soccer players, the concomitant pathology clouds interpretation of the relationship between hockey exposure and adverse outcomes. Furthermore, clinicopathological correlations (behavioral, cognitive, mood changes) of the former hockey players were not reported. This relationship between neuropathology and clinicopathological correlations is further confounded by the absence of CTE pathology during a recent autopsy of a 49-year-old retired professional hockey player who suffered from symptoms/conditions commonly said to be associated with CTE (i.e., self-reported memory loss, depression, and suicide) [77].

Investigations into long-term outcomes associated with participation in elite hockey have been limited. Esopenko et al. [78] examined cognitive and psychosocial functioning in 33 retired professional hockey players, with additional consideration of concussion exposure and genetic (apolipoprotein $\varepsilon 4$) influence. Results revealed no significant group differences across a wide array of cognitive functions (i.e., attention, verbal memory, visuospatial functioning, inhibitory control, and reaction time). Group differences were observed on select measures of psychosocial factors comprised of multiple psychiatric and behavioral inventories, executive function and visual reasoning, with a significant association between greater concussion history and lower cognitive performance on these measures. Increased psychosocial disturbances were associated with the *presence* of the apolipoprotein $\varepsilon 4$ allele. The degree to which differences were observed and further examination into how specific inventories differed was not performed.

Other limited-contact sports

The study of CTE in other common sports, such as baseball and basketball, is even more limited. A study of 66 individuals with sport exposure from a neurodegenerative disorder brain bank revealed the presence of Stage II CTE in a single former semi-pro baseball athlete. No information on baseball head impacts was provided [79]. AD-like (amyloid-β) pathology was also identified in this 87-year-old former semi-professional baseball. No antemortem cognitive or functional impairment was demonstrated at any point. CTE pathology was not observed in six other former baseball athletes (2 semi-professional, 1 college, and 3 high school level) with various other positive neuropathological findings in this sample. A single former high school basketball athlete was also identified in the sample as possessing stage I CTE, along with co-occurring ALS pathology. This is consistent with the established overlap of CTE and ALS pathology [75, 80, 81]. Concussion history for the sample was not reported and both the former baseball and basketball athletes had a positive history of reported alcohol use, of which the degree of consumption was unknown.

In conclusion, of the few larger series of case studies, CTE has been identified postmortem in a select number of non-football cases. CTE in non-football athletes was significantly obscured by confounding neurodegenerative disorders, the presence of neuropathology in athletes who were asymptomatic at death, and methodological flaws, such as not controlling for long-standing medical conditions and poor sampling/data recording techniques (e.g., remote retrospective query of behaviors and cognitive functioning rather than objective measures). Findings demonstrating adverse long-term cognitive, behavioral, and emotional outcomes in non-football sports have been mixed, with better-designed studies much less likely to demonstrate negative outcomes.

CTE IN AMERICAN FOOTBALL ATHLETES

Studies of CTE in football players

The earliest CTE study by the BU group was published in 2009 [43], where 5/51 (10%) of all CTE positive athletes were football players, and the remaining 46 were boxers. All 5 football players died in middle age (36–50 years) and played broadly similar positions (3 offensive linemen, 1 defensive lineman, 1 linebacker). Concussion information was obtained from next of kin and often included non-medically confirmed concussion history data. For example, one player's wife reported 8 NFL concussions, though only one was medically confirmed. Most common was a diagnosed mood disorder (depression), along with memory loss, paranoia, poor judgment, anger, irritability, apathy, confusion, and poor concentration. Cause of death was reported for 4 of 5 football players and included suicide (2), high-speed police chase resulting in motor vehicle collision, and accidental gunshot wound. The authors concluded that there was "overwhelming evidence" that CTE was the result of repeated sub-lethal brain trauma that often occurred well before the development of clinical manifestations [43]. The authors also

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mentioned that pathologically, CTE shared features with AD, and that the Aβ and NFTs found in CTE are immunocytochemically identical to those found in AD, suggesting a possible common pathogenesis [43].

Four years later, a second study from the BU group reported on brain autopsies of 85 individuals with repetitive mild head impacts. In this sample, 68 (80%) tested positive for CTE based on the presence of p-tau [76]. Of the 68 CTE positive players, 50 played football—35 professionally, 9 college, and 6 high school. The breakdown of CTE stage for the 35 football players included: 3 Stage I, 3 Stage II, 9 Stage III, and 7 Stage IV, with the remaining 11 having concomitant pathology and one player with no disease. Thirty-one of the 34 former professional football players had stage III–IV CTE or CTE plus co-morbid disease (89%). Positions played by NFL players positive for CTE included offensive linemen (26%), running backs (20%), defensive linemen (14%), linebackers (14%), quarterbacks (6%), defensive backs (6%), tight ends (6%), and wide receivers (6%). The authors concluded a positive correlation between years of football played and stage of CTE; yet number of concussions, years of education, and lifetime steroid use, all obtained secondarily from family, were not significantly associated with CTE stage. Moreover, none of the secondary analyses controlled for confounding variables, such as learning disorder, attention-deficit hyperactivity disorder, family history of neurocognitive disorders, or alcohol/substance abuse. Importantly, 17/50 had concomitant neurodegenerative diseases (AD, LBD, frontotemporal lobar degeneration, motor neurone disease, Pick's disease, and PSP). The authors concluded that repetitive TBI "might trigger molecular pathways that result in the overproduction and aggregation of other proteins prone to pathological accumulation in neurodegenerative disease such as those listed above," yet no justification was provided. The authors also cited literature showing that trauma was a risk factor for dementia, AD, ALS, and PD, yet these studies defined head injury as a hospitalized, closed head injury suffered during World War II, [82] lifetime head injury resulting in loss of consciousness and hospitalization, [83, 84] and one study assessed any traumatic incident, where only 7% (76/1131 participants) were concussions [85]. Perhaps most important, the study by Plassman et al. [82] of World War II veterans showed that mild TBI, irrespective of APOE ε4 allele status, was not a risk factor for dementia. Overall, these preliminary reports of CTE are exploratory in nature, and causal associations prove difficult to conclude given their nature as convenience samples, data collection methods, and comorbid pathologies.

In 2011, Omalu and colleagues [9] reported that 10 of 14 (71%) of professional male athletes (ages 18–52) were positive for CTE, including 7 of 8 professional football players and 1 of 3 high school football players. The single professional football player who tested negative for CTE was 24 years old. All three high school footballers died of acute brain and spine injuries suffered while playing. Importantly, the coexistence of severe TBI that resulted in these deaths has the potential to markedly affect pathological examination of these brains and diminish generalizability. No NFTs or neuropil threads (NTs) were identified in the 16- and 17-year-olds, but very sparse (1 to several) NFTs and NTs were seen in the cerebral cortex, subcortical nuclei/basal ganglia, hippocampus, and brainstem of the 18-year-old, which was interpreted as "incipient" CTE (abnormal tau protein). This individual had been playing football for approximately 6 years, but no other biopsychosocial or demographic information was provided. Though not separated into football players only, the authors noted that alcohol- and drug-related deaths were overrepresented in the CTE cohort, and AD-type atrophy was absent in all cases. The authors also cautioned against diagnosing CTE in the elderly (>65 years) to avoid confusing CTE changes with AD, normal aging, and chronic ischemic changes due to small vessel disease, in addition to the fact that amyloid may be found in cognitive normal elderly individuals [86]. Interestingly, the authors concluded by postulating four emerging and recurring histomorphologic CTE phenotypes based on the presence or absence of NFTs, NTs, and diffuse amyloid plaques in the cerebral cortex, subcortical nuclei/basal ganglia, hippocampus, and cerebellum, as well as the topographic distribution and predominance of NFTs, NTs, and amyloid plaques in those locations [9]. As stated above, this description of CTE differed from that proposed by McKee in 2013 [76] and the subsequent NINDS classification [44]. Omalu [9] did not find accumulation of tau-immunoreactive astrocytes, and the authors proposed that this difference may be due to difference in sport played (boxing versus football).

In 2013, Hazarati et al. [87] reported on a convenience sample of six retired Canadian Football League players, all of whom manifested progressive neurodegeneration prior to death. Three of 6 (50%) tested positive for CTE, while the remaining three

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were diagnosed with AD, ALS, and PD, respectively. The 3 CTE positive cases had comorbid pathology indicative of cancer, AD, and vascular disease. The authors concluded that the comorbid pathological findings may have contributed to the neurologic decline and that caution should be utilized in the diagnosis of CTE when other neurodegenerative diseases are present. The study by Hazrati and colleagues supports the notion that not all athletes with a history of repetitive head trauma developed CTE, and CTE alone is not responsible for any neurologic decline.

The largest study of football players and CTE was published in 2017 by Mez and colleagues [2] and was the most read article of JAMA in 2017 [88]. In a study of 202 former football players whose next of kin voluntarily donated their brains to the BU brain bank, 177 brains (87%) were positive for CTE, including 0/2 pre-high school, 3/14 high school (21%), 48/53 college (91%), 9/14 semi-professional (64%), 7/8 CFL (88%), and 110/111 NFL (99%) players. The diagnosis of CTE was divided into two principle categories, each made up of two stages: Mild CTE ($n = 44$), including Stage I (1 or 2 lesions) and Stage II (3 or more lesions). Severe CTE ($n = 133$) included Stage III ("multiple" lesions) and Stage IV ("densely distributed" lesions). The authors acknowledged their convenience sample of deceased football players, and postulated that it was unclear whether concussions or subconcussive impacts were responsible for the high proportion of CTE.

Several aspects of the study limit interpretation of the findings. First, ascertainment bias (families of players voluntarily donated their brains to comprise the study population) resulted in a biased sample that inaccurately estimates the prevalence of CTE. Quite accurately, the authors mentioned that 99% of this convenience sample of former NFL players had CTE. However, the conclusion was apparently misinterpreted by media outlets as, "99% of NFL players had CTE." Second, all NFL concussion and cognitive data were collected through family and next of kin on a postmortem basis. Recall bias has been shown to affect an individual athlete's ability to recall head injury information at the youth level, let alone in retired athletes [89]. Asking family members about the careers of retired players that took place 20–30 years prior may falsely represent specific facts about one's football playing career, as well as the onset, degree, and progression of clinical presentation/symptoms. Third, the study population from the BU brain bank was not representative of the American football population, limiting generalizability of the results. Fourth, substance use disorders were noted in 2/3 of the study population with mild or severe CTE. Not only can substance use cause neurologic injury and decline, but opiate abuse can also cause abnormal p-tau deposition, [90–92] and opiate abuse has been noted to be common among NFL retirees [93]. Fifth, whereas previous studies have utilized comparison groups with good control selection, no such comparison of all individuals exposed to football was present.

Sixth, and perhaps most important, the minimum threshold for CTE diagnosis consisted of a single p-tau lesion at the depths of a sulcus in the cerebral cortex, reportedly distinct from the lesions of aging-related tau astrogliopathy. Although this criterion is the solitary one postulated by the 2016 consensus criteria for a neuropathologic diagnosis of CTE, this low threshold maximizes sensitivity at the cost of specificity [94–96]. Whereas known neuropathologic diagnoses such as AD and LBD require many lesions to be present per a certain area, no such criteria exists for CTE. Moreover, in the Severe CTE group, accumulations of amyloid-β, a-synuclein, and TDP-43 were common, in addition to other comorbid neurodegenerative diseases, including AD, LBD, or frontotemporal dementia. It is possible that the p-tau seen were associated with these comorbid neuropathologies, and not CTE.

Reviews

Three systematic reviews on CTE in sports have been published; two exclusively on CTE in sports [45, 48, 97]. Though many excellent narrative reviews have been published, for the purposes of this chapter, only systematic reviews will be discussed.

In 2014, Gardner, Iverson, and McCrory summarized the 85 cases of CTE published to date, [9, 44, 76] drawing from 158 autopsy cases examined for CTE. First, the authors described the previously mentioned dichotomy of older, 'classic' description of CTE, which differed significantly from the 'modern' syndrome in the age of onset, natural history, clinical symptoms, and pathologic diagnostic criteria. The specific characteristics of modern CTE appeared to be the location of the neuropathology, in the gray matter and perivascular space at the depths of sulci, rather than the specific protein or lesion type [45]. Of the 85 cases of autopsies that have been performed in athletes over the past 10 years, 20% had 'pure' neuropathology consistent with CTE only, 52% had CTE plus other neuropathology,

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and 29% had no neuropathology or other neuropathology. The authors concluded that the strongly held causal assumptions relating to concussive and subconcussive brain impact exposure derived from the case studies were, "scientifically premature," especially given the absence of cross-sectional, epidemiological, prospective studies on the topic [45]. Furthermore, they summarized five important methodologic concerns when interpreting this literature: 1) the neuropathologic impact of drug/steroid abuse, alcohol abuse, psychiatric problems, cardiovascular/cerebrovascular disease is unknown; 2) the degree to which neuropathologic findings contribute to the clinical features witnessed; 3) genetic assessment of these individuals remains incomplete; 4) moderating and confounding variables between neuropathologic findings and neurologic symptoms are not known; and 5) a denominator of those at risk for CTE to accurately assess prevalence has not been adequately determined.

The second systematic review of CTE was conducted by Maroon and colleagues [97], which included a total of 153 neuropathologically confirmed cases of CTE in contact sports, 63 of which were football players. Of the 63 football players, substance abuse was seen in 9 players (14%). The authors concluded that the true incidence of CTE is unknown due to inconsistent definitions, a lack of longitudinal studies, and significant overlapping symptoms with common neurodegenerative disease. Furthermore, while McKee et al. [76] postulated that pathological findings in CTE were correlated with the numbers of years of football, three of seven high school football players in their review had CTE prior to the age of 20. It was also noted that a lower proportion of substance abuse was seen in football players compared to non-football players (14% versus 21%); however, the impact of substance abuse, namely opioid abuse in former NFL players, could not be determined as many studies did not assess these data points. The authors concluded that given the many limitations in the literature, conclusions regarding the prevalence of CTE among football and other contact sports cannot yet be determined.

Lastly, as part of the 2016 Concussion In Sport Group meeting in Berlin, Manley and colleagues [48] produced a comprehensive systematic review on potential long-term effects of SRC, of which CTE was discussed extensively. The authors correctly mentioned that prior to 2015, no agreed upon neuropathological criteria existed for identifying CTE, [44] and that the new consensus criteria excludes tauopathies associated with aging, such as age-related tauopathy [98] and age-related tau astrogliopathy [99]. Furthermore, tauopathy, amyloid-β, alpha-synuclein, and TDP-43 positive immunoreactivity occurs with normal aging, other neurodegenerative diseases, and in those with normal cognition [100]. After the exhaustive review, the international experts concluded, "A cause and effect relationship between CTE and concussions or exposure to contact sports has not been established," and additional case-control, cohort, and longitudinal studies are needed to understand the incidence, prevalence, extent to which the neuropathological findings cause specific clinical symptoms, the progressive nature of the disease, clinical diagnostic criteria, and other risk or protective factors.

Epidemiological studies

Though professional football players have been well studied, these elite athletes are not representative of the general population. Several epidemiologic investigations of high school football players and the subsequent development of neurodegenerative disorders have yielded important results, the most notable of which will be discussed here [79, 101–103].

In the largest population-based neuropathologic study with an outcome of pathologically confirmed CTE, Bieniek et al. [79] reported brain autopsy findings in 66 former athletes. In these former athletes, 21/66 (32%) had CTE, and only 1/66 (5%) had "pure" CTE, whereas the remaining 20 displayed mixed neuropathology with concomitant neurodegenerative diseases. Taken from the Mayo Clinic brain bank in Jacksonville, FL, inclusion criteria were presence of paraffin-embedded tissue, at least minimal medical documentation, and male sex, and subjects were excluded if they had a known neuropathologic diagnosis of PSP, corticobasal degeneration, Pick's disease, and a mutation in the microtubule associated protein tau gene (*MAPT*). Of note, cases of comorbid dementia, AD, or LBD were not excluded. Of the 1721 male remaining cases, 66 had a history of exposure to contact sports, and controls were men without documented exposure to contact sport. Within the contact sport exposure group, a total of 21/66 individuals (32%) tested positive for CTE, with 7 cases were classified as CTE Stage I, 7 CTE Stage II, 5 CTE Stage III, and 2 CTE Stage IV. Of the 43 patients with exposure to contact sports through football (football only and multiple sports), 16 (37%) had CTE pathology. Of these, 6 played

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up to high school level, 7 played to college, and 1 played professional football. In the 27 former football players without evidence of CTE pathology, 15 played up to the high school, 7 played up to college, and none played professional football. There were no significant differences in the proportion of football players with CTE pathology with respect to level of involvement (high school, college, professional; $p = 0.175$). On the 198 disease- and age-matched controls, no CTE pathology was seen, even in 33 controls with documented head trauma not related to contact spots. In terms of comorbid pathology, 20 of 21 (95%) confirmed CTE diagnoses met criteria for other neurodegenerative conditions. Overall, this study is important in that it serves as one of the few large scale, epidemiologic efforts to assess the prevalence of CTE. It should be noted that the rate of contact sports was exceedingly low, at 66/1721 (3.8%) male subjects, which raises concerns regarding the representativeness of the sample and results. A 2010 report concluded that more than 7.6 million students played high school sports, approximately 55.5% of all high school students [104]. Though the generalizability of these results is greater than the aforementioned studies of elite football players and athletes, the sample for the study was derived from brain bank predominantly dedicated to the collection of brains diagnosed with neurodegenerative disorders in the living, and in mostly Caucasians living in the Southeastern U.S. Furthermore, any descriptive assessment of head trauma or presentation/symptoms in the affected individuals could not be evaluated, creating ambiguity regarding the clinical meaningfulness of pathological findings.

In a study of high school football players from Rochester, MN from 1946–1956, Savica et al. [102] compared football players to a control group comprised of male students in the band, glee club, or choir. Using the records-linkage system of the Rochester Epidemiology Project from 2010-2011, the authors recorded later development of dementia, PD, or ALS and compared disease frequencies to the general population of Olmsted County, MN. The authors reported no increased risk of dementia, PD, or ALS among the 438 football players compared with the 140 non-football-playing male classmates. Compared to the general population, only PD was significantly increased; however, this was true for both groups, with a 2.4x greater risk for the football playing group and 5x greater for controls. Five years later, a follow-up study over a 40-year time period by Janssen et al. [101] of 296 high school football players compared to 90 swimmers, wrestlers, and basketball players from 1956–1970, assessing for the presence of dementia/mild cognitive impairment, ALS, and PD. The results indicated that varsity high school football players did not have an increased risk of neurodegenerative diseases compared with athletes engaged in other varsity sports.

The third long-term epidemiologic study estimated the association of playing high school football with cognitive impairment and depression at 65 years of age. After exclusions for missing data, Desphande et al. [103] studied 2,692 men, 834 (31%) of whom played football compared to 1,858 (69%) who did not. Two primary outcomes included a measure of cognitive impairment with a composite cognition measure of Letter Fluency and Delayed Word Recall. Depression was measured with the WLS-modified Center for Epidemiological Studies Depression Scale. After an extensive 1:1 matching process of baseline covariates, including adolescent IQ, family background, education level, and three different control conditions (all controls, non-collision sports, and no sports), playing football was not statistically associated with a reduced composite cognition score or increased depression score. Furthermore, after adjustment for multiple statistical tests, playing football did not have a significant adverse association with any of the secondary outcomes, such as the likelihood of heavy alcohol use at 65 years of age. Though other well-known neurodegenerative disorders were studied rather than CTE, which is understandable given its infancy and brain autopsy is the only means of diagnosis, these well done, prospective epidemiologic studies suggest no increased risk of neurodegenerative disorders or clinicopathological symptoms (e.g., cognitive impairment and depression) supposedly associated with CTE among high school football players compared to controls and the general population.

Additional studies

Though not specifically related to CTE or neurodegenerative disorders, four additional studies of American football players deserve mention [13, 105–107]. Baron studied 334 deceased former NFL players from 1959–1988 and concluded the rates of psychiatric illness and suicide were lower for former NFL players compared to the general population, and though the rate of neurodegenerative diseases was slightly higher than the general population (3.6% versus 2.9%), this was not a statistically

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significant difference. Using the same dataset of 334 deceased NFL players, Lehman et al. [106] found that overall player mortality compared to that of the US population was reduced, but neurodegenerative mortality was increased, including ALS and AD (NFL players were 3 times higher than that of the general US population). Two years later, the authors reported on 537 deceased NFL players and revealed that suicide among NFL players was significantly less than the general population (2.2% versus 4.8%), with no difference between speed and non-speed position players. Further, on the topic of suicide, Webner and Iverson [108] summarized 26 professional football players who committed suicide, with most deaths occurring in the last 15 years. The authors noted that most of the men suffered from multiple life stressors prior to their deaths, including retirement, loss of income, divorce, failed business ventures, family member estrangement, and substance abuse disorders, highlighting the multi-factorial nature of suicide in former NFL athletes.

Most recently in 2018, Venkataramani and colleagues [105] retrospectively studied mortality risk in 2,933 regular NFL players and 879 replacement players, who debuted between 1982–1992. A total of 144 NFL players (4.9%) and 37 replacement players (4.2%) died, and no statistically significant increase in mortality was associated with career players versus replacement players (HR 1.38, 95%CI, 0.95 to 1.99; $p = 0.09$). Neurodegenerative disorders were responsible for only 7% of deaths in the career players, which ranked 7th behind cardiometabolic (35%), transportation injuries (14%), unintentional injuries (10%), and others. Of note, all 7 deaths were due to ALS, and none due to CTE.

REMAINING QUESTIONS

How common is p-tau?

Recent focus has been directed toward the question of whether p-tau is unique to CTE. Multiple studies have previously demonstrated "CTE-like" p-tau pathology co-morbidly in a number of individuals without a history of head trauma, which include temporal lobe epilepsy [109, 110], ALS [111], the general population [112], and multiple systems atrophy [113]. Ultimately, this creates uncertainty around the specificity of the diagnostic consensus criteria put forth by the NINDS/NIBIB, which includes p-tau aggregates in neurons, astrocytes, and cell processes around small vessels in an irregular pattern at the depths of the cortical sulci [44]. Specificity of the criteria is called into question further based studies that have demonstrated head injury was not an independent predictor of p-tau pathology, when accounting for other comorbid pathologies or processes, such as ALS [80]. This is supported further by Noy et al. [112], who demonstrated that only a combination of head injury and alcohol and/or drug abuse, as well as age, were significant predictors of p-tau pathology in autopsies performed prospectively at a routine neuropathology service.

Lack of sociodemographic information

A crucial feature in studies on CTE to date is the paucity information regarding past psychiatric, socioeconomic, substance use, medical and family histories of the individuals examined. Whereas these demographic factors are routinely controlled in studies of SRC and TBI outcomes, they are largely missing due to the rarity and small samples of CTE studies. It has been empirically established that multiple factors have the potential to impact the development and presentation of CTE. Without rigorously controlling for such crucial biopsychosocial information, including age [114, 115], substance use [90, 93, 116, 117], psychiatric history [115], cardiovascular/cerebrovascular disease, or other health conditions, interpretation of any findings may be seriously compromised. In their systematic review, Maroon et al. [97] noted that very few studies took into account drug and substance use/abuse histories. Furthermore, study populations were homogenous and suffered from low numbers, making it difficult to draw generalizable information applicable to other populations. That said, some studies have made an effort to measure and control for confounding variables. In largest study of CTE to date, substance use disorders, suicidality, and family history of psychiatric illness were seen in 32 (67%), 22 (47%), and 23 (49%) cases, respectively [2]. Outside of this, few reports have considered demographics and comorbidities. Several authors have cautioned against over concluding without consideration of these important modifiers. Iverson and colleagues [46] contrasted CTE to AD, that when low levels of AD neuropathology are seen with major cognitive impairment, it is possible that other comorbid diseases may substantially contribute to the clinical decline.

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The importance of comorbid pathology

A common theme among the previously mentioned studies is the concomitant neuropathology that often accompanies a CTE diagnosis, including LBD and AD, among others. In some of the more notable studies, the following rates of comorbid pathology were found: 32 of 111 in Mez et al. [2], 20 of 21 by Bienek et al. [79], and 3 of 3 by Hazrati et al. [87] The review by Gardner and colleagues concluded that 20% of reports had "pure" CTE and 52% had CTE plus other neuropathology. Given the prevalence of comorbid diagnoses, what can we conclude about the high rate of comorbid pathology?

One interpretation is that the relationship between contact sport and neurodegeneration is strengthened by multiple neuropathologic entities seen together. A cascade of neuropathologic abnormalities has been noted, contributing to a neurologic decline and possibly premature death. However, an alternative explanation questions the existence and importance of CTE—is it possible that the p-tau in cortical sulci depths is just an artifact of normal aging and/or part of the other comorbid neuropathologic diseases, and not unique to CTE? The truth remains elusive, but several authors have weighed in. In 2011, Omalu et al. [9] acknowledged the coexistence of CTE and other pathologic findings, and recommended that, "these additional findings be enumerated as separate diagnoses accompanying CTE." The same authors go on to caution the diagnosis of CTE in those older than 65 years, "to avoid confusing CTE changes with AD pathology, normal age related, changes in the brain, and/or chronic ischemic changes/small vessel disease changes in the brain," and that NFTs and amyloid plaque, "may be found in low densities . . . in cognitively normal elderly individuals [9]. Hazrati et al. [87] and Gardner et al. [45] emphasized that the comorbid pathology may have contributed to the clinical signs and symptoms witnessed prior to death, and that differentiation of which pathologic diagnoses was responsible for clinical decline cannot be determined. As with most controversial aspects of CTE, research remains ongoing.

FUTURE DIRECTIONS CTE RESEARCH

Clearly, chronic traumatic encephalopathy has garnered significant scientific and media attention with the publication of numerous studies aiming to demonstrate or disprove a relationship between contact sports and neurodegeneration. Unfortunately, both the public and the scientific community are left unsure how to proceed. Questions regarding the acceptable age of contact sport participation and even whether football can remain a viable athletic venture have been raised. How can and how should the public and scientific community move forward?

In order to expand and enlighten our understanding of CTE, the challenge is reconciling these positive and negative studies while simultaneously separating from an emotional belief that has taken hold in the public's eye—the now seemingly well-accepted notion that repetitive mild head injury leads to chronic degenerative disease. Allowing oneself to make this leap bypasses the necessary initial questions of scientific research: What are the mechanisms of pathogenesis? What is the prevalence and frequency? Are there specific risk factors? Reconsidering CTE as a novel, underdeveloped neuropathologic entity presents opportunities to truly answer these questions, while viewing CTE as a well-defined entity induced by head injury in a causal manner stymies critical studies to better understand pathogenesis and risk profiles. Simply restating the question, "*Do contact sports lead to CTE?*" to the broader questions of, "*What is the prevalence of diffuse perivascular tauopathy in the general population? and What risk factors exist?*" eliminates the emotional, highly charged topic of head injury in sports. This question attempts to hone in on general aspects of the disease, which largely remain unknown as CTE research quickly moved past the early steps of disease study.

For both the public and especially those with the opportunity to add to the literature surrounding CTE, *moving forward* means taking every opportunity to think critically about one's own data and other published studies. Critical appraisal can prompt methodological improvement. For example, the majority of autopsy studies [2, 76] are cross-sectional in nature with few participants providing prospective data, while the few longitudinal/long-term follow-up studies [103, 118] do not seem to suggest any differences in in risk for neurodegeneration between contact and non-contact athletes. How do we reconcile this? Does a high proportion of positive autopsies in very well-defined symptomatic samples suggest a high overall prevalence? Critically assessing these studies by asking questions helps to define the critical attributes of an ideal study of CTE.

Based on a thorough critique of the literature, it seems that future studies of CTE should strive to include as many of the following components as

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possible. First, the study will prospectively collect extensive medical, family and social histories from participants. Additionally, prospective sign, symptom and head injury exposure data collection will improve accuracy and assist in delineating a more precise phenotype of CTE. Recall bias clouds any retrospective collection, especially with retired players, data collection from next of kin, and often in the context of litigation. Second, these data will be collected longitudinally over an extended period (decades), which will help establish the natural history and correlate signs and symptoms to neuropathologic findings if they exist following the methodology of Braak et al.'s landmark work with AD [119–122]. Finally, studies should aim for wide enrollment including a variation in degree of sport participation, head contact propensity and when applicable neuropsychiatric symptoms (from asymptomatic to severely symptomatic). Also of interest would be those with similar neuropsychiatric symptoms but without any history of head trauma. Keeping these key aspects in mind during study development and implementation will lead to studies that move the literature forward and answer key unanswered questions regarding CTE.

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The Neuropathological and Clinical Diagnostic Criteria of Chronic Traumatic Encephalopathy: A Critical Examination in Relation to Other Neurodegenerative Diseases

Benjamin L. Brett[b,c,*], Kristin Wilmoth[b], Peter Cummings[d], Gary S. Solomon[a,e], Michael A. McCrea[b,c] and Scott L. Zuckerman[a,e]
[a]*Vanderbilt Sports Concussion Center, Vanderbilt University Medical Center, Nashville, TN, USA*
[b]*Department of Neurology, Medical College of Wisconsin, Milwaukee, WI, USA*
[c]*Department of Neurosurgery, Medical College of Wisconsin, Milwaukee, WI, USA*
[d]*Department of Anatomy and Neurobiology, Boston University Medical Center, Boston, MA, USA*
[e]*Department of Neurological Surgery, Vanderbilt University Medical Center, Nashville, TN, USA*

Abstract. This work critically reviews chronic traumatic encephalopathy (CTE), with a specific focus on the single criterion necessary and sufficient for diagnosis. Herein, CTE is compared to other well-established neurodegenerative entities including Alzheimer's disease and dementia with Lewy bodies. Each neurodegenerative disorder is reviewed in five pertinent areas: 1) historical perspective, 2) guideline formation process, 3) clinical diagnostic criteria, 4) pathological diagnostic criteria, and 5) validation of previously described diagnostic criteria (e.g., sensitivity and specificity). These comparisons indicate that CTE is a disease in the earliest stages of formation and has yet to undergo rigorous development and refinement similar to other neurodegenerative diseases. Suggested future revisions to the diagnostic criterion of CTE include establishing a lower threshold for accumulation of pathology, as well as accounting for the presence of concomitant neuropathology and comorbid neurodegenerative disorders. Currently, while initial efforts have been attempted, agreed upon antemortem clinical criteria do not exist. As has been the scientific standard with similar neurodegenerative disorders, antemortem diagnostic guidelines should first be refined through subcommittees of neuroscientists from diverse institutional backgrounds with a subclassification of levels of diagnostic certainty (possible, probably, and definite). Validation studies should then assess the predictive value and accuracy of proposed antemortem diagnostic criteria in relation to potential pathological criteria.

Keywords: Chronic traumatic encephalopathy, concussion, football, neurodegenerative diseases, sports, traumatic brain injury

INTRODUCTION

The potential for long-term neurologic impairment and chronic traumatic encephalopathy (CTE) from contact sport participation has taken hold in the public narrative. The perceived risk of CTE in youth, high school, and collegiate athletes exposed to

*Correspondence to: Benjamin L. Brett, PhD, Departments of Neurosurgery & Neurology, Medical College of Wisconsin, 8701 West Watertown Plank Road, Milwaukee, WI 53226, USA. E-mail: bbrett@mcw.edu.

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concussive and sub-concussive impacts has resulted in policy changes at the local, state, and national level [1, 2]. Based on a small number of case reports and convenience samples [3, 4], it appears to have become widely accepted that repetitive neurotrauma of any kind suffered in sport or military activities has deleterious effects on long-term neurologic function.

Players, parents, coaches, educators, and healthcare providers represent key stakeholders impacted by this potential relationship, which has far-reaching public health implications. Experts have postulated that we may be prematurely assuming a causal relationship between sporting head impacts and neurodegeneration [5–7]. Popularized accounts of CTE in professional athletes [8, 9] may be responsible for the current public and scientific disconnect.

A more measured and scientific approach calls for a step back in order to better understand all that CTE represents. Relevant questions include: how long has CTE existed? How accurately can a diagnosis be made ante- and postmortem? How does CTE compare to other neurodegenerative disorders? Though first mentioned in 1928 by Martland, the 21st century version of CTE bears little resemblance to its original description. Recent reports have acknowledged a "classic" [3] versus "modern" form of CTE [10]. Currently, the only pathologic criterion required to diagnose CTE is accumulation of a single focus of abnormally phosphorylated tau protein (*p*-tau) in an irregular pattern at the depths of one or more cortical sulci. Moreover, few to no qualifiers in the current diagnostic criteria [10] are given for the fact that abnormal tau proteins are also found in other neurodegenerative processes or if CTE is seen in tandem with other neurodegenerative diseases [6, 7, 11].

Upon further examination, one finds that modern CTE is a young, complex, and often confounded disorder. A recent review by Iverson and colleagues [12] called for the separation of CTE neuropathology from the various clinical correlates often attributed to the disorder. The authors meticulously highlight that an extensive variety of cognitive, behavioral, and emotional sequelae are often attributed to CTE without observational relation to neuropathology, and narrowing of the clinical symptoms associated with its presentation is needed [13, 14]. Moreover, a recent study reported that the wide range of mood, behavioral, and cognitive symptoms were reported at similar rates among CTE-negative individuals (97.14%) compared with CTE-positive individuals (96.67%) [15].

To fully understand the science of CTE amidst the new wave of public health concern, a critical examination is warranted. The objective of the current review was to critically compare CTE to similar neurodegenerative diseases in the following areas: 1) historical diagnosis, 2) guideline formation, 3) current clinical and 4) pathologic diagnostic criteria, and 5) validation studies. By better understanding CTE among its peer neurodegenerative disorders, the public and scientific community can begin to contextualize the implications of CTE.

METHODS

To compare diagnostic criteria across multiple neurodegenerative disorders, a selective review of each disease entity was performed. Each neurodegenerative disorder selected for this review has well-established, consensus-driven diagnostic criteria that have undergone at least one revision. Secondly, while all of these diseases are neurodegenerative in nature, these diagnoses possess some diversity in epidemiology, neuropathology, laboratory/neuroimaging techniques involved in arriving at the diagnosis, and clinical presentation/symptomology, which allows for determination of common elements often utilized in development of diagnostic criteria. In addition to CTE, the two neurodegenerative disorders included for comparison were: Alzheimer's disease (AD) and dementia with Lewy bodies (DLB).

For each degenerative disorder, five distinct areas were reviewed:

1. Historical diagnosis – first characterization of the disease until formation of initial diagnostic guidelines, including early case reports and initial case series proposing the clinical entity.
2. Guideline formation process – description of how the first official diagnostic guidelines were proposed, including number of iterations, number of experts, types of specialties included, different institutions involved, number of subcommittees, and how committees were assembled.
3. Clinical diagnostic criteria – description of current clinical criteria needed to establish a diagnosis.
4. Pathologic diagnostic criteria – description of current pathologic findings needed to establish a diagnosis.
5. Validation studies – discussion of validation studies that have examined the efficacy of the current

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diagnostic guidelines (e.g., correlational studies, sensitivity and specificity, etc.).

In completing each of the five sections, selective reviews were performed. In the absence of a systematic review for each neuropathologic entity, the largest studies and those cited frequently as guideline formations were included. For validation studies, commonly cited efforts with large sample sizes were chosen. References of all current and former guidelines were searched for relevant articles that may have been missed.

RESULTS

Alzheimer's disease

Historical diagnosis

In 1906, Alois Alzheimer first described AD pathology, including neurofibrillary tangles (NFTs) and neuritic plaques, in the autopsy case report of Auguste Deter [16]. Interestingly, his index patient was in her early 50s and presented with primary psychiatric symptoms. The 1980s marked the initial establishment of a standardized approach to diagnosing AD. In the fall of 1983, 23 experts met as part of the first National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) panel [17]. The group's purpose was to establish clinical diagnostic criteria for AD. Their efforts yielded specific clinical criteria (published in 1984) that estimated probabilities of "possible", "probable", and "definite" AD. The number of cases studied to arrive at such proposed criteria was not mentioned. However, at this very early stage, the group responsibly noted that such criteria could not be fully operationalized due to insufficient knowledge about the disease. With the first AD clinical diagnostic criteria proposed, the experts regarded the criteria as tentative and subject to modification pending the emergence of empirical studies. In 1985, a group of 37 experts, convened by the same NINCDS and ADRDA aimed to further identify the most pressing areas for study, and to outline clinical and technical issues within research on AD diagnosis. The multi-disciplinary group was organized into 6 panels representing neurochemistry, neuropathology, neuroradiology, neurology, neuropsychology, and psychiatry [18].

That same year (1985), the original pathologic guidelines for diagnosing AD were published. Referred to as the *Khachaturian criteria*, these neuropathological guidelines were based on age-dependent numerical cutoffs of senile plaque counts and the density of plaques per field. In 1986, 24 institutes (and under the auspices of the National Institute of Aging [NIA]) prospectively followed 1,094 patients, collecting information such as clinical symptoms, neuropsychological symptoms, and neuropathologic findings. This approach set the standard for establishing a diagnosis of AD for many years to come. These criteria were subsequently challenged in the following decade due to a lack of clinical correlation with the Khachaturian criteria—postmortem studies show that some individuals with pathologic findings of AD often had no clinical manifestations of disease [19, 20]. Additional critiques of the Khachaturian criteria were that while based on the density of neuritic plaques, they did not consider NFTs, nor did they consider correlations with the varying stages of clinical dementia in AD patients.

Guideline formation process

Following publication of the Khachaturian criteria, findings from further clinical-pathological studies facilitated the criteria published by the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) in 1991. A group of neuropathologists from nine university medical centers were tasked with creating a neuropathological protocol consisting of an illustrated guidebook and data entry form to be linked with clinical information on demented and non-demented patients. From 15 CERAD centers, neuropathology data from 142 consecutive brain autopsies of patients clinically diagnosed as having probable AD, and from 8 subjects deemed as having no evidence of cognitive impairments (150 total), were analyzed and validated in the formation of a standardized protocol for the neuropathologic assessment of AD [21]. CERAD criteria were based on the semi-quantitation of neuritic plaque density in key topographic regions of the brain. The semi-quantitated plaque score, in consideration of patient age, would then permit estimation of the likelihood that a patient's antemortem dementia was in fact due to AD, versus other dementing illnesses. The CERAD effort revolved solely around pathologic diagnosis, no clinical correlation, and also had a poor balance of cases (142) to controls (8).

Recognizing the need for a comprehensive, unified clinical-pathologic criteria, Braak and Braak [22] published a combined staging system based on the distribution of NFTs in key topographic regions of the

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brain that demonstrated differential and hierarchical vulnerability to AD. This system permitted classification of AD into six stages, correlating to three groups of progressive symptomatic manifestation (discussed below). This Braak and Braak study examined 83 brains, which included patients with a clinical diagnosis of dementia, but whose neuropathologic exam did not show features of developed AD ("demented old-aged patients"), as well as patients with a clinical diagnosis of dementia who did demonstrate sufficient isocortical neurofibrillary changes to substantiate the clinical diagnosis of AD [22].

Subsequently in 1997, 17 experts from 16 different U.S. and European institutions met as part of the NIA and Reagan Institute Working Group. Following a two-day workshop, recommendations for the postmortem assessment of AD were published. Incorporating both CERAD and Braak and Braak criteria (described above), protocols were proposed that considered cases encountered in daily routine practice settings, as well as in AD research settings [23].

Clinical diagnostic criteria

The clinical diagnostic criteria for AD have traditionally revolved around the progressive dementia, namely memory loss and global cognitive decline. However, it has been increasingly recognized over the past three decades that symptomatology and neuropathologic findings exist on a heterogeneous spectrum and, while pure AD may be present, clinical and pathologic overlap with other neurodegenerative entities may occur [24]. In recent years, mild cognitive impairment (MCI) has been described as a form of prodromal AD, and it has been acknowledged that a better understanding of conversion from MCI to AD is needed. Conversely, AD neuropathologic changes in the brain may occur in the absence of clinical cognitive impairment and may possibly reflect AD early in clinical evolution [25].

Revision of the 1984 NINCDS-ADRDA clinical AD criteria occurred in 2009 when the NIA and Alzheimer's Association sponsored an international group from academia and industry. The guideline formation involved 4 separate committees tasked with formulating diagnostic criteria for both AD dementia and symptomatic predementia of AD, revised pathologic criteria for AD, and a research agenda to improve knowledge of asymptomatic preclinical AD. The process consisted of committee members meeting in person and via conference calls. Feedback gathered from public symposia held during the 2010 International Conference on Alzheimer's Disease were incorporated. A fifth subcommittee was tasked with reviewing biomarker recommendations and harmonizing discussion across workgroups in the fall 2010. The final, peer-reviewed documents were published in early 2011.

The revised NINCDS-ADRDA criteria expanded beyond memory loss to include a diagnosis of all-cause dementia, which required impaired abilities in at least two cognitive domains (memory, executive functioning, visuospatial abilities, language, and/or social comportment) established by clinical examination and confirmed by neuropsychological testing or other objective cognitive assessment. Deficits must impact social or occupational functioning, represent a decline from a prior level of neurobehavioral functioning, and symptoms cannot be due to delirium or psychiatric conditions. Probable AD criteria additionally include an insidious onset, worsening of cognitive symptoms, and impairment in a core cognitive domain (i.e., memory, language, visuospatial abilities, and executive functioning) and one other cognitive domain. Features of cerebrovascular disease, DLB, and frontotemporal dementia must be absent. Finally, clinical symptoms cannot be attributable to another medical condition or medication. The possible AD distinction is given when all other criteria for probable AD are met, but the course is atypical or there is an etiologically mixed presentation [26].

Biomarkers were omitted from clinical diagnostic guidelines but included for research purposes. Probable AD dementia with evidence of the AD pathophysiological process is applied when biomarkers or autopsy confirms AD pathology in an individual meeting clinical criteria for probable AD. Individuals with AD-positive biomarkers or autopsy confirmed AD pathology, but who meet criteria for a non-AD dementia are designated as possible AD dementia with evidence of the AD pathophysiological process [17].

Prior to the first consensus meeting in 1983 described above, the Diagnostic and Statistical Manual of the American Psychological Association (DSM) independently attempted to describing clinical criteria for AD in its third edition, published in 1980 [27]. The diagnosis of dementia was solely based on clinical presentation and required impairment in memory and 1 other cognitive domain. Currently in its fifth edition published in May of 2013 (DSM-5) and its third iteration of AD clinical diagnostic criteria, these revisions classify

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AD as a subtype of Major Neurocognitive Disorder [28]. The new criteria mirror those outlined by NINCDS-ADRDA for all-cause dementia; however, impairment in only one cognitive domain is required (complex attention, executive function, learning and memory, language, perceptual-motor, or social cognition). The DSM-5 defers description of subtype-specific cognitive, behavioral, and functional symptoms to the respective consensus group criteria.

Pathologic diagnostic criteria

The most recently established criteria and guidelines for diagnosing and reporting AD were published in 2012, approximately 27 years after publication of the original Khachaturian criteria. A total of 562 cases selected from autopsies collected in the National Alzheimer's Coordinating Center Uniform Data Set from 2005 to 2010 were stratified and analyzed on the basis of CERAD scores, Braak stage, and Clinical Dementia Rating Scale (CDR) by experts from the U.S. and Europe. These cases came from approximately 30 NIA-funded AD Centers across the U.S. Reflecting the collaboration of the NIA and the Alzheimer's Association, the standardized criteria proposed recognize pre-clinical stages of AD, and incorporate three key pathologic parameters into the AD assessment. Referred to as the "ABC" score, they reflect the amyloid-β (Aβ) plaque score based on Thal phases (A), the NFT score based on Braak and Braak stages (B), and the CERAD neuritic plaque score (C), with each parameter assessed on a scale of 0 to 3 and reported regardless of clinical history [29].

ABC scores are used to classify "None," "Low," "Intermediate," and "High" pathologic evidence of AD. None is designated in the absence of Aβ plaques (no Thal phase). When Aβ deposition is confined to the neocortical or allocortical regions (Thal phases 1-2) and neuritic plaques are infrequent (CERAD score of none to sparse), the Low classification is used. Low also captures Braak stages 0 to II. Intermediate designation is given to Braak stages III to VI, except for those with Thal phases 4-5 and CERAD scores of moderate to frequent, which are instead classified as High [29].

Validation studies

The above discussion illustrates how diagnostic criteria for AD evolved progressively over several decades, not only with accumulation of clinical pathologic correlative data, but also in applying and building upon the progressive foundation of previous working groups. This, in effect, forms the basis of continuous validation, which started in 1983 by a group of experts convened by the NINCDS and ADRDA. Throughout the last 35 years, with the collaboration of additional work groups and institutes, accrual and maturation of robust, generalizable patient registries led way to enhancement of diagnostic criteria, thus reflecting further validation and refinement of existing criteria and standards.

A synthesis review of several meta-analyses revealed that the original NINCDS-ADRDA clinical criteria confirmed against neuropathological AD diagnosis yielded variable sensitivity (76–93%) and specificity (55–91%) [30]. Using the updated NINCDS-ADRDA criteria described above, preliminary studies have revealed good diagnostic accuracy of 90% or greater [31]. Regarding efficacy of neuropathological criteria, evaluation by 10 AD Center neuropathologists revealed excellent agreement in ABC score classification (weighted $\kappa = 0.88$) [32].

Dementia with Lewy bodies

Historical diagnosis

DLB was first characterized by Fritz Jakob Heinrich Lewy in 1912 while studying Parkinson's disease (PD) neuropathology in Munich, Germany [33]. At this time, Lewy recorded cellular inclusions outside of the substantia nigra (dorsal motor nucleus of the vagus nerve, the nucleus basalis of Meynert, and some thalamic nuclei of PD patients) that were characteristic of PD [34]. The inclusions were first termed Lewy bodies (corps de Lewy) in 1919 by Konstantin Nikolaevich Tretiakoff, who postulated that nerve cell loss due to these inclusions may be associated with motor symptoms of tremor and rigidity [35]. The further connection between Lewy body (LB) pathology and current clinical features of DLB was presented by Okazaki and colleagues in 1961, who reported postmortem findings of diffuse LB-like pathology in two individuals with dementia [36]. One of these cases included a 69-year-old male, who exhibited gradual deterioration in mental status, along with visual hallucinations, and flexion rigidity. Postmortem examination revealed numerous LBs in the brain stem and diencephalon; however, what was particularly unique about this case was the cerebral cortical presence of intracytoplasmic, eosinophilic, and argentophilic inclusions (i.e., Lewy-like-bodies).

A thoroughly presented case study by Kosaka et al. [37] could be considered as the first report of DLB, listed as an "unclassifiable dementia" in an autopsy

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of a 65-year-old female with gradual progression of memory difficulties, rigidity, and psychomotor "restlessness." Kosaka and colleagues [38] then published a case series of three individuals demonstrating similar subcortical (basal ganglia) and cortical distribution of LBs, most especially in the anterior frontal, temporal, insular, and cingulate cortex. Two years later, Kosaka published a series of 20 individuals with similar neuropathological distributions where he introduced the term Lewy body disease [39] and proposed three main classifications based on distribution location: 1) brain stem type (now corresponding best with PD), 2) transitional type, and 3) diffuse type. Prior to the current day diagnostic term of DLB, the disease was known as "diffuse Lewy body disease" [40] and cerebral type of Lewy body disease [41].

The next era or major step in the formation of DLB as a distinct diagnostic entity occurred approximately a decade later with the formation of the first consensus guidelines for clinical and pathological diagnosis of DLB as part of the First International Workshop of the Consortium on Dementia with Lewy Bodies [42]. Given the heterogeneity of findings from case reports described above, the consortium set out to establish concrete diagnostic criteria for the clinical symptoms (antemortem) and postmortem identification of DLB. Additionally, the consortium established strict pathological criteria for a diagnosis of DLB, which included guidelines regarding: 1) the morphology, 2) sampling distribution, and 3) and frequency of LBs and a diagnostic rating protocol (also described below). Simultaneously with the formation of the first consortium, another significant event in the evolution of Lewy body disease was also taking place, as α-synuclein was identified as the hallmark of the disease by Spillantini and associates [43], who demonstrated similar staining of α-synuclein in LBs across idiopathic PD and DLB patients.

Guideline formation process

The initial collective attempt at establishing consensus diagnostic criteria for DLB first occurred in 1995 as part of the First International Workshop of the Consortium on DLB mentioned above. This consensus meeting attempted to improve upon and synthesize two prior efforts to operationalize criteria for DLB as a distinct clinical and neuropathological diagnostic entity [44, 45]. This earlier attempt involved retrospective reviews of 21 neuropathologically confirmed LB cases and 37 neuropathologically confirmed AD cases as a comparison group; however, this effort towards operationalization was limited by a lack of institutional and subject matter diversity. Attempting to expand upon this initial attempt, the first consensus meeting consisted of 26 subject matter experts across diverse disciplines (neurology, neuropathology, psychiatry, neuropsychology, neuroscience) from 21 different institutions. While the number of cases evaluated was not listed, the initial consensus criteria aimed to critically evaluate prior attempts to establish criteria, as well as peer-reviewed studies based on these criteria. Since the original consensus meeting in 1995, three additional meetings have taken place as part of the continual refinement and improvement of the DLB diagnostic criteria [46–48]. Review of all consensus criteria and changes with each subsequent iteration is beyond the scope of the current paper. The following content within the section will review the process in which the most recent guidelines were formulated.

The most recent consensus meeting was conducted in 2015 and organized by the Mayo School of Continuous Professional Development (MSCPD). Meeting support and co-sponsorship included organizations such as the Lewy Body Dementia Association, the Lewy Body Society, Alzheimer's Association, the National Institute on Aging, and the National Institute on Neurologic Disease and Stroke. Sixty-three invited multi-disciplinary subject matter experts from over 40 institutions/organizations and 10 countries attended the consortium and participated in one or more working groups in some capacity during the international DLB conference.

The process of guideline formation was completely transparent and involved four stages of development; this included 1) pre-conference scoping, 2) pre-conference working groups, 3) conference activity, and 4) post-conference activity. Pre-conference scoping involved two individuals from two different institutions and countries inviting a group of international experts to review the current literature around DLB, as well as determining the agenda for the DLB consortium meeting. Based on these pertinent topics, four pre-conference working groups consisting of international experts were established in order to review the current consensus guidelines. The four work groups, which met multiple times by email or teleconference, included areas of 1) clinical diagnosis, 2) clinical management and trial design, 3) pathology, genetics, biofluids and basic science and 4) global harmonization. In examining these four areas, work groups were instructed to adhere to a consistent review framework of 1) identifying elements in need of amendment, and those not needing to be changed;

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2) potential new topics for inclusion, and 3) identify anticipated future developments.

Guideline formation continued through conference activities, in which the four working groups conducted live review sessions. An educational program also took place in conjunction with the consensus meeting and guideline formation, and any individual attending that portion of the event was able to register and participate in the discussions of the four different working groups. Drafts of recommendations for each of the four live review sessions were constructed and led by chairs of working groups. Following the conference (post-conference activities), working group chairs circulated final reports among members for review and solicitation of feedback. Final drafts of working groups were submitted to the conference chair, who drafted an integrated consensus report. The draft of the integrated report was circulated to members for review and a final revised version was submitted for publication [48].

Clinical diagnostic criteria

As highlighted above, the consensus guidelines for the diagnosis and management of DLB have undergone three iterations and the fourth edition, most recently published in 2017, maintains several aspects of the previous versions with several modifications/additions [48]. According to the consensus, a diagnosis of DLB requires the presence of a "*progressive cognitive decline of sufficient magnitude to interfere with normal social or occupational functions, or with usual daily activities*" [48]. Levels of diagnostic certainty are delineated as probable and possible DLB, similar to AD. Specific combinations of core clinical features, supportive clinical features, indicative biomarkers, and supportive biomarkers can be used in determining a diagnosis at different levels of diagnostic certainty.

A diagnosis of probable DLB requires the presence of two or more core clinical features with or without the additional presence of indicative biomarkers. A diagnosis of probable DLB can also be made for instances in which there is the presence of a one core clinical feature and one or more indicative biomarker. According to the guidelines, probable DLB should not be diagnosed solely on the presence of biomarkers. A diagnosis of possible DLB can be made if only one core clinical feature is present with no indicative biomarker evidence. Possible DLB can also be diagnosed if one or more indicative biomarkers are present, but no core clinical features. Below, we further describe the core clinical features, indicative biomarkers, and supportive features.

Core clinical features

Four core clinical features of DLB were listed as: 1) fluctuating cognition with pronounced variations in attention and alertness, 2) recurrent visual hallucinations that are typically well formed, 3) REM sleep behavior disorder (RBD; may precede cognitive decline), and 4) one or more spontaneous cardinal features of parkinsonism (bradykinesia, rest tremor, or rigidity). Aspects of each of the core clinical features, as they relate to DLB, are reviewed in depth by the consensus guidelines. Additionally, likelihood of each symptom is provided based on prevalence studies (e.g., visual hallucinations observed in 80% of DLB patients and parkinsonian features in 85%). Suggestions for specific instruments/inventories and laboratory studies that can aid in the identification of core clinical features are also provided.

Indicative biomarkers

Three indicative biomarkers in the diagnosis of DLB which are provided include: 1) SPECT or PET demonstrating reduced dopamine active transporter (DAT) uptake in basal ganglia; 2) Low uptake on 123iodine-MIBG myocardial scintigraphy; 3) Polysomnographic confirmation of REM sleep without atonia. These biomarkers are classified as indicative, rather than supportive, due to their clinical utility and diagnostic sensitivity and specificity. For example, PET reduced DAT uptake in the basal ganglia on PET imagining has demonstrated effective sensitivity (78%) and specificity (90%) in differentiating DLB from AD [49]. Similarly, reduced uptake on ^{123}iodine-MIBG myocardial scintigraphy has also demonstrated clinically useful sensitivity (77%) and specificity in differentiating DLB from AD (94%) [50].

Supportive DLB features

Supportive clinical features and biomarkers are also provided by the guidelines in order to aid clinicians in diagnosing DLB. These indicators were not included as part of the required criteria, as the experts responsibly acknowledged the lack of diagnostic specificity [51–53]. Supportive clinical features that are commonly observed in DLB include 1) general sleep disturbance (hyper- and hyposomnia), 2) transient episodes of unresponsiveness, and 3) severe sensitivity to antipsychotic medication. Supportive biomarkers are also provided and

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include 1) relative preservation of medial temporal lobe structures on CT/MRI scan, 2) generalized low uptake on SPECT/PET perfusion/metabolism scan with reduced occipital activity accompanied by the cingulate island sign on FDG-PET imaging, and 3) prominent posterior slow-wave activity on EEG with periodic fluctuations in the pre-alpha/theta range. Additionally, clinical features that indicate decreased likelihood of DLB presence are discussed in the guidelines in order to assist with differential diagnosis. For example, when parkinsonism occurs at or after the onset of dementia, or is the sole clinical feature, DLB is regarded as less likely.

Pathologic diagnostic criteria

The First International Workshop of the Consortium on DLB in 1995 [42] provided a descriptive morphology of LBs, highlighting conventional characteristics and locations of the disease pathology. For example, the authors distinguish "classic" LB inclusions with a hyaline core and pale halo typically seen in the brain stem from cortical LBs, which typically involves less well-defined spherical inclusions seen in cortical neurons. Further, the guidelines provide direction for effective detection of LBs such as effective histological stains (i.e., brainstem LB is H-E and for cortical LB H-E and/or ubiquitin with tau immunostaining to differentiate cortical LBs from small tangles). Classification of co-occurring pathology (i.e., CERAD guidelines for AD and vascular pathology, especially as they relate to the presence of LBs) is also discussed. Since the initial guidelines, immunohistochemistry for α-synuclein has been a major advancement in this area and is now the preferred method of identification of LBs [43].

Comprehensive guidelines for brain sampling were put forth by the original guidelines, which included sampling of specific cortical sections at appropriate coronal levels (see McKeith et al. [42] for full description of sampling guidelines). The CERAD guidelines for brainstem sampling were adopted. The protocol for frequency scoring of LBs involves a numerical score ranging from 0 to 2 for each region sampled. Scores are assigned 0 for no observed LB count, 1 for up to 5 LBs, and 2 for greater than 5 LBs. Scores for each area are then summed based on distribution or density classification for five main cortical regions. These summed scores provide the basis for a severity grading system across three subtypes (neocortical, limbic, brainstem-predominant), which correspond with Kosaka's original subtypes [41].

Since the formation of the initial guidelines, subtypes of LB pathology have expanded to five classifications (diffuse neocortical, limbic [transitional], brainstem-predominant, amygdala-predominant, and olfactory bulb only) [48]. Additionally, the most recent guidelines provide a system of assessment, which aids in the attribution of dementia-related symptoms to LB pathology findings. Specifically, the system grades the likelihood that postmortem pathology findings are associated with DLB clinical observations as low, intermediate, and high. The five aforementioned LB subtypes are classified based on the location of recorded pathology and the presence of AD pathology based on the NIA-AA guidelines described above. These two factors determine the low, intermediate, and high classification of pathology as predictor of clinical presentation (probable DLB). For example, diffuse neocortical LB pathology in the presence of none/low AD pathology (Braak stage 0–II) would be classified as "high," essentially predicting a high likelihood of antemortem diagnosis of probable DLB, assumed to be associated with the observed pathology.

Validation studies

Given that the most current guidelines were published very recently, validation studies have yet to be performed. However, multiple validation studies for all prior DLB consensus criteria have been performed in order to continually refine and sharpen each iteration of diagnostic criteria. A recent systematic review and meta-analysis by Rizzo and colleagues [54] investigated the course of DLB criteria, examining changes in accuracy criteria with each subsequent consensus conference. Pooled sensitivity, specificity, and accuracy were calculated from 22 selected studies that consisted of 1,585 patients. Based on the systematic review [54], the first consensus criteria for possible DLB [42] yielded pooled sensitivity, specificity, and accuracy of 72.3%, 64.3%, and 66%, respectively. Pooled sensitivity, specificity, and accuracy for probable DLB were 48.6%, 88%, and 79.2%, respectively. The revised iteration of guidelines' criteria for possible DLB yielded pooled sensitivity, specificity, and accuracy of 91.3%, 66.7%, and 81.6%, respectively. Studies examining probable DLB criteria produced pooled sensitivity, specificity, and accuracy of 88.3%, 80.8%, and 90.7%, respectively. Based on the pooled diagnostic metrics, changes and refinements to the first consensus guidelines improved overall diagnostic accuracy of the second iteration.

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Chronic traumatic encephalopathy

Historical diagnosis

Many believe the first mention of long-term brain damage induced by contact sport was in 1928 by Harrison Martland who published "Punch Drunk" in the *Journal of American Medical Association*, detailing the neurocognitive and neurobehavioral condition of 23 professional boxers [55]. The term CTE was coined in 1940 by Bowman and Blau in the case report of a 28-year-old boxer [56]. Roberts et al. [57] in 1969 and Corsellis et al. [3] in 1973 provided the foundation for what is now referred to as "classic" CTE. From a list of 16,781 retired boxers in the UK, Roberts selected an age-stratified random sample of 250; he was able to locate and clinically examine 224 of these men, and found that 17% exhibited some form of neurologic dysfunction (11% with a mild form, and 6% with a severe form). Four years later, Corsellis et al. published the first gross, neuropathologic criteria for diagnosing CTE based on autopsies of 15 boxers, which included: cerebral atrophy, enlarged lateral/third ventricles, thinning of the corpus collosum, a cavum septum pellucidum with fenestrations, cerebellar scarring, and agyrophilic neurofibrillary degeneration. Roberts et al. [58] later examined the 15 specimens used by Corsellis and nearly all had amyloid beta deposition suggestive of AD, confounding aspects of the initial formulated diagnostic criteria.

After a nearly 30-year period, it was not until 2005 that a second wave of CTE studies came about, but for the first time in non-boxing athletes, subsequently dubbed "modern" CTE [6]. Omalu and colleagues [59] published case reports of former NFL players in 2005 [59], 2006 [60], and 2010 [61], and one former wrestler in 2010 [62] presenting with CTE; however, the pathological and clinical presentations were substantially different from the earlier accounts published by Roberts in 1969 and Corsellis and colleagues in 1973. As CTE in non-boxers became more acknowledged, the "modern" CTE entity was established. Though the "classic" and "modern" CTE entities are not formally recognized in any guidelines, further discussion is necessary to contextualize the history of modern CTE.

Dr. Ann McKee and colleagues of the Boston University Center for the Study of Traumatic Encephalopathy (BU-CSTE) group published their first case series of modern CTE in 2009 [63]. The BU-CSTE group proposed the first contemporary CTE staging system based on four primary criteria: 1) perivascular foci of *p*-tau immunoreactive NFTs and astrocytic tangles (ATs); 2) irregular cortical distribution of *p*-tau immunoreactive NFTs and ATs at the depth of cerebral sulci; 3) clusters of subpial and periventricular NFTs in the cerebral cortex, diencephalon, basal ganglia, and brainstem; and 4) NFTs in the cerebral cortex preferentially in the superficial layers [63]. Based on these criteria and autopsies of 51 individuals (convenience sample of athletes and civilians) with a history of mTBI, of which 80% possessed the criteria described above, McKee and colleagues described a progressive, 4-tiered neuropathologic staging system of CTE, and each stage had a corresponding clinical signs and symptoms description.

As the BU-CSTE diagnostic criteria was proposed, a dissimilar set of diagnostic criteria was proposed by Omalu et al. [64] based on autopsies of 17 contact sport athletes. Omalu et al. proposed four non-progressive, distinct neuropathologic phenotypes of CTE. Unlike McKee's classification, "stages" of disease progression were not described. Additionally, Omalu, did not include clinical presentation as part of his criteria, and did not propose that clinical signs and symptoms were associated with a particular phenotype.

Guideline formation process

The first consensus guidelines for CTE were first published in 2016 based on a National Institute of Health (NIH) consensus meeting aimed towards determining the nature of CTE and long-term TBI outcomes [10]. Central to this aim was to determine whether CTE was a distinctive tauopathy that could be reliably distinguished from other tauopathies. Four neuropathologists not involved in the examination and grading of subjects (two from the BU-CSTE group, one from Mt. Sinai, and one from the Mayo Clinic Jacksonville) selected 25 cases of various tauopathies, all of which originated from the BU-CSTE brain bank. Seven neuropathologists from seven different institutions (Washington University School of Medicine, Mayo Clinic Jacksonville, Brigham and Women's Hospital, University of Washington School of Medicine, Uniformed Services University of Health Sciences, Boston University, and Columbia University) rated each specimen as *1-unsure, 2-possible, 3-probably,* or *4-definite* after microscopic inspection. All cases (according to the staging criteria of BU-CSTE) were late-stage disease, with 8 being presumptive stage IV CTE and 2 being presumptive stage III CTE. No stage I or stage II cases were evaluated. When assessing agreement for

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any tauopathy in the 25 cases, which included 6 other tauopathy diagnoses (AD, argyophilic grain disease, corticobasal degeneration, Guamanian Parkinson's dementia complex, primary age-related tauopathy, and progressive supranuclear palsy), the agreement level was 67%, meaning that 33% of tauopathy experts disagreed on a tauopathy diagnosis. Even with examination of only late-stage cases—the most severe forms—agreement for CTE was 78%. A closer inspection of the online supplement of published consensus guidelines shows that all CTE cases were diagnosed with other disease "co-morbidities" by some respondents. Further, CTE was the sole diagnosis in only 27 out of 70 interpretations.

After initial evaluations, evaluators were then provided with the gross findings and clinical summaries for each case, and asked to revisit their initial diagnosis and provide a second level of diagnostic certainty. On the aforementioned 1–4 scale, the degree of diagnostic certainty rose from 3.1 to 3.7 when a clinical history and pathologic summary was provided. In contrast, greater agreement was observed among non-CTE cases. For example, there was 97.1% agreement among reviewers for AD cases.

This seminal publication represents the initial and only official clinical guideline formation process, involving samples taken from a single institution and evaluated by 7 neuropathologists [10]. Further, there has yet to be any follow-up multi-institutional meetings for refinement of the criterion based on new research since this initial consensus conference held in 2015 and published in 2016. Of additional concern is the lack of academic transparency or listing of any discussions that occurred during the process of guideline formation outside of the final publication. It is common practice for consensus papers to highlight discussions that occurred during the consensus meeting in narrative form, especially those in which there may have been disagreement among participants. Both consensus guidelines described above (AD and DLB) have adhered to that practice. For example, as part of the NIA-AA consensus meeting for AD, the paper highlighted that "a major point of discussion among committee members was the relative value of evaluation Aβ/amyloid plaque phase and neuritic plaque score in the assessment of AD neuropathological change;" and go on to discuss this in further detail [29]. The CTE consensus paper is solely a discussion of pathology, not consensus dialogue, and contains little content regarding the implications, or lack thereof, the diagnosis actually possesses. Without knowledge of these discussions, the reader is left uncertain regarding consensus members discussion around a potential lower pathological threshold, disagreements on staging, and the purpose of omitting stage I and II cases from the rating procedure.

Clinical diagnostic criteria

No consensus clinical diagnostic criteria currently exist for CTE. However, several authors have attempted to coalesce clinical and/or research diagnostic criteria based on earlier studies. In the first contemporary staging system published in 2013 [65], McKee and colleagues attached clinical symptoms to each one of their I–IV CTE Stages: Stage I: asymptomatic, mild short-term memory difficulties, depression, and mild aggression; Stage II: mood lability, explosivity, loss of attention, and depression; Stage III: visuospatial difficulties, memory loss, executive dysfunction, cognitive impairment, and apathy; and Stage IV: profound attention loss, language difficulties, paranoia, dysarthria, and parkinsonism. These clinical variants were based on anecdotal inferences from case studies rather than expert consensus or higher order statistical analyses.

In their 2011 study, Omalu and colleagues [64] outlined 9 syndromic profiles of CTE positive cases; however, no attempt was made to correlate symptoms with specific neuropathology due to the postmortem, retrospective nature of their study. The 9 syndromic domains were: deterioration in (1) social and (2) cognitive functioning, (3) mood and (4) behavioral disorders, (5) deterioration of interpersonal relationships, (6) criminal/violent tendencies, (7) alcohol/drug abuse, (8) religiosity, and (9) generalized head and body aches. No attempts to validate or replicate these syndromic profiles have been made since their proposal in 2011.

Based upon the work of Omalu, McKee, and review of available literature, Jordan [13] attempted to delineate between symptom types of CTE as either behavioral/psychiatric, motor, or cognitive in nature. Additionally, the same review provided a diagnostic classification system of improbable, possible, probable, and definitive based on numbers and clusters of symptoms. For example, 'probable CTE' consisted of any neurological process characterized by two or more of symptom types described above, which is also distinguishable from any known disease process (e.g., not consistent with PD). Considerable overlap between other neurogenerative disorders and this classification system exists. In fact, possible CTE is described as "any neurological process that is consistent with the clinical description of CTE, but can be

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potentially explained by other known neurological disorders." This classification has yet to be empirically examined or validated.

Another attempt was made to describe the clinical symptoms of CTE by Stern et al. [66], with 36 CTE positive male subjects without comorbid neurodegenerative or motor neuron disease. After retrospective reports from next-of-kin informants, two distinct clinical presentations were proposed: 1) younger age with behavioral/mood disturbances, and 2) older age with cognitive impairment. In addition to the methodological limitations of retrospective reports, these clinical presentations entailed interpretations and impressions from both next-of-kin and clinical evaluators. Additionally, only simple descriptive reports were provided, rather than utilizing rigorous statistical methods such as factor analyses or other data reduction methods that have been used elsewhere [67–69].

Further classification of clinical presentation for research, termed traumatic encephalopathy syndrome (TES) was attempted by Montenegro [14], based on a range of clinical symptoms associated with the disorder. This classification system was based on reviews of prior literature and not an independent sample of subjects. A diagnosis of TES required five core general criteria (e.g., history of multiple head impacts), one core clinically-determined cognitive, behavioral, or mood feature, and at least two supportive features (e.g., impulsivity, anxiety, headache). Montenegro also attempted to establish a sub-classification system, delineating TES into four variants: TES behavioral/mood, TES cognitive, TES mixed, and TES dementia. Another review was performed by Reams et al. [70], who modified the previous TES criteria. These modified criteria contained seven required features and three variant-based (emotional dysregulation, behavioral change, and mood disturbance) supportive features. Again, empirical investigation and validation of these criteria have yet to be performed.

In the largest and most recent report to date, Mez and colleagues [4] attempted to correlate clinical symptoms to pathologic disease stages I–IV by dichotomizing groups into mild (Stage I-II) and severe (Stage III-IV) CTE. Retrospective next-of-kin interviews were also employed to collect antemortem cognitive, behavioral, and mood symptoms. These were essentially descriptive reports with minimal pathologic correlation and no mention of sensitivity and specificity compared to controls. For example, the authors note in the discussion that "Participants with mild CTE pathology often had these symptoms [impulsivity, depressive symptoms, apathy, anxiety, explosivity, episodic memory symptoms, and attention and executive function symptoms] despite having relatively circumscribed cortical pathology and absence of p-tau pathology in the hippocampus, entorhinal cortex, or amygdala." Additionally, patients in both the mild and severe CTE groups exhibited the majority of reported symptoms at virtually the same rate.

Pathologic diagnostic criteria

Based on the established consensus guidelines, a diagnosis of CTE requires meeting only a single criterion: at least one perivascular p-tau lesion consisting of p-tau aggregates in neurons, astrocytes, and cell processes around small blood vessels found at the depths of cortical sulci. There are other supportive findings, such as p-tau pretangles and NFTs in superficial cortical layers (layers II/III) of the cerebral cortex; pretangles, NFTs, or extracellular tangles in CA2 and CA4 of the hippocampus; subpial p-tau astrocytes at the glial limitans; and dot-like p-tau neurites [4]. Furthermore, CTE stages of worsening severity are also reported. However, the only necessary and sufficient pathologic criterion required is a solitary perivascular p-tau lesion located in the depth of a sulcus.

Validation studies

There is currently an absence of validation studies for any of the staging systems or clinical phenotypes described above. Ideally, validation of any of these antemortem classification systems would involve prospective enrollment of former contact sport athletes of all playing levels (youth, high school, collegiate, professional) and controls (non-contact sports and/or non-athletes) in order to measure symptoms and changes over time and then utilize these recordings to attempt to predict postmortem neuropathological findings. Prospective studies of this nature could also be effective validation of the staging system or phenotypes presented in prior work. This would involve investigating the sensitivity and specificity of antemortem clinical criteria, or even characteristics in differentiating those exhibiting pathological findings, especially as compared to controls. With this, further refinement of the first iteration of pathological and proposed clinical criteria can occur. For example, DLB diagnostic criteria have demonstrated improvement in sensitivity and specificity with each iteration and refinement of consensus

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guidelines (see above). However, pooled specificity of the newest guidelines is still approximately 66% and continued improvement is likely to occur with further consensus efforts and refinements.

Prospective multi-institution studies of this nature would also allow for a more precise estimate of CTE prevalence. The generalizability of the current proposed clinical diagnostic criteria is extremely limited, as they are derived primarily from professional male athletes. The degree to which these apply to women, or those who played contact sports at lower than elite levels is unknown. Furthermore, while the reports continue to diagnosis and attribute proposed clinical symptoms of CTE across all populations (i.e., high school and collegiate athletes) [71, 72], the prevalence of and degree to which the pathological diagnostic and staging criteria apply to those below the age of 60 is particularly unclear, as none of the 10 cases that comprised the "CTE as submitted diagnosis" group used to develop the pathological criteria was younger than 60 [10]. Additionally, at this time, the single current diagnostic criterion is based upon 25 cases, only 3 of which did not contain AD pathology (Aβ). This raises concerns regarding the true prevalence of CTE, as that degree of sparseness of non AD-related cases is derived from an institution, which currently possesses the largest repository of donated CTE cases. Multi-center prospective enrollment would offer the opportunity to determine the true prevalence of the disease.

DISCUSSION

Although CTE has been described in various forms for approximately 90 years, its neuropathologic delineation remains in evolution, and the efforts at arriving at consensus agreement remain in infancy. To date, a single, preliminarily agreed upon consensus diagnostic criterion based on few selected late-stage cases of CTE is available, and this criterion awaits cross-validation and empirical extension. Without a universally accepted, independently established diagnostic staging system, clinical-pathologic correlations of CTE is likely to be fraught with error. Unlike AD or DLB, a diagnosis of CTE currently requires only a single microscopic focus of perivascular tau at the depth of a sulcus. At present, the presence of a solitary (or even multiple foci) of *p*-tau has been invoked to explain a wide range of neuropsychiatric symptoms ranging from headache to suicidality [4, 14, 65, 66].

Development of reliable pathological criteria levels

Under the currently proposed staging method, where there is no lower threshold for the diagnosis of CTE, brains with one, two, or three foci of p-tau accumulation are counted as CTE cases. This potentially leads to an overestimate of CTE case numbers, a circumstance where specificity is sacrificed for sensitivity. This problem was highlighted by a recent prospective case series of 111 brains published by Noy and colleagues [73], who demonstrated that only 4% of cases were positive for CTE. When the authors included 'tiny' amounts of pathology consistent with stage I CTE, the number of CTE-positive cases jumped to 30.6%, resulting in a sample prevalence of over one-third. The inclusion of these minimal cases in CTE populations clouds the ability to accurately detect signs and symptoms which may in fact be specific to CTE, thus leading to over-inclusion of symptoms that are common and nonspecific in psychiatric and neurodegenerative disorders. Current efforts are underway to identify other possible neuropathological features that may be more prevalent in CTE, such as astrocytic degeneration [74], which could provide more desirable neuropathological specificity of CTE. However, the current pathological criterion (≥single *p*-tau lesion at the depths of a sulcus) may be over inclusive and any attempts to develop diagnostic clinicopathological criteria in relation to neuropathology will likely be fruitless.

In other neurodegenerative diseases where tau is a prominent feature, cognitive impairment and behavioral changes are associated with the distribution of tau throughout the brain, which forms the basis of the Braak and Braak staging in AD. However, as stated above, staging approaches in AD take into account the significant diagnostic overlap with individuals who display no cognitive symptoms despite high loads Aβ plaques and tau-positive NFTs [25]. Unlike CTE, the autopsy diagnosis of AD is based on an age-adjusted numerical score with an age-related minimum number of lesions in key regions of the brain from the limbic system to the neocortex. A 4-grade scale counting classic AD lesions from none to frequent is then integrated into an age-related score where the diagnosis of AD is said to be possible, probable, or definite. This approach not only relies on autopsy findings, but the age of the patient and the clinical suspicion of AD.

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Development of reliable pathological criteria levels helps to minimize some of the subjectivity in disease diagnosis (i.e., interpretation of neuropathological findings). Postmortem diagnoses involve interpretation of neuropathological findings and not a quantified diagnostic test that definitively computes and classifies disease presence. These neuropathological interpretations can be subject to individual preferences/biases, as well as other factors [75]. Within the field of pathology, this is commonly referred to as "diagnostic review bias." As part of this bias, the pathologist is aware of a final result and may search more thoroughly for evidence, or be more likely to interpret borderline histological findings [76]. The subjectivity in the pathological interpretation of the presence of a disease was observed in the NINDS/NIBIB consensus meeting, as at least one expert on the panel diagnosed CTE in 8 of the non-CTE cases. In contrast, one expert failed to identify CTE as the sole diagnosis in any of the cases, which was still true for 4 of the 10 cases, even after unblinding. Given this subjectivity in pathological interpretation, it is important to highlight that most of the evidence linking, contact sport participation, a neuropathological diagnosis of CTE, and adverse long-term outcomes have all been derived from a single institution/disease related study center. By developing reliable pathological criteria that includes pertinent factors (e.g., age, co-occurring pathology) derived from standard clinical protocols, reduction of subjectivity in neuropathological interpretation can be achieved. As highlighted above, inter-rater agreement for CTE diagnosis (78% agreement among the original consensus panel), is inferior to AD, based only on a sequential accumulation of NFTs in the cortex without taking into clinical variables (i.e., age), which has yielded a kappa of 0.90 [77].

Consideration of concurrent neurodegenerative diseases

The classification of neurodegenerative diseases such as AD also take into account the presence of other coexisting pathologies. Even after decades of research, the diagnosis of AD is often still problematic due to the diverse morphologic heterogeneity of AD, including the numerous subtypes and presence of coexisting neurodegenerative findings such as LBs, cerebrovascular disease, and hippocampal sclerosis. In these situations, AD may be the primary diagnosis, but the comorbid neurodegenerative pathologies and diseases are recognized as existing and possibly contributing to dementia. As such, the standardized approach to reporting of autopsy findings are reflective of this and not only describe the scores, but also clinical correlations and the description of other coexisting pathologies. The experts involved in these sister diseases acknowledge and embrace this uncertainty. To date, such is not the case in CTE. Instead we see the converse, where scientific experts (and the lay public) embrace preliminary and immature data, all with the presumption of scientific certainty.

While the CTE consensus panel recommended that the presence of pathology consistent with another primary, comorbid neurodegenerative disease excludes CTE as a single diagnosis, cases in the literature are still reported or emphasized as solely CTE [4, 15, 78]. There is little to no consideration given to the other neurodegenerative disease processes that may be present and contributing to symptom burden. For example, in a most recent 2017 publication, 98 of the 177 brains with CTE diagnoses had other primary neurodegenerative diseases [4]. However, this is not reflected in the case reporting, and all clinical findings of subjects are attributed to CTE and no other comorbid neurodegenerative disease processes.

Refinement of criteria through diverse sample and panels

Unlike the lengthy processes and large case numbers used in AD and DLB described above, none of the CTE cases were acquired prospectively and all clinical information relied on self-reporting or recollections of next of kin, which reflects study design limitations; including the potential for heuristic errors, such as confirmation bias and congruence bias. Valid and reliable pathological and clinical diagnostic criteria should be based on the rigorous processes broadly implemented by the two other neurodegenerative disorders discussed in this paper. While the two degenerative disorders varied in the degree to which they adhered to the established consensus process, the development of each criteria set adhered to a few common principles. These include 1) multiple iterations or refinements of originally developed criteria, 2) samples taken from large registries of specimens, in order to obtain a clearer estimation of disease characteristics (e.g., true prevalence, risk factors, etc.), 3) consensus meetings consisting of diverse subject matter experts from different institutions and disciplines, and 4) validation studies as

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Table 1
Disease history and consensus derived criteria for AD, DLB, and CTE

	Alzheimer's Disease	Dementia with Lewy Bodies	Chronic Traumatic Encephalopathy
Evidence			
a) Earliest year	1906	1912/1961	2005
b) Iterations	8	4	1
c) Years	35 y	22 y	3 y
d) Cases	562	Literature Reviewed	25*
e) Experts	18	63	7
f) Institutions	18	>40	7
g) Subcommittees	Yes	Yes	No
Pathologic Diagnostic Criteria	• ABC score of Aβ, NFTs, CERAD neuritic plaques, each with scale of 0 to 3 • ABC score classified as None, Low, Intermediate, High pathologic evidence of AD based on 0 to 3 in all 3 aspects.	• Score from 0 to 2 in each region based on number of LBs • Scores summed based on distribution and density in five main regions • Low, intermediate, and high classification based on above and concomitant AD pathology	At least 1 perivascular p-tau lesion consisting of p-tau aggregates in neurons, astrocytes, and cell processes around small blood vessels found at the depths of cortical sulci
Clinical Diagnostic Criteria	• Meets requirements for All-cause dementia (function decline, poor activities of daily living, psychiatric ruled out, cognitive/behavioral impairment) • **Probable AD:** insidious, worsened cognition, two impaired 'core' cognitive domains • **Possible AD:** most above criteria met, atypical course, mixed presentation	• Progressive cognitive decline that interferes with function; attention, executive, visuoperceptual deficits; persistent memory impairment later • **Probable DLB:** 2 + clinical features with or without biomarkers (reduced dopamine transporter uptake in the basal ganglia on PET or SPECT); or, 1 + clinical feature and 1 + biomarker • **Possible DLB**: 1 + biomarker without clinical features	NA
Validation			
a) Sensitivity	76–93%	88%–91%	NA
b) Specificity	55–91%	67%–81%	NA
c) Accuracy	90%	82%–91%	NA

*25 cases of tauopathies; cases of stage III/IV CTE = 10; cases of stage I/II CTE = 0.

a means to evaluate and refine the previously established criteria. Perhaps the most important of these principles is number 4, as it involves refinement and attempts to improve proposed criteria. Given that consensus statements inherently suggest that the science on the issue is not yet settled, results are often provisional and will likely require modification, which has been observed among the large majority of consensus statements. The CTE diagnostic neuropathologic criterion (single focus of p-tau), in its current form, will continue to lack diagnostic specificity until a more comprehensive consensus process is performed that at least broadly adheres to these common principles of neurodegenerative disease criteria development, particularly further refinement.

The study of CTE is in its infancy. Despite wide use of the terminology in publications, there are still no universally accepted or validated diagnostic and/or staging criteria. The neuropathologic diagnostic criterion currently in use is based on small numbers of cases, many with other prominent neurodegenerative pathology and/or diseases, and are the products of just a select group of neuropathologists. The CTE literature lacks a nomenclature that is reflective of the other more prevalent neurodegenerative diseases, and there is no use of terminology such as 'unlikely,' 'possible,' or 'probable', which is typical of that used in other neurodegenerative disease classifications. Additionally, antemortem clinical criteria with distinct profiles for each stage of CTE should involve rigorous processes that involves refining

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specific clinical symptoms associated with each classification, rather than efforts involving simple correlational or frequency-based retrospective neurobehavioral observations. However, as highlighted above, attempting to establish clinical criteria without further refinement of the single neuropathological criterion will likely prove futile. The use of pathologic staging to infer disease progression and explain clinical symptoms suffers from a lack of a lower threshold for diagnosis and as result, lacks diagnostic specificity. The lack of a lower threshold inflates the number of CTE cases and inhibits the clarification of purported clinical symptoms associated with this disease. Until consensus- and collaboration-derived diagnostic and staging criteria are established by the international medical community, we will not have a clear understanding of CTE. Going forward, we suggest further involvement with international consensus panels with the inclusion of cases reflecting a range of disease severity and cases from more than one study center in order to aid in the development of the optimal neuropathological and clinical criteria.

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No Evidence of Chronic Traumatic Encephalopathy Pathology or Increased Neurodegenerative Proteinopathy in Former Military Service Members

Rudy J. Castellani[a,c,*], Arushi Tripathy[a], Ashley Shade[a], Brittany Erskine[a], Kristi Bailey[a], Abigail Grande[a], George Perry[b] and Joyce L. deJong[a]
[a]*Center for Neuropathology, Western Michigan University Homer Stryker MD School of Medicine, Kalamazoo, MI, USA*
[b]*College of Sciences, University of Texas at San Antonio, San Antonio, TX, USA*
[c]*Departments of Pathology and Neuroscience, West Virginia University School of Medicine, Rockefeller Neuroscience Institute, Morgantown, WV, USA*

Abstract. It is presently unknown whether military service members are at risk for chronic traumatic encephalopathy (CTE) or Alzheimer's disease (AD) pathology, due to traumatic brain injury (TBI). Studies with respect to AD have had mixed results with respect to mild TBI, although an increased risk of clinical AD with moderate and severe TBI is more consistently demonstrated. No studies to date have demonstrated a longitudinal progression from TBI to autopsy. We therefore initiated a cross-sectional survey of former military service members. 18 brain specimens have been examined to date that had extensive sampling, with an additional 64 specimens with limited sampling. The mean age was 68.4 years across all cases. Of these 82 cases, 26% were combat veterans. 13% noted a TBI history, either on active duty or in civilian life. 53% had a history of psychiatric problems, including 20% with post-traumatic stress disorder (PTSD). 17% reported neurological problems. No cases had CTE pathology. Assessment for proteinopathy by Braak staging and modified CERAD plaque scores showed averages of 2.41 and 0.78, respectively, which was essentially identical to age-matched controls (2.46 and 0.77, respectively). In the extensively sampled cases, there was no relationship between p-tau in the amygdala and psychiatric signs, including PTSD. These data suggest that military service per se is not a risk factor for CTE pathology or neurodegenerative proteinopathy. More research is needed to study the relationship, if any, between TBI and neurodegenerative proteinopathy.

Keywords: Alzheimer's disease, chronic traumatic encephalopathy, tauopathy, traumatic brain injury, veterans

INTRODUCTION

Chronic traumatic encephalopathy (CTE) is conceptualized as a progressive neurodegenerative proteinopathy due to repetitive head trauma [1]. The concept was initially hypothesized in boxers (dementia pugilistica) [2], although in recent years it has expanded to include participants in other high energy collision sports, especially American football [3]. In parallel with the more recent literature in athletes, the concern that military serviced-related traumatic brain injury (TBI) may serve as a nidus for neurodegeneration has been raised [4]. Military service members are vulnerable to TBI because of the nature of armed conflict and military training, and because of the increased use of improvised explosive devices

*Correspondence to: Rudy J. Castellani, MD, 64 Medical Center Drive, Morgantown, WV 26506, USA. Tel.: +1 304 293 1625; Fax: +1 304 293 1627; E-mail: Rudolph.castellani@hsc.wvu.edu.

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(IEDs) in recent conflicts. Most military service-related TBIs since 2006 have been associated with IED blasts [5]. Case reports and small case series have likewise described CTE, hypothesized to be due to blast-related TBI, in former military service members [6, 7]. Reports have gone so far as to suggest that post-traumatic stress disorder (PTSD), which may or may not have co-morbid TBI, may share common neurobiological underpinnings with CTE [6–8]. If a progressive degenerative proteinopathy caused by service-related TBI, with or without PTSD, exists, this would be of interest to the Department of Defense, and may prompt policy changes and allocation of resources dedicated to prevention and therapeutic intervention.

Large scale epidemiological studies, meta-analyses, and reviews have mixed results as to whether TBI represents a risk for neurodegenerative proteinopathy [9–17], although moderate to severe TBI has more consistently demonstrated higher relative risk for clinical Alzheimer's disease (AD) [11, 18]. A causal relationship between TBI and neurodegenerative proteinopathy, however, has not been demonstrated. Risk between TBI and CTE pathology is difficult to ascertain epidemiologically, since putative human cases to date have limited TBI history, other than that inferred from participation [1, 3]. Lack of a clinical substrate for protein deposits also hampers TBI risk assessment [19]. Studies evaluating AD risk may also be confounded by reverse causality [10]. Definitions of TBI, including mild TBI, vary widely, with as many as 50 different definitions pointed out in one study [20], which, along with heterogeneous study designs, make comparisons between studies difficult.

The TBI-proteinopathy paradigm is therefore complex and hypothetical. Of concern is that conclusions are increasingly suggested about risk or cause of progressive proteinopathy commensurate with military service [4]. The overall goal of this study is to determine whether or not progressive proteinopathy emerges as over-represented in former military service members compared to non-military controls with no TBI history, and if so, the nature of that proteinopathy. Our overall working hypothesis is that proteinopathy of whatever extent is more age-related, possibly with a genetic component, than trauma- or military-service related, and that the extent of proteinopathy per se correlates poorly with clinical signs. The latter is apparent in case series in athletes, as well as the collective literature on AD, other neurodegenerative disorders, and aging-related entities. We further hypothesize that in order to demonstrate the existence of a clinicopathological entity associated with TBI, structural brain changes (e.g., neuronal loss, encephalomalacia from trauma) are required, irrespective of the presence or extent of localized p-tau immunoreactivity.

METHODS

Veteran brain procurement

All brain tissue was procured and medical records obtained with consent from legal next-of-kin. In most cases, next-of-kin were approached for donation when death investigators in the various counties under the jurisdiction of Western Michigan University School of Medicine Pathology Department came across veteran status. Brain tissue from a subset of cases was obtained following decedent donation to the medical school. Veteran status was the only criterion. The catchment area included 12 counties in western Michigan. The donations took place between 2016 and 2018. 18 cases were sampled extensively for proteinopathy studies. 64 additional cases were obtained under a separate protocol (protocol number 20111080) by informed consent of next of kin, with more limited sampling and immunohistochemistry.

For age-matched control tissue, a series of 108 brains with an age range comparable to the veteran sample over the same time period of the study were also obtained. Exclusionary criteria included circumstances suspicious for homicide, deaths in police custody, hepatitis, human immunodeficiency virus, meningitis, specific brain abnormalities (e.g., stroke, brain tumor), seizure disorder without a psychiatric history, or prolonged artificial life support.

A questionnaire was provided to the next-of-kin, with data points regarding medical history; TBI history; neurologic, neurocognitive, and psychiatric function; and social history. The study protocol was presented to the Institutional Review Board at Western Michigan University Homer Stryker MD School of Medicine and was exempted from formal application. Information with respect to contact sports was not sought.

Gross examination

Postmortem interval and pH of the brain was recorded. The brain was photographed externally, the Circle of Willis was dissected and photographed, the

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dura mater oriented, examined, and photographed, and the brain examined externally by a board-certified neuropathologist for documentation of structural pathology. 18 cases were examined with extensive sampling. In these cases, the left cerebrum, hemibrainstem, and hemi-cerebellum were saved frozen at –80°C. The right half was fixed in formalin and processed for histopathology. 64 cases were examined with more limited sampling, per a separate protocol. In these cases, all tissue was frozen apart from the samples processed for histopathology.

Routine histopathology

After gross examination, the following samples were obtained for histopathology for the 18 extensively sampled cases: prefrontal cortex (dorsal, lateral, medial, gyrus rectus), pyriform cortex, insula, superior/middle temporal gyrus, promotor cortex (dorsal, lateral, cingulate), parietal cortex (dorsal, lateral, medial), occipital cortex (area 17, area 18), anterior hypothalamus with basal forebrain, hypothalamus with mammillary body, basal ganglia, thalamus, anterior hippocampus and adjacent cortex, posterior hippocampus and adjacent cortex, amygdala, midbrain, pons, medulla at inferior olivary nucleus, medulla at obex, cerebellar hemisphere with dentate nucleus, and cerebellar vermis. The brain regions sampled encompasses all brain regions utilized in the NINDS/NIBIB recommendations [21]. Specifically, the publication suggests: "... we recommend wider p-tau screening to capture CTE and other tauopathies. In addition, if there is a high index of suspicion of CTE, we recommend taking extra sections of frontal and temporal cortices, and hypothalamus including the mammillary body." To this point, we took extra sections of frontal and temporal cortices, and specifically sampled posterior hypothalamus and mammillary bodies.

For the 64 cases with limited sampling, superior frontal, middle temporal, inferior parietal, calcarine cortex, hippocampus, midbrain, and cerebellar cortex were obtained. The cortical sections specifically targeted sulcal depths. Cortical sections and hippocampus were immunostained for p-tau. Cortical sections only were immunostained for Aβ. Following two weeks or more in 10% neutral buffered formalin, the above post-fixed samples were processed through graded ethanol and xylene solutions, and embedded in paraffin. Five-micron thick sections were prepared and stained with hematoxylin and eosin.

Immunohistochemistry

Antibodies: p-tau (AT8), amyloid-β, TPD-43, alpha-synuclein were utilized in the extensive protocol (see Table 1). The assessments generally followed the 2016 NINDS/NIBIB recommendations for CTE diagnosis [21] and modified NIA-AA 2012 consensus guidelines for assessment of AD neuropathologic change [22]. The CERAD component of the NIA-AA 2012 guidelines was modified with the use primarily of Aβ immunohistochemistry to assess Aβ burden and plaque frequency, otherwise semiquantitated into sparse, moderate, and frequent per schematic illustrations in the original CERAD paper [23]. This approach was chosen to examine for proteinopathy per se, rather than attempt to assess for "neuritic plaque" frequency, as the latter is poorly defined with no standards for methodology (e.g., silver impregnation versus thioflavin versus immunohistochemistry). Braak and CERAD plaque scores were obtained using similar methodology and consent criteria were compared to 108 donors between the ages of 59 and 95 (mean age 68.9 years ± 10.1 years). For calculating averages, Braak I through VI was assigned a numerical value of 1 to 6. Modified CERAD scores of sparse, moderate, and frequent, were assigned numerical values of 1, 2, and 3.

For control samples, p-tau and Aβ burden was assessed by immunohistochemistry similar to the veteran cases with limited sampling.

RESULTS

Clinical data (Table 2)

Clinical data for the extensively sampled cases are provided in Table 2. Of these 82 veteran cases overall, 26% were combat veterans. 13% noted a TBI history, either on active duty or in civilian life. 53% had a history of psychiatric problems, including 20% with (PTSD). 17% reported neurological problems.

Gross neuropathology

Gross neuropathological changes included the spectrum of atherosclerotic disease from no disease to advanced atherosclerosis. No cases demonstrated mammillary body atrophy, disproportionate expansion of the 3rd ventricle, or septal fenestrations. Metastatic lesions were present in two decedents. There was no hippocampal sclerosis in the series. Aside from cases with AD pathol-

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Table 1
Antibodies

Antibody	Clone	Vendor	Dilution	Antigen retrieval
Phospho-tau	AT8	Thermo Scientific	1:250	ThermoScientific Dewax and HIER L Buffer, 20 mins, 98°C
Amyloid-β	BAM01	Thermo Scientific	1:50	95% formic acid, 30 min
TDP-43	TARDBP	ThermoFisher Scientific	1:50	95% formic acid, 30 min
Synuclein	syn211	Thermo Scientific	1:200	ThermoScientific Dewax and HIER L Buffer, 20 min, 98°C

Table 2
Summary of demographic data, TBI history, neurology and psychiatric history, and p-tau burden by Braak stage

Age	Gender	Branch	TBI	Blast	PTSD	Other Psych	Neuro	Braak
36	M	Army	Yes	Yes	Yes	DEP	ALZ	VI
68	M	Army	No	No	Yes	A, DEP	No	I
90	M	AR/NA	No	No	No	No	No	IV
55	F	Marine	Yes	No	Yes	A, BP, DEP, SI	No	III
68	M	Army	No	No	No	A, IN, SC	SZ	I
94	M	Army	No	No	No	No	No	V
89	M	?	?	?	?	DEP	DEM	III
69	M	Marine	No	Yes	Yes	BP, SCA	No	I
81	M	Army	No	No	No	No	No	V
63	M	Army	No	No	No	DEP	No	II
66	M	Army	No	No	Yes	DEP	No	I
37	M	?	No	?	Yes	?	?	0
71	M	?	?	?	?	No	ST	III
66	M	Marine	?	?	?	No	No	II
68	M	?	?	?	Yes	BP, DEP, IN	ST	I
66	M	Army	No	No	Yes	DEP	No	I
88	M	Army	Yes	No	Yes	No	No	IV
32	M	SF	?	?	Yes	DEP	No	0

A, anxiety; ALZ, Alzheimers' disease; BP, bipolar disorder; DEM, dementia; DEP, depression; EP, epilepsy; IN, insomnia; Neuro, neurological history; Other psych, psychiatric history apart from PTSD; PTSD, post traumatic stress disorder; SC, schizophrenia; SCA, schizoaffective; SI, suicidal ideation; ST, stroke; SZ, seizure disorder; TBI, traumatic brain injury.

ogy, the extent of atrophy showed age-related variability.

P-tau neuropathology (Table 3)

No subjects had changes demonstrating the required criterion according to NINDS/NIBIB consensus recommendations (p-tau in neurons, astrocytes, and cell processes around small blood vessels at the depths of cortical sulci).

In the extensively sampled cases, even with much more limited numbers, Braak stages varied according to age (R = 0.74 (Pearson correlation coefficient) after excluding the case of familial early onset AD). Supportive neuropathological features of CTE according to the 2016 consensus guidelines were variable, and included superficial layer (II-III) p-tau pretangles and neurofibrillary tangles (NFTs) (seven cases), as well as pretangles and NFTs in areas CA2 and CA4 of the hippocampus (seven cases), subcortical p-tau (six cases) (Fig. 1), p-tau in thorny astrocytes at glia limitans (five cases) (Fig. 2). Two subjects showed evidence of Braak stage III or IV neurofibrillary change, with sparse or absent amyloid-β, consistent with primary age-related tauopathy (PART) [24]. Six cases showed primarily astrocytic p-tau immunoreactivity consistent with aging-related tau astrogliopathy (ARTAG) [25]. P-tau in the amygdala varied from absent to abundant (Figs. 3 and 4), with no relationship to neurological or psychiatric history. Patchy astrocytic tauopathy at sulcal depths around small blood vessels was noted in one 88-year-old veteran with PTSD and TBI history, but did not qualify for the required CTE criterion since no neuronal p-tau was present (Fig. 5).

Since CTE is hypothesized as a progressive tauopathy, we were also interested in whether overall p-tau "burden" correlated with TBI history or PTSD. Although the numbers are too small for statistical analysis, p-tau ranged from Braak 0 to Braak VI in two veterans with a TBI history reported by next-of-kin, and from Braak 0 to Braak VI in two veterans with a PTSD history reported by next-of-kin; the Braak stage VI case was familial early-onset AD in both instances.

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Table 3
Summary of proteinopathy findings

Age	ABC	SL	CA2	SP	PV	SE	SC	PL	Amyg	PART	ARTAG
36	A3B3C3	+	+	–	–	–	+	–	++	–	–
68	A0B1C0	+	min	–	–	–	–	–	–	–	–
90	A2B2C2	+	+	+	+	+	+	–	+	–	+
55	A1B2C1	+	+	–	–	–	+	–	+	–	–
68	A0B1C0	–	–	–	–	–	–	–	–	–	–
94	A2B3C2	+	+	+	–	–	+	–	++	–	+
89	A1B2C1	+	–	–	–	–	–	–	+	+	–
69	A0B1C0	–	–	–	–	–	–	–	–	–	–
81	A2B3C2	+	+	–	–	–	++	–	++	–	–
63	A1B1C1	–	–	+	–	+	–	–	+	+	+
66	A0B1C0	–	–	–	–	–	–	–	–	–	+
37	A0B0C0	–	–	–	–	–	–	–	–	–	–
71	A1B2C1	–	+	+	+	+	–	–	+	min	+
66	A0B1C0	–	–	–	–	–	–	–	NA	–	–
68	A0B1C0	–	–	–	–	–	–	–	–	–	–
66	A0B1C0	–	min	–	+	+	–	–	+	–	+
88	A3B2C3	+	+	–	–	–	+	–	++	–	–
32	A0B0C0	–	–	–	–	–	–	–	–	–	–

ABC, AD criteria according to Montine et al. [22]; SL, tau in superficial cortical laminae (present, absent, or minimal); SP, subpial p-tau; PV, perivascular p-tau; SE, subependymal p-tau; SC, subcortical p-tau; PL, pathognomonic CTE lesion according to McKee et al. [21]; Amyg, relative abundance of amygdala p-tau; PART, primary age-related tauopathy; ARTAG, aging-related tau astrogliopathy.

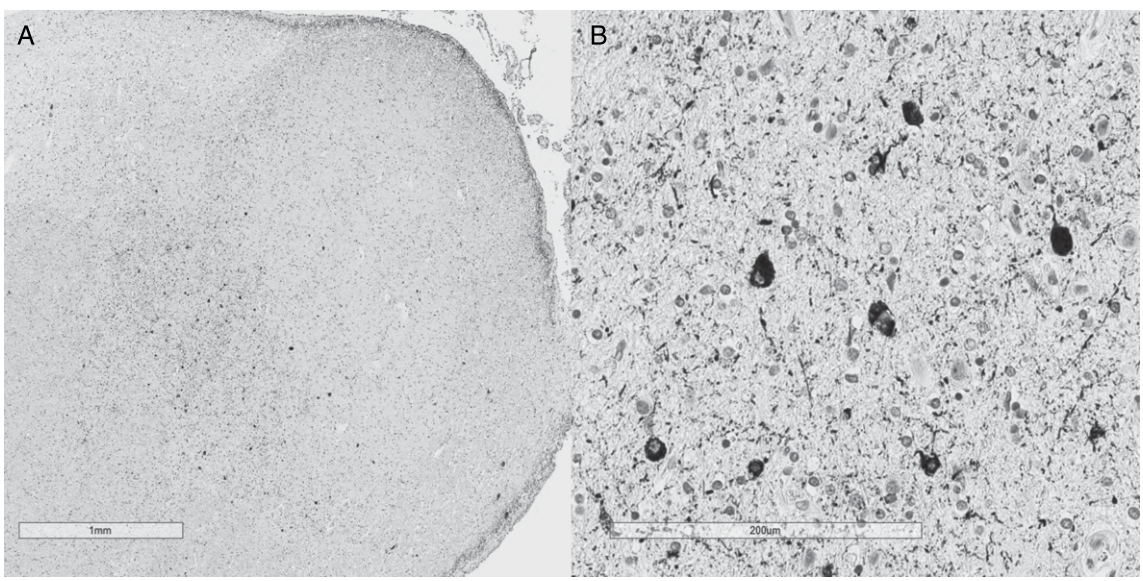

Fig. 1. P-tau immunohistochemistry of the hypothalamus and mammillary body at low magnification (A) and high magnification (B) shows significant subcortical p-tau including neurofibrillary tangles and neuropil threads. Despite the extensive p-tau pathology, this veteran had no cognitive complaints during life.

In the both the extensive and limited sample cases, the average Braak score was 2.41 (Braak scores I through VI assigned a numerical value 1 through 6) and the average modified CERAD score was 0.78 (sparse = 1, moderate = 2, frequent = 3). This compared to age-matched controls with an average Braak score of 2.46 and average modified CERAD plaque score of 0.77 (p = NS, student t-test).

TDP-43 neuropathology

TDP-43 immunohistochemistry variably reacted with neurofibrillary pathology in the extensively sampled cases. Neuronal cytoplasmic inclusions and dot-like structures in the hippocampus, anteromedial temporal cortex, and/or amygdala were variably noted.

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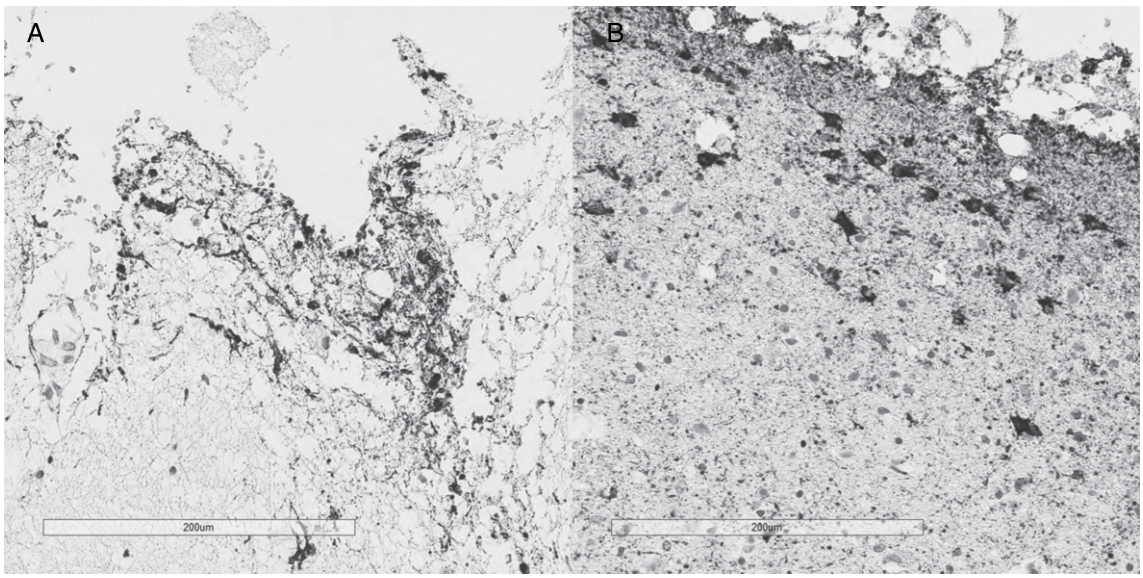

Fig. 2. Representative p-tau immunohistochemical stains shows subpial astrocytic tau at the depth of a sulcus (A) and at the cortical surface (B). Although considered a supportive feature for CTE pathology, this finding is also encountered in aging-related tau astrogliopathy.

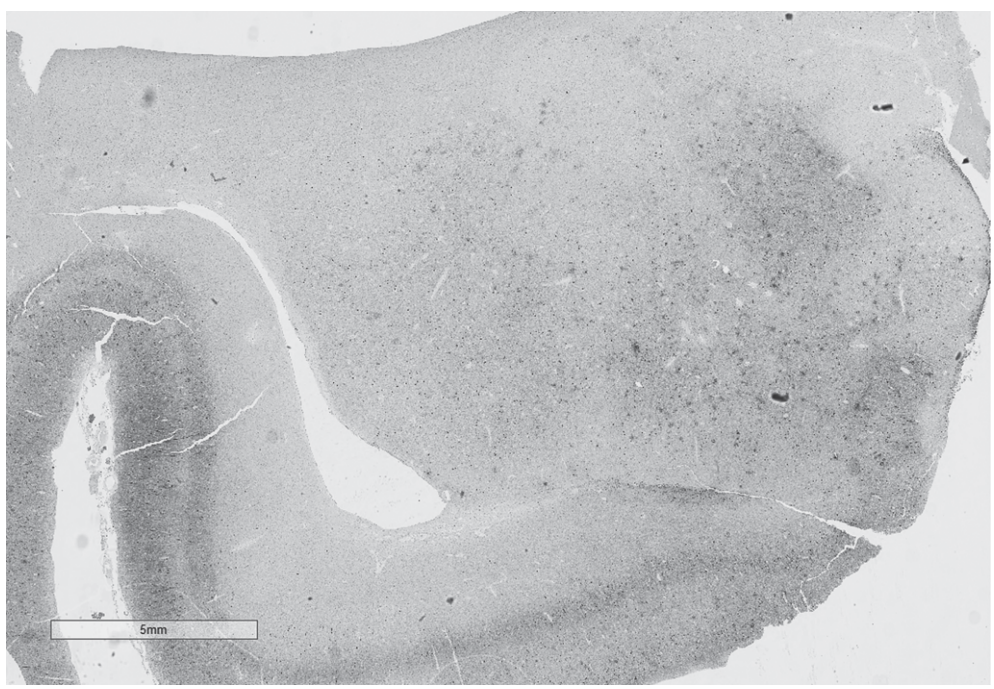

Fig. 3. P-tau is often abundant in the amygdala and is involved early in the aging process, as it was in this case shown at low magnification. It is noteworthy that abnormal amygdala function is implicated in post-traumatic stress disorder (PTSD). In our series, however, the presence or extent of p-tau pathology did not correlated with PTSD. Despite the extensive p-tau "burden" in this case, this veteran had no cognitive complaints during life and did not suffer PTSD.

Amyloid-β neuropathology

In the extensively sampled cases, Amyloid-β immunohistochemistry varied from absent to advanced (modified CERAD frequent, Thal phase V), which correlated with age even in this limited sample (R = 0.68 (Pearson correlation coefficient), when excluding the case of familial early onset AD

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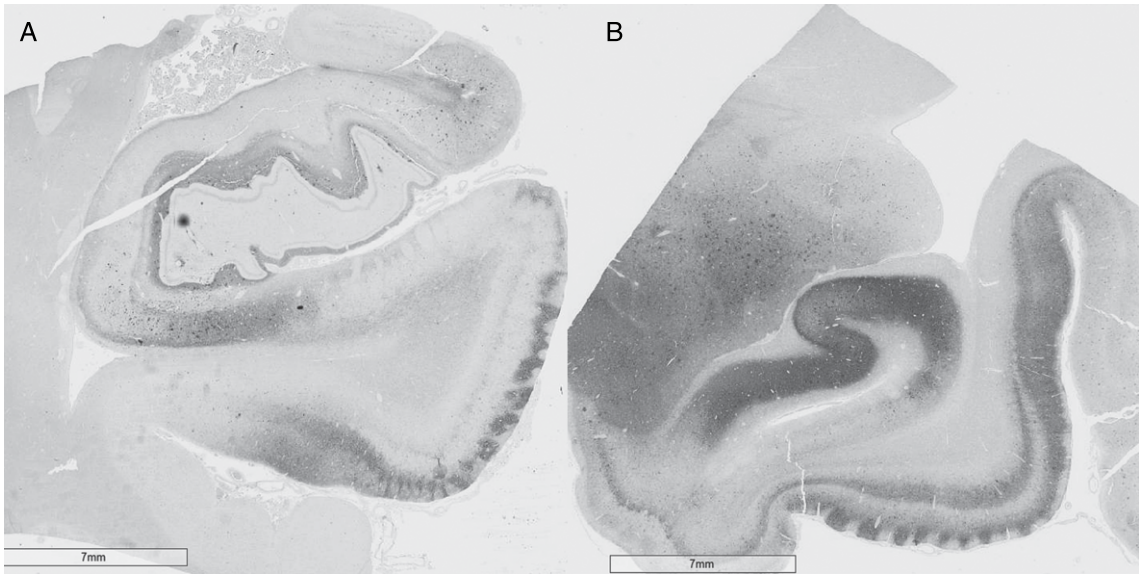

Fig. 4. Abundant p-tau in the medial temporal lobe invariably accompanies the aging process (A: 81-year-old Army veteran; B: 88-year-old Army veteran). Note that such pathology often includes involvement of neurons, astrocytes, and cell processes around small blood vessels at the depth of cortical sulci, with or without the presence of significant amyloid-β, the only possible difference from CTE pathology being that p-tau in the aging process is more diffuse. The overlap between aging-related p-tau and CTE pathology is nevertheless considerable, suggesting that some caution is indicated before interpreting any given p-tau pattern as specific for a TBI, sport, or military service history.

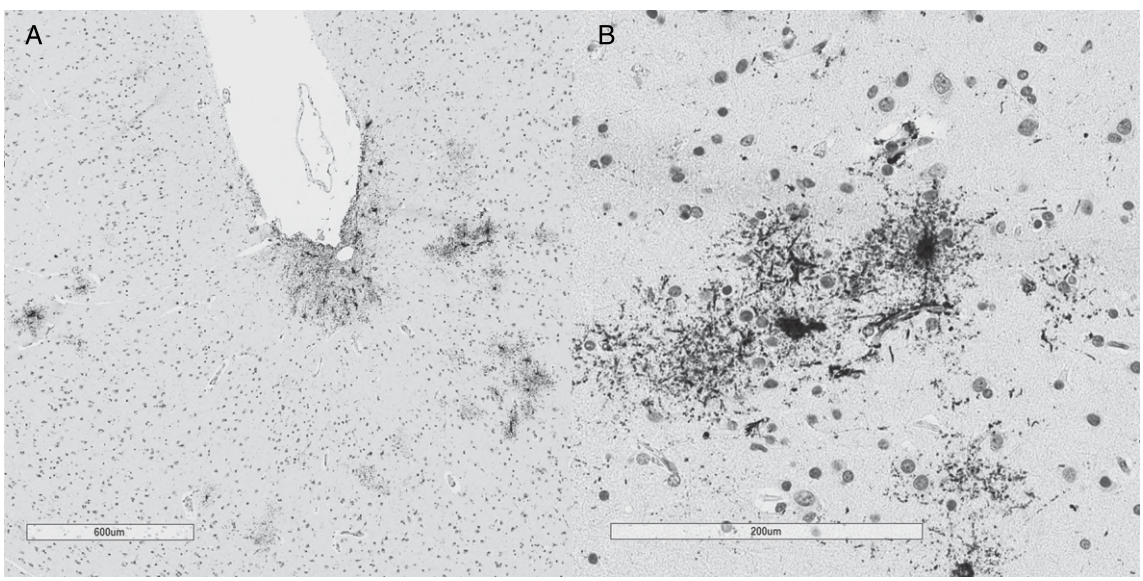

Fig. 5. This case emphasizes the difficulty in distinguishing aging-related p-tau with CTE pathology (A: low magnification of a sulcal depth; B: high magnification of the same sulcal depth). In this elderly veteran, there is p-tau in astrocytes and cell processes in an irregular distribution around small blood vessels at the depth of a sulcus. The morphologic characteristics indicate that the p-tau is entirely astrocytic; the required CTE criterion is not met because of the absence of neuronal involvement. This marginal difference, which may be simply due to sampling, indicates an aging-related change that could be present in any given person, yet it is nearly indistinguishable from a change regarded as pathognomonic for a progressive neurodegenerative disease. These findings, again, indicate that more research is needed before concluding that particular patterns of p-tau are specific to sport- or military-related TBI or a putative neurodegenerative disease.

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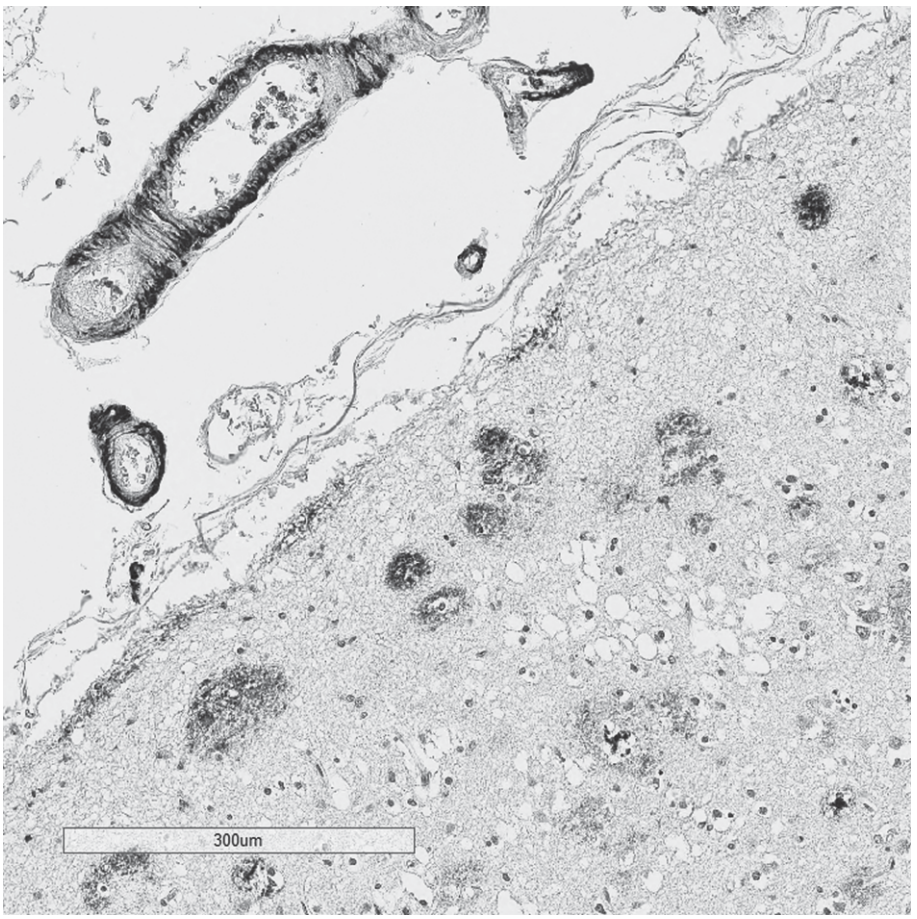

Fig. 6. Amyloid-β immunohistochemistry shows abundant amyloid-β and cerebral amyloid angiopathy in this 36-year-old veteran with a family history of dementia. This combination of findings most likely indicates familial, early-onset Alzheimer's disease. Knowledge of genetic information is therefore critical before concluding that a progressive proteinopathy is causally related to TBI, sport, or military service.

(Fig. 6). Modified CERAD plaque scores were similar in the series with limited sampling compared to age-matched controls, as noted above.

Alpha-synuclein neuropathology

Immunohistochemistry showed no evidence of synucleinopathy in the extensively sampled cases. Thus, there was no evidence of brainstem-predominant, limbic, amygdala-predominant, or neocortical (diffuse) Lewy body disease in this series. Although the lack of synucleinopathy in this series is somewhat at variance with the incidence of synucleinopathy in the general population, signs of parkinsonism was not reported in medical records available or by the next-of-kin.

DISCUSSION

In this study, we note a spectrum of age-related and neurodegenerative proteinopathy in former military service members. AD pathology was variably present, including one probable familial autosomal dominant, early-onset AD case. Age-related p-tau and Aβ pathology was also present, the extent of which was essentially identical to age-matched controls. With regard to features supportive of CTE according to the 2016 consensus paper, variable p-tau was noted in superficial cortical laminae, CA-2, and glia limitans in some of the extensively sampled cases. However, since supportive CTE pathology overlaps considerably with PART and ARTAG [24, 25], and since there are no specific guidelines on the diagnostic significance of supportive CTE features

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in the absence of the required criterion [21], these changes were interpreted as aging-related. The extent of proteinopathy, or immunohistochemical burden, was not significantly different from age-matched control tissues. In short, we did not uncover evidence of progressive proteinopathy, increased AD pathology, or increased CTE pathology, in the veteran population compared to controls with no history of TBI or military service.

While we found no convincing CTE pathology in any of our veteran cases irrespective of trauma exposure, it is important to note that we did find age-related neuronal and astrocytic p-tau, which overlaps considerably with required and supportive CTE criteria. CTE-like pathology is increasingly identified in brain tissue of subjects with no trauma history, including people with temporal lobe epilepsy, schizophrenia status post leucotomy, amyotrophic lateral sclerosis, multiple system atrophy, and a variety of other neurodegenerative diseases [26–30]. As such, CTE criteria appear far more aging-related than TBI specific.

Since CTE is hypothesized as a progressive tauopathy, we also examined whether overall p-tau "burden" correlated with demographic features, TBI history, or PTSD. The most striking correlation, not surprisingly, was the association of p-tau burden with age. P-tau burden was minimal in young decedents (with the exception of the familial AD case) and often abundant in older decedents. P-tau burden in the amygdala, a neuroanatomic region involved in fear conditioning and implicated as functionally altered in PTSD, did not correlate with a PTSD history. Further discussion of PTSD is beyond the scope of this study, other than to note that it appears unlikely that PTSD, or PTSD subsets, correlate with tauopathy.

One case warrants elaboration. Case 2 in our series was an Army veteran with a history of TBI, blast exposure, and PTSD, who died at age 36. Following discharge from the army, he developed progressive neurological decline and was diagnosed with early-onset AD. There was also a history of dementia in the decedent's father. Autopsy examination revealed advanced AD pathology including cerebral amyloid angiopathy. Although genetic analyses are not available, the decedent almost certainly had familial, autosomal dominant AD, possibly with a presenilin mutation [31]. This suggests genetic lesions should be considered before extrapolating anecdotal cases to a TBI-progressive proteinopathy construct. Interestingly, the first case linking boxing-related neurotrauma exposure to neurodegenerative pathology was a similar case with advanced AD pathology in a relatively young man [32], indicative of familial early-onset AD based, having nothing to do with repetitive neurotrauma.

Some studies have suggested that TBI is a risk for AD specifically. One study examining World War II veterans suggested that AD risk was increased in subjects with a history of moderate or severe TBI in a dose dependent fashion, with moderate TBI conferring a roughly two-fold risk, and severe TBI conferring a roughly four-fold risk [18]. The study did not find an AD risk with mild TBI, which is in line with systematic reviews concluding the same [9]. The study also did not examine subjects for AD neuropathology. One recent, large-scale case cohort study of US veterans concluded that mild TBI without loss of consciousness conferred a modest risk for dementia as well as AD specifically [11]. The risk was higher in mild TBI with loss of consciousness, and higher still in moderate or severe TBI. A recent large cohort study of civilians in Denmark likewise concluded that mild TBI conferred a modest risk for both dementia and AD [33]. Neither of these studies assessed subjects for AD neuropathology.

Causal assertions from epidemiological studies remain problematic. The risks are overall modest, as noted. The repeatedly demonstrated dose–response relationship between AD and TBI severity is also interesting in that severe TBI causes dementia and reduced life expectancy [34], while AD increases exponentially with age. Small relative risks in this setting raise problems of reverse causality (increased vulnerability to TBI because of neurologic dysfunction) and interpreting TBI-related dementia (encephalomalacia from traumatic brain damage) as AD. For example, Lewin et al. [34] studied 75 severely head-injured patients and found that patients often had dementia from TBI, with few surviving more than a decade. Accurately assessing AD risk in the moderate and severe TBI setting may require rigorous pathological examination (generally not available in large scale epidemiological studies) since moderate and severe TBI often include trauma-induced encephalomalacia (e.g., contusion, diffuse axonal injury). To date, no longitudinal study demonstrating a sequence from TBI, to a period of latency, to clinical neurological deterioration, to autopsy confirmed AD is available. The issue of cognitive reserve, which plays a role in clinical assessment of AD [35], has also not been adequately addressed in the setting of TBI. It is possible, and even likely, that more severe TBI with encephalomalacia may reduce reserve to the

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point where less cell loss, age-related or otherwise, is required to manifest as dementia. More research is needed before the null hypothesis, or the conclusion that the reported dose-response relationship with TBI and AD is a statistical artifact, can be rejected.

Importantly, blast-related TBI has emerged as a major cause of morbidity and mortality in military service. Blasts have been the most common cause of injury in American soldiers since 2006, while of the ∼ one million veterans screened for TBI between 2007 and 2015, 8.4% reported TBI, the majority of which were mild and associated with blast [5]. Injury to the brain associated with blasts is heterogeneous [36]. Primary blast injury is due to positive and negative pressure waves, secondary injury is due to shrapnel, tertiary injury is due to acceleration of the head and body across the war theater, and quaternary injury is any further downstream pathology, including burns, lung injury, mass effect from brain swelling, ischemic brain injury, etc. Neuropathological sequelae of primary blast injury are unclear, although early data suggests astroglial scarring at sites of differing tissue density (gray-white interface, periventricular tissue, perivascular areas, subpial areas) [37]. P-tau proteinopathy was inconsistent in this series, arguing against the hypothesis of blast-induced CTE. In the current study, two subjects with reported blast exposure showed no evidence of CTE pathology, although the specific details of the blast exposure and associated TBI were unknown to the next-of-kin.

It is finally noted that most decedents in this series had one or more psychiatric problems, with many carrying a history of PTSD. In case series in athletes, psychiatric problems are also common, including completed suicide, over-represented because of sample convenience [3]. In recent years, suicidality has been suggested as a core clinical feature of CTE in athletes, although such an association has been refuted [38]. In a recent analysis by Iverson [39], the suicidality claim appears to be based on self-citing of anecdotal cases (circular reasoning), citing of reports that do not discuss suicide at all, and citing of reports in which the cause of death was cardiovascular disease and the manner of death natural. In this study, people with psychiatric signs had a range of proteinopathy from very little (Braak 0, no amyloid) to abundant (Braak VI, frequent plaques), while the only decedent with advanced dementia had advanced AD pathology at a young age. This is consistent with the neuropathological literature, in which common psychiatric conditions are not defined by proteinopathy on post mortem examination, and in which proteinopathy does not predict severity of cognitive dysfunction or other neurological signs with any degree of precision [22, 24, 40–43]. The notion, therefore, that proteinopathy drives neurological or psychiatric signs, is not supported by the human data, including the current series.

This study has the obvious limitations of incomplete TBI histories and variably available medical records. The findings are therefore subject to modification as additional cases and more complete medical records become available. Despite the limitations in medical records, it is nevertheless interesting how frequently psychiatric problems are reported by next-of-kin in postmortem questionnaires, combined with the absence of CTE pathology.

In conclusion, we performed neuropathological assessment of brain specimens donated by former military service members, as an initial attempt at brain surveillance of proteinopathy in this cohort. We found no differences between former military service members and controls, in terms of burden of proteinopathy. Psychiatric problems including PTSD were prevalent, but showed no relationship with p-tau, amyloid-β, or TDP-43 proteinopathy, which is expected since psychiatric disorders often lack structural pathology. No CTE pathology was detected in this series, as CTE pathology is currently conceptualized. These findings are in line with a recent presentation at the National Institutes of Health by Dr. Daniel Perl, in which only two out of one hundred veterans had minimal CTE pathology, and in which the diagnostic yield did not improve with extensive sampling. More research is needed before concluding that military service provides a plausible bridge between the pathogenically distinct processes of TBI and neurodegeneration, including so-called CTE. This is in line with a recent position from the US Department of Defense [4].

DISCLOSURE STATEMENT

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/18-1039r1).

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What is the Relationship of Traumatic Brain Injury to Dementia?

Mario F. Mendez[a,b,*]
[a]*Department of Neurology, David Geffen School of Medicine, University of California Los Angeles (UCLA), Los Angeles, CA, USA*
[b]*Department of Neurology, Neurobehavior Unit, V.A. Greater Los Angeles Healthcare System, Los Angeles, CA, USA*

Abstract. There is a long history linking traumatic brain injury (TBI) with the development of dementia. Despite significant reservations, such as recall bias or concluding causality for TBI, a summary of recent research points to several conclusions on the TBI-dementia relationship. 1) Increasing severity of a single moderate-to-severe TBI increases the risk of subsequent Alzheimer's disease (AD), the most common type of dementia. 2) Repetitive, often subconcussive, mild TBIs increases the risk for chronic traumatic encephalopathy (CTE), a degenerative neuropathology. 3) TBI may be a risk factor for other neurodegenerative disorders that can be associated with dementia. 4) TBI appears to lower the age of onset of TBI-related neurocognitive syndromes, potentially adding "TBI cognitive-behavioral features". The literature further indicates several specific risk factors for TBI-associated dementia: 5) any blast or blunt physical force to the head as long as there is violent head displacement; 6) decreased cognitive and/or neuronal reserve and the related variable of older age at TBI; and 7) the presence of apolipoprotein E ε4 alleles, a genetic risk factor for AD. Finally, there are neuropathological features relating TBI with neurocognitive syndromes: 8) acute TBI results in amyloid pathology and other neurodegenerative proteinopathies; 9) CTE shares features with neurodegenerative dementias; and 10) TBI results in white matter tract and neural network disruptions. Although further research is needed, these ten findings suggest that dose-dependent effects of violent head displacement in vulnerable brains predispose to dementia; among several potential mechanisms is the propagation of abnormal proteins along damaged white matter networks.

Keywords: Alzheimer's disease chronic traumatic encephalopathy, dementia, traumatic brain injury

INTRODUCTION

Dementia and traumatic brain injury (TBI) are among the most prevalent neurological disorders [1]. Alzheimer's disease (AD), the most common form of dementia, is a virtual epidemic, increasing in prevalence as populations achieve greater longevity. Likewise, TBI is a major public health problem, occurring in as many as four million people every year in the U.S. [2]. There is a long literature associating TBI and dementia [3, 4], from early descriptions of the "punch-drunk" syndrome and "dementia pugilistica" among boxers to the current burgeoning information on chronic traumatic encephalopathy (CTE) among athletes and others exposed to multiple, repetitive mild TBIs [5]. These developments, along with a series of epidemiological studies, have solidified TBI as a risk factor for neurocognitive syndromes; however, in the process

*Correspondence to: Mario F. Mendez, MD, PhD, Neurobehavior Unit (691/116AF), V.A. Greater Los Angeles Healthcare Center, 11301 Wilshire Blvd., Los Angeles, CA 90073, USA. Tel.: +1 310 478 3711/Ext. 42696; Fax: +1 310 268 4181; E-mail: mmendez@UCLA.edu.

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they have led to many questions about how TBI and dementia interrelate [6].

Although this is a complex relationship, the current literature has begun to answer these questions and unravel the relationship between TBI and dementia. A review of the literature suggests a number of tentative conclusions, subject to many reservations such as the potential for recall bias in reporting head injuries and referral bias in research participation [7]. The nature of TBI itself is broad and can range from single severe injuries to multiple small subconcussive TBIs and to concussion, a term often used synonymously with mild TBI. Likewise, the term dementia refers to a decline in two or more areas of cognition that can be associated with a range of pathologies beyond that of AD. Both TBI and dementia each vary in a number of other ways, including symptoms, course, other associated factors, biomarkers, animal modeling, and others. While unable to determine all variables that affect the TBI-dementia relationship, a current review of this evolving literature does point to ten potential emerging trends and preliminary conclusions regarding the complex relationship.

GENERAL TBI-DEMENTIA CLINICAL RELATIONSHIPS

Increasing severity of a single moderate-to-severe TBI increases the risk of subsequent AD, the most common type of dementia

Studies report a relationship between a prior moderate-to-severe TBI and the eventual development of AD [8–13]. Although a number of studies have failed to document the association [13–16], most reports, and three meta-analyses of 11–19 well-characterized studies [3, 8, 17], indicate that, in the aggregate, there is evidence to conclude that a single moderate-to-severe TBI is a risk factor for AD (See Fig. 1) [3, 8, 17–19]. The largest and most recent meta-analysis of the TBI risk for AD versus controls found an overall odds ratio (OR) of 1.4 (95% Confidence Interval [CI]: 1.02–1.90), and an increased risk of TBI for mild cognitive impairment, which is often a precursor to dementia [17]. In the two earlier meta-analysis, males, but not females, were at increased risk of AD from TBI [3, 8], but this gender difference does not persist in other studies [17, 20]. In addition, the limited number of prospective studies show a relationship between moderate-to-severe TBI and AD or other dementia [9–11]. In one of the most important ones, investigators evaluated the risk for dementia among 548 U.S. World War II veterans 50 years after confirmed TBIs, compared to 1,228 with other types of injuries [10]. They found that a record of moderate or worse TBI was significantly associated with AD or other dementia [10].

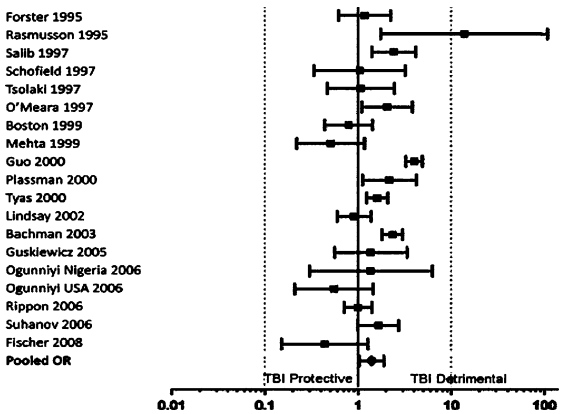

Fig. 1. Odds ratios and confidence intervals for Alzheimer's disease. Reprinted from the *Journal of Neurosurgery*, Perry et al., 2016 [17], with permission.

In sum, a single moderate or severe TBI, especially if recent rather than remote, is essentially the major known environmental risk factor for AD [3, 8, 21], and this risk increases with increasing severity of the head trauma. These studies indicate that a when TBI is of moderate severity, there may be a two-fold increased risk of AD or other dementia, and when TBI is severe, there may be a four-fold increased risk [10].

Repetitive, often subconcussive, mild TBIs increases the risk for chronic traumatic encephalopathy (CTE), a type of dementia

The vast majority of TBIs are mild (loss of consciousness <30 minutes; post-traumatic amnesia < 24 hours) [2]. Recent studies now show that mild TBI, particularly if repetitive, can also increase the risk of dementia [22–28]. The long-term risk of dementia from repetitive mild TBIs is a crucial question for athletes and among veterans [29, 30], as mild TBIs are common from participation in contact sports and from military deployment [31–33]. A Taiwanese study of one million people with three years follow-up found a three-fold increased risk for dementia in those with a history of mild TBIs [23], and the California statewide health database found an increased risk of dementia from mild TBIs in elderly individuals [34].

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The repetitive occurrence of mild TBIs appears to be much more of a risk for later dementia from CTE, a neuropathological disorder associated with TBI, than just one prior episode. We have long known of the association of TBI with the "punch drunk syndrome" or dementia pugilistica on neuropathology in boxers, now known at CTE [35]. Clinically, CTE often resembles AD in the delayed deterioration of cognition [36, 37], although one form of CTE manifests primarily with behavioral changes such as depression and suicidality, apathy and aloofness, poor impulse control, and behavioral disinhibition [5, 38]. Recently, there is widespread attention of the risk of CTE to professional football players and other athletes with increasing years of exposure to possible mild and repetitive TBIs [39, 40]. The clinical and neuropathological aspects of CTE have occurred not just in athletes but, despite a scarcity of neuropathological cases, among veterans, who are often prone to repetitive mild TBIs [5, 39–41]. This is further implied from epidemiological data from the VA National Database [24]. Of 188,764 veterans older than 54 years of age and followed for about 9 years, the presence of any TBI, including blast-force TBI, increased their risk of developing dementia by 60% (OR: 1.57; 95% CI: 1.35–1.83) [24].

TBI may even increase the risk for other neurodegenerative disorders that can be associated with dementia

Epidemiological studies report that TBI may increase the risk of developing non-AD neurodegenerative disorders potentially associated with cognitive impairment or dementia. Examining a California statewide administrative health database, investigators assessed the risk of dementia among 51,799 patients 55 years of age or older who had had a TBI during the preceding 5–7 years [34]. They found that moderate-to-severe TBI can increase the risk of different types of dementia with a minimum hazard ratio adjusted for age of 1.26 (95% CI: 1.21–1.32) [34]. In the prospective study of U.S. World War II veterans, there was an increased risk of both AD and non-AD dementias after a single moderate or severe TBI [10]. In addition to AD, specific neurodegenerative conditions linked to TBI include frontotemporal dementia (FTD) and to Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS), two conditions with the potential for associated cognitive decline.

TBI appears to be a risk factor for FTD. A study from the National Alzheimer's Coordinating Center (NACC) database of autopsy-confirmed cases of FTD found that a history of a remote TBI with five or more minutes of loss of consciousness was more common among 1,016 cases with FTD pathology compared to 2,015 normal controls (OR: 1.67; 95% CI: 1.00–2.78) [42]. In a prior retrospective case-control study of 80 patients with FTD compared to controls, investigators found 19 (23.8%) who had had a history of TBI [43], and in another retrospective case–control study involving 845 veterans, a history of prior TBI was association with a 4.4 times greater risk of developing FTD compared to controls [44]. Furthermore, in a large retrospective cohort study involving 147,510 patients in Taiwan, those with evidence of a prior TBI had a 4.13 times greater risk of developing FTD compared to non-TBI controls [45].

Studies have also reported TBI as a risk factor for PD. In a case-control study of 196 persons with PD in the Rochester, Olmsted County Epidemiology Project [46], there was a greater history of prior TBI among the PD cases than among matched controls (OR: 4.3; 95% CI: 1.2–15.2), and this was most significant among those who had more severe TBI or were previously hospitalized with a TBI. In a large retrospective cohort study of inpatient or emergency room data of patients presenting with TBI, there was an increased risk of PD in the subsequent 5–7 years of 44% (adjusted hazard's ratio of 1.44; 95% CI: 1.31–1.58), and the risk was greater with increased severity and frequency of the TBI [47]. An interesting case-control study of 93 twins reported that prior TBI increased the risk for PD (OR: 3.8; 95% CI: 1.3–11) [48]. The risk of PD after a history of TBI was also increased in the prospective study of U.S. veterans from World War II [10]. Additionally, one meta-analysis of 22 case studies found an increased risk of PD from TBI of 57% [49], and a second meta-analysis of 15 studies found an increased association of TBI with PD (OR: 1.45; 95% CI: 1.18–1.78) [17].

After reports of an unusually increased risk of ALS after TBI among football (soccer) players in Italy [50, 51], investigators examined this association in other populations. In one case-control study of 109 patients with ALS compared to matched controls, investigators found an increased risk for ALS among those with a history of two or more TBIs (OR: 3.1, 95% CI: 1.2–8.1) or if a single TBI had occurred in the prior 10 years (OR: 3.2; 95% CI: 1.0–10.2) [52]. In another case-control study of 77 patients with ALS compared to 185 controls, those with a TBI between age 30–40 had an OR for ALS of 14.2 (CI 1.04–194.42) [53]. Among veterans, including 241 with ALS and 597

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controls, those with TBIs in the prior 15 years had an adjusted OR of 2.33 (95% CI: 1.18–4.61) [54], and this association was strongest among those with had an APOE ε4 allele. One meta-analysis reported a moderately increased risk of ALS with prior TBIs (OR: 1.7; 95% CI: 1.3, 2.2) [52], but a subsequent meta-analysis of four studies did not quite find an elevated risk (OR = 2.07; 95% CI = 0.94–4.56) [17]. In contrast, some case-control studies did not find an association between TBI resulting in hospitalization and subsequent ALS, including in the Oxford Linkage study and among 4,004 ALS patients from the Swedish Patient Register [55, 56]. Furthermore, one meta-analysis of 12 studies did not find that a single prior TBI was a risk factor for ALS [57]. More epidemiological studies with neuropathological correlation would help resolve some of these discrepancies.

TBI appears to lower the age of onset of dementia, potentially adding "TBI cognitive-behavioral features"

There is evidence that TBI may lower the age of onset of any dementia or permit crossing of the threshold into dementia at an earlier age [20, 25, 58–60]. There is a particularly high prevalence of early-onset dementias among populations that have a high incidence of TBIs, such as U.S. and other veterans [58, 59, 61]. Among Swedish young men who had median follow-up of 33 years, those with a history of prior TBI had an increased risk for early-onset dementia of 50% (OR: 1.5; 95% CI: 1.1–2.0), which increased with the severity and frequency of TBIs [22]. Moreover, in a meta-analysis, the relative risk for AD was 1.63 with a TBI 10 or more years prior to onset, but increased to 5.33 with a TBI within 10 years, suggesting that more recent TBIs accelerate the emergence of AD [3].

In a large study, investigators used the NACC Uniform Dataset (UDS) to compare the prevalence of a history of TBI between AD patients with an early age of onset (<65 years; $n = 1{,}429$) versus those with a later age of onset (>65 years; $n = 4{,}292$) [20]. This study also examined the prevalence of TBI in a cohort of 115 early-onset AD patients attending a university subspecialty clinic. Despite potential recall bias, the prevalence of a history of TBI among the early-onset AD patients from both cohorts, was 13.5–13.9%, compared to 7.8% for the NACC late-onset cohort ($p < 0.001$). The NACC early-onset AD patients with a history of TBI, compared to those without, were more likely to have disinhibition and irritability, clinical signs of frontal lobe dysfunction often associated with "TBI cognitive-behavioral features" (see Fig. 2) [20].

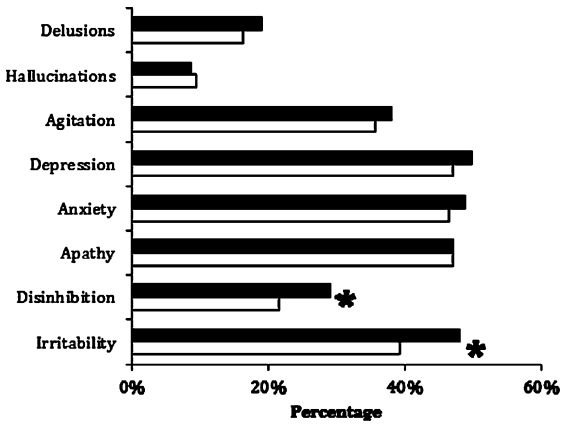

Fig. 2. Prevalence of neuropsychiatric inventory symptoms among early-onset Alzheimer's disease patients with ($n = 178$) and without ($n = 1{,}234$) a history of traumatic brain injury. *Significance to $p = 0.01$ level for disinhibition (29.0% versus 21.6%; $\chi^2 = 6.19$) and for irritability (48.1% versus 39.3%; $\chi^2 = 6.40$). Reprinted from the *Journal of Alzheimer's Disease*, Mendez et al., 2015 [20], with permission from IOS Press.

TBI not only lowers that age of onset of dementia, but also alters the clinical manifestations. Frontal injury from TBI may cause patients to have executive deficits, and orbitofrontal and anterior temporal injury may manifest as disturbances in social awareness and impulse/emotional control. In addition, dementia patients with a history of TBI have had better memory and verbal fluency but more depression and neurological symptoms in gait and motor speed [62]. This "TBI cognitive-behavioral profile" includes disproportionately impaired cognitive tests of attention, processing speed, and executive functions, compared to memory and language functions, as well as a greater psychiatric history (depression, apathy, irritability, poor impulse control and social-emotional disturbances, post-traumatic stress disorder, and substance abuse) [63–65].

SPECIFIC RISK FACTORS FOR TBI-ASSOCIATED NEUROCOGNITIVE DECLINE AND DEMENTIA

Any blast or blunt physical force to the head as long as there is violent head displacement

Most data are on blunt-force or impact TBI, but recent studies on blast-force TBI have implications

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for the relationship of TBI to neurocognitive decline and dementia. The majority of mild TBIs from recent combat deployments have been due to exposure to explosive devices such as mortar blasts or improvised explosive devices [66], and this blast-force mild TBI has become the "signature" injury of recent U.S. military engagements [67]. In addition to persistent post-concussive symptoms [32, 33, 68–70], blast-force mild TBI may be associated with post-traumatic cognitive impairment [71, 72], and there may be late cognitive, behavioral, and neuroimaging changes [63, 65]. As indicated in the VA National Database study, blast-force TBI may increase the risk for the eventual development of dementia [24]. This cognitive sequalae from blast-force TBI may resemble that from blunt-force TBI [73].

The clinical, as well as neuropathological, aspects of CTE have occurred in those exposed to blast-forces [39, 40, 72, 74]. A case series found CTE in postmortem brains from U.S. veterans exposed to blast-force as well as blunt-force TBI [72, 74]. Investigators have replicated this evidence of blast-force CTE among mice exposed to blast winds of 330 miles/hour [72], and studies on blast impact on mice show that, although it is a different brain force than from brain impact, the brain pathology resembles that of CTE and of blunt-force trauma [72]. Furthermore, L.E. Goldstein and colleagues indicate that there may be an absence of the neuropathology of CTE when the animal's head is kept stationary during experimental head trauma, hence equating the risks from both blast and blunt TBI with violent head displacement [72, 75]. Other studies indicate that most TBI has in common a sliding of the brain relative to the skull with rotation and deformation stress at anchoring points [76].

Decreased cognitive and/or neuronal reserve and the related variable of older age at TBI.

Prior mild TBIs may permit the development of AD not by the direct promotion of AD neuropathology but by lowering neuronal or cognitive reserve. The concept of "reserve" refers to resiliency in the face of brain damage, with "brain reserve" suggesting neuronal number and vulnerability to pathology and its clinical expression and "cognitive reserve" suggesting neuroplasticity, connectivity, and the capability for alternate, cognitive strategies. Common indicators of cognitive reserve include level of education, occupational attainment, and baseline intelligence. Epidemiological studies show that the lower the education and occupational ranking, the greater the risk for AD and other dementias [77, 78]. The attainment of high occupational achievement appears to delay the onset of cognitive and behavioral decline and the clinical aspects of CTE [79]. Conversely, a lower intelligence quotient before penetrating TBI among Vietnam veterans may be associated with greater cognitive decline on 30-year follow-up [80].

Neuronal and cognitive reserve decline with age, suggesting that age at the time of the TBI is a risk factor. The older the age at the time of TBI, the more likely the person is to develop dementia, as well as to develop amyloid-β plaques, a hallmark pathology of AD [81]. In another study, mild TBIs increased the risk of developing dementia by 20% if they occurred at age 65 or older, but not at a younger age [34].

The presence of apolipoprotein E ε4 alleles, a genetic risk factor for AD

Investigators have proposed that APOE ε4 alleles may be synergistic or additive with TBI in increasing the risk for developing AD [82, 83]. The presence of one or two APOE ε4 alleles is the major known susceptibility gene for the development of AD, increasing the risk by 3 or 12 fold, respectively [84], and shifting the age of onset of dementia to a younger age [85]. APOE ε4 impairs the efficiency of proteolytic breakdown and clearance of Aβ [84, 86, 87], and this may link TBI to the development of AD [85, 88, 89]. Several studies indicate that TBI particularly increases risk for AD if the patient has an APOE ε4 allele [82, 83]. After an acute TBI, those with an APOE ε4 allele have a greater frequency of deposition of Aβ at autopsy than those without an APOE ε4 allele, especially if they are homozygous for the gene [87]. In addition to AD, among boxers and football players, the presence of an APOE ε4 allele may also increase the likelihood of dementia pugilistica or CTE on neuropathology [90, 91]. But not all studies show a TBI-APOE association. In one case-control study of 230 AD cases and 390 controls with at least one APOE ε4 allele, there was no significant variation in the TBI-AD risk by APOE ε4 genotype [92].

After a TBI, the major effect of APOE ε4 has been poor post-traumatic outcomes [93]. In a series of studies of brain injury, including a meta-analysis of 14 prospective TBI studies, there were poorer neurological outcomes if the patient had an APOE ε4 allele than if they did not have an APOE ε4 allele [93–97].

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Among boxers with 12 or more professional bouts, those with an APOE ε4 allele had worse cognitive-behavioral outcomes than those without an APOE ε4 allele [90], and among 53 professional football players, those with an APOE ε4 allele had worse cognitive test scores than those without an APOE ε4 allele [91]. Moreover, in one study, the presence of an APOE ε4 allele increased the risk of unfavorable outcomes at six months or more after a TBI [93, 98], and other studies showed that this poorer outcome was particularly evident among younger patients or children [99, 100]. The reason of an association of APOE ε4 with poor post-traumatic outcomes is unknown but may relate to impaired mechanisms of repair.

NEUROPATHOLOGICAL FEATURES RELATING TBI TO DEMENTIA

Acute TBI results in amyloid pathology and neurodegenerative proteinopathies

Acute TBI results in multiple neuropathological processes that may predispose to dementia, including inflammation, microglial activation, and oxidative stress [101, 102], but, as evidenced in CTE, one of the most notable mechanisms is a predisposition to abnormal proteins associated with neurodegeneration [103]. A single moderate-to-severe TBI may increase both Aβ and hyperphosphorylated tau deposition, the abnormal protein components that are the substrates of the neuritic plaques and neurofibrillary tangles of AD [104, 105].

After an acute TBI, there has been strong evidence for increased Aβ production and accumulation, both in humans and mice (see Fig. 3) [103, 106–112]. Although not established, this post-traumatic Aβ may be in a prefibrillar, non-aggregated, oligomeric form and may also be detected in the cerebrospinal fluid (CSF) up to 72 hours after acute TBI [113]. Among 152 TBI brains from people ranging from 8 weeks to 85 years old and from 4 hours to 2.5 years after TBI, 20% of those <50 years of age and 60% of those 51–60 had Aβ deposition, compared to none of the controls [81]. Investigators have discovered non-plaque Aβ intracellularly in 80% of temporal cortex samples surgically excised from 18 patients 2–16 hours after a TBI [114]. Additional CSF findings after an acute TBI not only include alterations Aβ, but also in tau, S100b, glial fibrillary acidic protein, neuron-specific enolase, neurofilament light protein, ubiquitin C-terminal hydrolase, and others [115], but the post-TBI levels of all these proteins usually return to normal and have not proven to predict later neurocognitive decline. Genetic polymorphisms are associated with Aβ degradation and clearance and may predispose a subset of post-TBI patients to greater Aβ accumulation and aggregation [110, 116, 117]. In mouse models, the increased production of Aβ after an acute TBI can be decreased by preventing amyloid precursor protein secretase enzymes and the prevention of Aβ accumulation [118, 119].

In contrast to AD, most of the post-traumatic axonal Aβ may not be in fibrillar, thioflavin-S positive neuritic plaques characteristic of AD [120]. On autopsy, amyloid plaques do not occur in most patients with TBIs (70%) [81, 114]; only 30% of patients, who die of TBI, have actual Aβ plaques similar to those of AD [121].

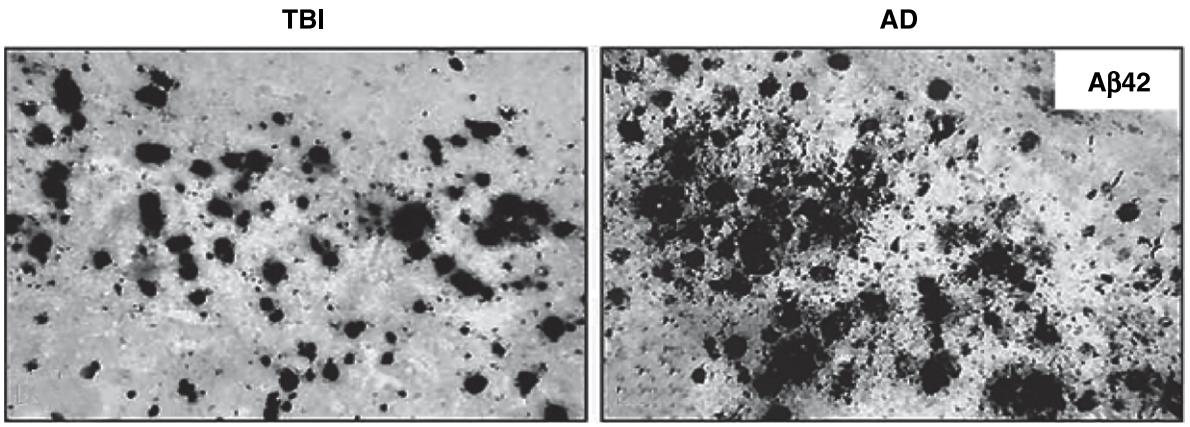

Fig. 3. Amyloid (Aβ) deposition in traumatic brain injury (TBI) and Alzheimer's disease (AD). Left: Tissue resected from temporal cortex of acute severe TBI patients showing Aβ staining. Right: Tissue form patient with AD showing similar Aβ staining. Reprinted from *Experimental Neurology*, Ikonomovic et al., 2004 [114], with permission from Elsevier.

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In addition to the accumulation of Aβ, there is production of abnormal tau protein after a TBI [107, 122]. Although most tau transgenic mice exposed to multiple TBIs per day have not shown an accumulation of phospho-tau [123], which is seen in AD, investigators frequently find tau pathology among players of contact sports even in the absence of frank CTE [5, 39, 72, 124]. After an acute TBI, there is tau hyperphosphorylation as well as increased tau levels in the CSF, but not the tau-derived neurofibrillary tangles of AD [114]. Among 39 patients surviving severe TBI for up to 47 years, 34% of those less than 60 years of age have had tau pathology compared to only 9% of 47 control brains, and the tau among the TBI patients appears more widespread in the sulcal depths and superficial layers of the cortex, whereas it is limited to the entorhinal cortex and hippocampus in the control brains [120].

CTE shares features with neurodegenerative dementias

As previously noted, repetitive mTBI, including blast-force mTBIs, can result in CTE, which is characterized by phospho-tau containing neurofibrillary tangles irregularly located in perivascular neurons and glia in the depth of sulci [5, 58, 72, 125, 126]. In CTE, there is progressive deposition of phospho-tau and other neurodegenerative proteins in the brain, similar to neurodegenerative dementias [5, 58, 61, 120, 123, 125, 127, 128]. The neuronal hyperphosphorylated tau deposits in CTE have a3 R/4 R tau isoform ratio similar to AD [129]. Furthermore, among neuropathologically-diagnosed cases of CTE [126], over half also have Aβ deposition, which is present in AD, but which may not have a primary pathophysiological role in CTE [5, 130]. Patients with CTE may have TAR DNA-binding protein 43 and some have α-synuclein, which are other abnormal proteins seen in neurodegeneration [5, 39, 130]. Additional pathology in CTE can include neuroinflammation with monocytes and activation of brain microglia, and traumatic blood-brain barrier disruption.

CTE is a TBI-related neurodegeneration that is distinct despite sharing features with other neurodegenerative disorders. There is a predominant involvement and deposition of abnormal tau in cortical layers II and III rather than layers V and VI as in AD [131]. There is axonopathy from small blood vessel injury and extensive tau-immunoreactivity perivascularly in superficial cortical layers, especially in the depth of sulci in the frontal lobes [5, 39]. Many patients with a history of TBI have orbitofrontal and anterior temporal injury, and CTE starts as a focal neuropathology in frontal neocortex rather than mesolimbic or parietal changes as in typical AD [5, 132]. Positron emission tomography (PET) with radioligands that bind to fibrillary insoluble protein aggregates, although not established in terms of tau specificity or CTE neuropathology, are beginning to document increased PET binding in TBI patients with clinically probable CTE [133–135]. Investigators have also associated the neuropathological findings of CTE with the clinical and neuropathological changes of FTD, PD, and ALS [5].

TBI results in white matter tract and neural network disruptions

Acute brain damage is associated with diffuse axonal injury (DAI) and white matter pathway disruption [29, 30, 63, 136, 137], evidenced by diffusely distributed and discontinuous areas of white matter abnormalities [138, 139]. During a TBI, the cortex moves at different speed relative to subcortical white matter, with consequent shear forces and deformation stress, axonal and vessel stretching, and microbleeds with resultant DAI. Post-TBI, DTI shows stretches of axons with damaged axonal membrane permeability and ionic shifts, with calcium damage to mitochondria, and resultant energy failure and breakdown of microtubules. Hence, where there is DAI, there is failure of axonal transport, increased axonal Aβ and other abnormal protein accumulation, and released Aβ and hyperphosphorylated tau [37, 103, 110, 120, 121, 140]. Recent work suggests, but does not establish, that these abnormal proteins could then spread in a prion-like fashion along white matter connections and functional neural networks [141]. Clearly, much more research is needed in order to establish this putative mechanism.

Similar TBI and AD effects on white matter tracts on diffusion tensor imaging (DTI) suggest a reduction of functional long-distance connectivity between frontal and caudal brain regions and a possible mechanism for the synergy of TBI with AD and other dementias (see Fig. 4). DTI measures the integrity of white matter, and, although functional anisotropy measures of DTI may decrease, increase, or remain unchanged after TBI [142–144], there are frequent reductions of functional anisotropy in the corpus callosum, cingulum, and other major fiber bundles [145]. On DTI, some investigators report diffusely

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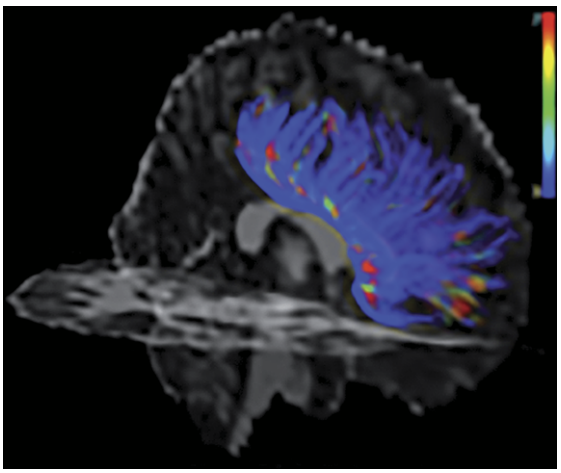

Fig. 4. Diffusion tensor imaging showing axon injury and white matter tract disruption in frontal corpus callosum region due to traumatic brain injury. Blue indicates greater disruption. From Emily Dennis, Laboratory of Neuro Imaging Keck School of Medicine, and Paul M. Thompson, Ph.D., University of Southern California.

distributed and discontinuous areas of white matter abnormalities in patients with mild TBIs [125], as well as in veterans who have had blast-force TBIs [139, 146, 147]. Among boxers, investigators report abnormalities in alternate DTI measures of white matter integrity (mean diffusivity, axial diffusivity, radial diffusivity) compared to controls [148, 149], and among soccer players and other athletes, there may be increased (abnormal) axial diffusivity in the corpus callosum and other major tracts [145, 150, 151]. Investigators have found significantly more DTI abnormalities among veterans heavily exposed to bombardment compared to those with limited exposure, demonstrating a potential cumulative effect of injury [151]. In AD and mild cognitive impairment, DTI has also shown early disruption of whole-brain white matter connectivity and long-range tracts such as the cingulum, uncinate fasciculus, superior longitudinal fasciculus, inferior fronto-occipital fasciculus, and corpus callosum [152–157].

There is growing evidence that both TBI and AD target functional neural networks with disconnection occurring before other neuropathology [158–164]. In AD, resting state functional MRI classically shows early dysfunction of the default mode network (DMN) in the precuneus/posterior cingulate and the mesial temporal lobe, even before any clinical symptoms [165–173], with an eventual loss of cross-network relations as the severity of AD increases [174]. After mild TBI, resting state functional MRI also shows abnormalities in neural networks [125, 175]; there is decreased connectivity of the DMN immediately after TBI which then increases during later stages, possibly as a form of compensation for injury to other neural networks. Investigators report the development of hyperconnectivity of the DMN associated with repetitive subconcussive mTBI [176]. In contrast, there may be hyperconnectivity in frontally-based executive and salience networks in AD [177–179]. A possible explanation for these findings is that TBIs promote AD through enhancing the early impairment of the DMN, but later diverge with subsequent opposite effects as other neural networks become involved.

CONCLUSIONS

This literature clarifies several aspects of the TBI-dementia relationship. First, the risk of dementia increases with increased severity, frequency, and repetitiveness of TBIs, but does not dependent on the type of physical force to the head. This finding suggests a cumulative effect of violent head displacement with consequent brain deformation, shear forces on axons, and DAI. Second, the impact of these forces depends on individual vulnerabilities of the patient's brain. Factors affecting vulnerability include cognitive and neuronal reserve, age at time of TBI, and the presence or absence of an APOE4 allele. Third, the effects of TBI are more general than effects on AD, extend to other neurodegenerative disorders, and may suggest a less specific pathophysiological mechanism. TBI is a risk factor for FTD, PD, and possibly ALS as well for AD. This is also suggested by a general effect of lowering of the age of onset of dementia and the addition of TBI cognitive-behavioral features. Fourth, the pathophysiology of TBI is reflected in the neuropathology of CTE, the occurrence of abnormal proteinopathies, and the disruption of white matter tracts and neural networks. One putative mechanism from these results is the release and accumulation of Aβ, hyperphosphorylated tau, and other abnormal proteins along white matter tracts and networks. Additional potential mechanisms include neural network disruption, neuroinflammation, abnormal calcium signaling, vascular abnormalities, and others.

This synthesis of research on the relationship of TBI and dementia is a preliminary assessment of a rapidly growing literature. The conclusions from this review are mitigated by significant reservations, including recall bias on reporting the presence,

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number, and severity of TBIs as well as major methodological hurdles in establishing causality and not just association between TBI and dementia. Future research is need in order to establish whether dose-dependent effects of head displacement in vulnerable brains damages axons and, thereby, initiates a neurodegenerative process. More work is specifically needed on post-traumatic biomarkers that may predict cognitive decline, the relationship of clinical dementia syndromes with the neuropathology of CTE, and the effects of neural network disruption and potential abnormal protein propagation. In sum, this review can stimulate future studies on the important relationship between TBI and AD, CTE, and other forms of neurodegeneration with potential dementia.

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Brain Injury in the Context of Tauopathies

Jose F. Abisambra* and Stephen Scheff
Sanders Brown Center on Aging, University of Kentucky, Lexington, KY, USA

Abstract. Traumatic brain injury (TBI) is the most common form of head injury and is a leading cause of death worldwide. Due to the vast variability in the types and severity of trauma, the cellular consequences of head injury are not completely understood. The development of reliable models of TBI will aid in understanding the molecular consequences of head trauma, and they will assist in identifying biological surrogate markers of the degree of damage and prognosis. In doing so, effective therapeutic strategies can be applied. Current *in vivo* experimental models yield important information, but they too have a significant amount of variation. The goal of this review is to re-evaluate the use of these *in vivo* models of TBI and assess whether they correlate with the consequence of TBI in humans from the perspective of tau, an axonal microtubule-stabilizing protein. We present and discuss the current models of traumatic head injury, and we focus on those that assess changes in tau. We evaluate reports of TBI in humans that measured changes in tau and that were detectable in serum and cerebrospinal fluid, and as a pathological consequence in brain tissue.

Keywords: Alzheimer's disease, chronic traumatic encephalopathy, tau, tau post-translational modification, traumatic brain injury

INTRODUCTION

The variety and severity of head trauma results in multiple sequelae, which are difficult to diagnose. As a result, there is a vigorous effort to understand the pathological consequences of head injury. In doing so, biological markers that will correlate and predict clinical outcomes for head injury could be identified. Successful detection of these biomarkers will help establish differential diagnoses and therefore propel more appropriate interventions for the improvement of clinical outcomes and quality of life.

Cognitive impairment of American football players who suffered head trauma has raised public awareness to this issue. Similarly, the havoc of women and men in the military exposed to blast injuries has drawn attention to head injury prevention and research. In conjunction, head injury is a risk factor for Alzheimer's disease (AD), the most common form of dementia.

Tau aggregates and neurofibrillary tangles that are characteristic of AD and other tauopathies have been identified in the brains of head injured patients [1]. Therefore, understanding the pathological consequences of tau in tauopathies like AD might shed light on the molecular mechanisms of tau-mediated cell death in head injury. Currently, 50% of the population 85 and older has AD. Moreover, the number of AD diagnoses is predicted to rise from 5.2 to 13.8 million people in the United States by 2050 [2]. This is partly due to successful treatments for other leading causes of death such as cardiovascular disease and cancer [2]. Despite autosomal dominant mutations as pathogenic cues for the familial form of AD, the vast majority of patients present an idiopathic and sporadic neurodegenerative process.

The study of the molecular mechanisms resulting from head injury may help explain the sporadic presentation of AD and other neurodegenerative disorders to which tau is linked. The involvement of tau in the pathogenesis of AD and other tauopathies

*Correspondence to: Jose F. Abisambra, Sanders Brown Center on Aging, University of Kentucky, 800 S Limestone Street, Lexington, KY 40536-0230, USA. Tel.: +1 859 323 6040/Ext. 488; E-mail: joe.abisambra@uky.edu.

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has attracted attention of this protein as a protagonist in the spectrum of brain injury. Tau's localization, function, and aberration in disease, as well as its bioavailability post injury, make it a potential therapeutic target and a candidate to predict head injury outcome.

Studies focusing on tau and brain injury show inconsistent data. The reason for these discrepancies is unclear, but it may reside in the many types and severity of head trauma, the variety of research models, and the tools available for identification of tau. Nonetheless, the strides achieved in understanding tau biology, the evolution of imaging techniques, and sensitivity of diagnostic instruments for protein detection warrants a new perspective on biomarker identification for head injury.

In this review, we (1) provide a brief introduction of brain injury by discussing its complexity and how it is classified into different subtypes. We also introduce fundamental concepts of tau biology that will help explain the difficulty in tracking changes in tau after brain injury. Then, we (2) present and discuss the different animal models of a specific form of head trauma classified as traumatic brain injury (TBI). This section will compare the most commonly used models. In addition, (3) we discuss all the brain injury-mediated changes to tau that have been described to date; this section is divided into five subsections that describe how TBI affects tau in the brain and tau modifying proteins, TBI's differential effects on amyloid and tau, and tau changes in the CSF and in serum.

BRAIN INJURY

It is difficult to differentially diagnose the multiple subtypes of head injury due to the vast and diverse forms of symptoms. This is partly due to the different types of injury, location of trauma in the head, types of impacting object, force, and even confounding genetic risk factors for the long-term consequences of brain injury [3]. In general terms, head injury is divided in two categories (summarized in [4]).

The first group corresponds to single events of head trauma, and they include concussion, acute brain injury, and mild TBI. Concussions are the mildest form of trauma in this group, and patients often present cephalalgia, vertigo, nausea, hypoprosexia, and memory problems, yet they do not show associated pathology. More severe concussions have all of the above accompanied by loss of consciousness. Post-concussive syndrome is the continuing stage where patients have more pronounced and longer lasting concussive symptomatology [5]. In the most severe cases, a single injurious event will result in subdural hematoma and death; this is termed catastrophic head injury.

The second classification of head injury corresponds to cases of repetitive head trauma and concern chronic head trauma and chronic traumatic encephalopathy (CTE, also known as dementia pugilistica). The clinical outcome in these patients is very diverse. As a consideration feeding into the variability is the individual's profession and the inherent risk of head injury; for instance, head trauma is prevalent in athletes. A unique group of brain injury-prone athletes are boxers, as data directly related to head trauma is a part of the athletes' performance. Correlation studies in professional boxers show that pathology is directly proportional to the number of bouts and length of career [6]. Pathological signs include cortical and hippocampal atrophy and dilated perivascular spaces. Professional athletes who suffered repetitive injuries show symptoms only years after retirement, and 30% of these cases are progressive [7].

The primary sign of TBI is diffuse axonal injury (DAI), which consists of axonal shearing [8]. Axonal shearing might distort and break microtubules thereby releasing tau; complemented by compromise of plasma membrane integrity, tau might exit into the extracellular milieu before it aggregates intracellularly (Fig. 1). This idea supports that the extent of DAI correlates with severity of the injury; moreover, it correlates with the abundance of tau in cerebrospinal fluid (CSF) and serum. Therefore, tau levels in CSF and serum are proportional to the severity of damage [6]. However, the link between severity of injury or injuries and serum biomarkers is less consistent.

TAU

Much of tau research focuses on a group of diseases that share the same pathological hallmark: abnormal aggregation of tau inside brain cells. These diseases, tauopathies, are composed of about 17 neurodegenerative disorders of which AD is the most common form. In 1973, Corsellis et al. discovered neurofibrillary tangle (NFT) deposits in the brains of professional boxers with CTE, linking this disorder to the list of tauopathies [9]. However, it is not clear

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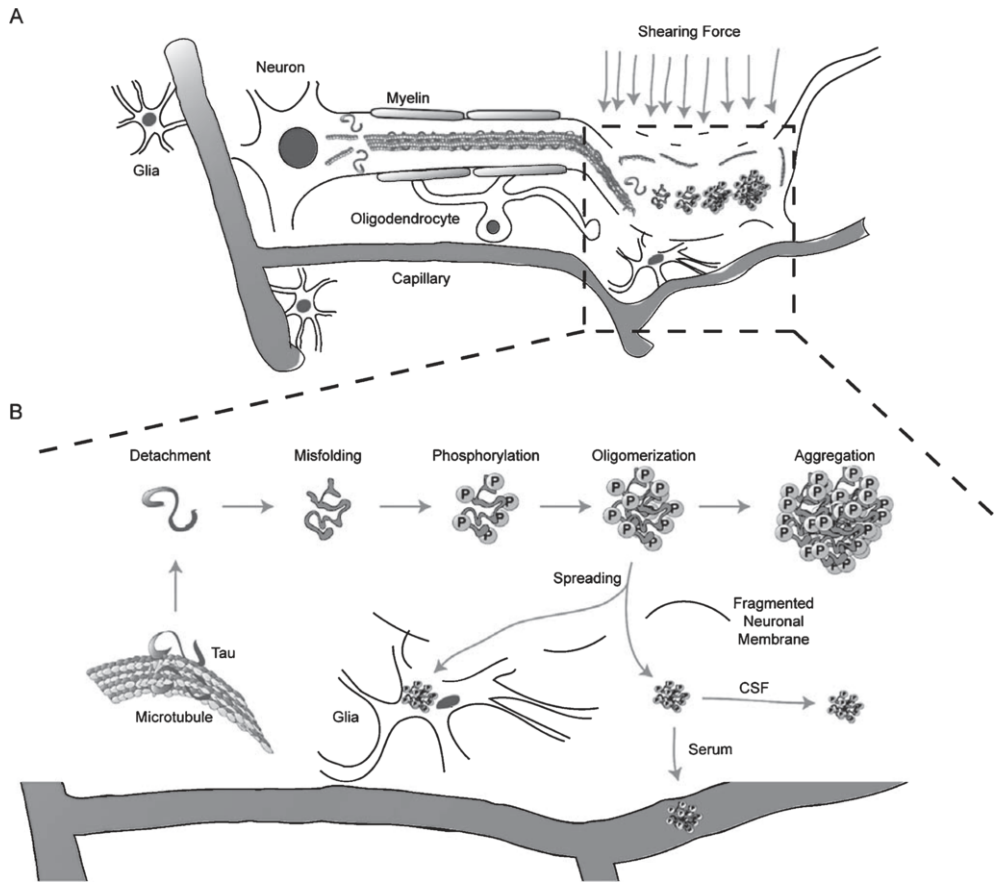

Fig. 1. Proposed molecular effect of head injury on tau in neurovascular areas of the CNS. A) Shearing forces that occur in DAI after a head injury damage neurons, glia, oligodendrocytes, and blood vessels. Damage to any or all of these cells leads to trophic uncoupling, and the entire unit bears dysfunctional consequences. Axons contain a high amount of tau that is normally associated with microtubules. Shearing forces destabilize the integrity of the cell membranes, and in the axon, the microtubule network. B) Closer view of the damaged area. Upon microtubule destabilization, tau detaches from the microtubule. Tau can then lead into a pathological pathway where it can be misfolded, phosphorylated (and hyperphosphorylated), adopt a soluble oligomeric conformation, aggregate, and/or spread. Tau spreading can infiltrate other cells (such as neighboring glia), the serum, and/or CSF.

whether tau participates in the neurological damage initiated by the traumatic event. During AD pathogenesis, the levels of specific tau species change over time in brain tissue, CSF, and serum, and this is also true of TBI and TBI models [10–25]. In TBI, the events leading to tau release may be varied and complex, and the type of modified tau species may vary as well.

The normal function of tau is to promote elongation and stabilization of microtubules, and this is dependent on post-translational modifications. While in a low or no phosphorylation state, tau stably binds to microtubules; contrarily, phosphorylated tau has lower affinity for microtubules [26–28]. These modifications on tau are in the protein sequence flanking the microtubule-binding region, the latter of which is composed of four repeats that are coded by exons 9–12 of the *MAPT* (microtubule associated protein tau) gene (Fig. 2). Changing the phosphorylation state will alter tau's affinity for the microtubule. Therefore, microtubule stability is at the mercy of kinases and phosphatases that act on tau [29–32].

Another important tau modification is alternative splicing, which yields differently sized tau isoforms. *MAPT* codes for six splice variants in the adult human brain [33]. Excision of the exon 10-coded transcript leads to production of three-repeat tau (3R tau), while expression of exon 10 generates four-repeat tau (4R tau). Normally, the ratio of 3R to 4R is 1:1 in the adult brain [33]. However, the balance may shift in particular diseases.

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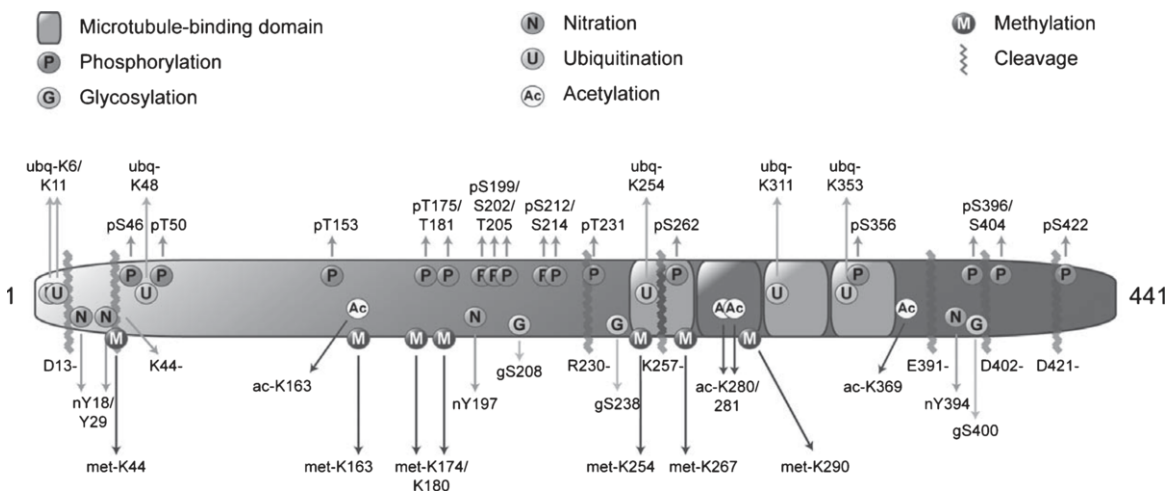

Fig. 2. Schematic of tau post-translational modifications that have been validated *in vivo* and in mammalian cell culture models. Post-translational modifications include four microtubule-binding domains, the second of which is subject to alternative splicing. Schematic depicts 4R0 N tau. Amino terminal splice variants are not shown.

Tau pathology is almost exclusively composed of 4R in patients suffering from progressive supranuclear palsy, corticobasal degeneration, and argyrophilic grain disease [34–38]. Meanwhile, 3R tau is predominant in deposits of brains with Pick's disease [39, 40]. Yet, an imbalance in tau sub-pools is not necessary for disease. In AD, tau tangles are made up of both 3R and 4R tau at a similar ratio [38, 41]. However, there is a frank shift in the ratio of 3R to 4R tau mRNA in specific brain sites such as in cholinergic basal forebrain neurons within the nucleus basalis and CA1 hippocampal neurons [42]. Whether this translates into changes in the protein levels is not yet clear, but it is possible that localized changes in gene expression that alter the 3R/4R ratio may underlie pathogenesis. Therefore, changes in the ratio of 3R to 4R tau may serve as correlates of specific disease states in specific locations, and assessment of changes in the ratio could be utilized to monitor the course of brain trauma recovery.

In addition to phosphorylation and splicing, tau is subject to glycosylation, glycation, ubiquitination, prolyl isomerization, nitration, poly-amination, sumoylation, oxidation, acetylation, and methylation (Fig. 2) [43–58]. Cleavage enzymes also process tau yielding truncated fragments, and these activities may be enhanced in disease [49, 59–64]. For instance, cleaved tau species are detectable in brain and CSF of individuals who suffered brain trauma [65]. Together, these post-translational modifications may correlate with different degrees of severity of the traumatic event and the course of disease. Part of the complexity is rooted in the implications of the post-translational modifications on tau's structural properties. For instance, insoluble tau will be more prone to deposit within brain cells. This insoluble pool of tau is best characterized to date in postmortem tissues of individuals who suffered tauopathies compared to bioavailable tau in CSF and serum.

Tau aggregation status also determines its ability to spread between cells. For instance, oligomeric tau species spread between cells, while aggregated insoluble tau does not. Tau spreading occurs in disease [66, 67], and this feature may also be shared in pathological processes initiated by head injury. It is possible that pathways for protein degradation like autophagy and the proteasome become saturated due to the sudden TBI-initiated protein excess [68, 69]. Tau could exit the cell as in recently described mechanisms of tau spreading [70, 71]. In a damaged cell where the plasma membrane is destroyed, remaining cytoplasmic proteins like tau might exit the cell and be taken up by neighboring cells, further substantiating trophic uncoupling. Depending on the location of the damaged, tau-releasing cell, tau may cross into the CSF and the vasculature (Fig. 1).

Tau pathology is inherently linked to CTE. Histologically, 133 of 140 reported cases of CTE show tau pathology. Interestingly, tau deposition was the reason for CTE diagnosis among many of the 140 cases. Unfortunately, biochemical data are only available from two cases of boxers with CTE [5]. When

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compared to *bona fide* AD tangles, these inclusions were distinct in form and tau isoform ratio. Therefore, tau has become a critical aspect of CTE postmortem diagnosis, yet the mechanism of tau deposition may be unique to CTE compared to well-established tauopathies. These mechanisms are poorly understood, showing that the study of tau in TBI is in its infancy.

ANIMAL MODELS OF TRAUMATIC BRAIN INJURY

Since many models of head injury involve rodents, it is important to highlight the differences between human and rodent tau, which are coded by the *MAPT* and *Mtapt* genes, respectively. Mouse and human tau protein sequences are 88% identical for the longest central nervous system (CNS) tau protein isoform, 4R2N [72]. Interestingly, the microtubule-binding domain between both species is 98% identical.

The human adult expresses both 3R and 4R tau isoforms; in contrast, the adult rodent brain only expresses the 4R tau isoforms [73–75]. This is interesting because wild-type mice, AβPP transgenic mice, and even transgenic mice that express human *MAPT*gene (coding for all 3R and 4R isoforms) do not have tangle pathology (reviewed in [76]). However, tau tangles are present in transgenic mice that express disease-associated mutant forms of tau or human wild-type tau while endogenous mouse tau expression is knocked out [77–80]. The greatest divergence between human and mouse tau is in the amino terminus where the human has 14 additional residues coded throughout exons 1, 2, and 5, and two fewer coded by exon 7. These amino-terminal and microtubule binding domain differences might play a role in the protective effect of mouse tau over human tau in transgenic models.

A large number of different paradigms have been developed for animal experimental modeling of TBI. These have included the use of iron pendulums [81–83], humane stunners [84–88], lead tipped darts [89], modified rat trap [90], reflex hammer [91], rotary striker [92], blasting caps [93] and gas pistons [94]. Much of the very early studies involved non-human primates, mongrel dogs, and cats. Other studies involved rabbits, guinea pigs, and miniature swine. The use of small rodents such as rats for experimental TBI [85, 95, 96] did not really occur until the late 1960s and early 70s. This is somewhat surprising since a great deal of work in the area of learning and memory involving ablation studies in rats was popular in the 1940s and 50s. There are several excellent reviews of these animal models of TBI [97–99].

The primary goal of any animal model of TBI is to produce an injury that is reproducible and has at least some relevance to the clinical condition. Because the cascades attributed to human TBI are extremely complex and in many aspects unique to human anatomy/physiology, no animal model can totally reflect all the attributes. For any animal model to be truly useful, the extent of the morphological/physiological injury must be proportional to the mechanical force applied. It must also have the ability to mimic a wide range of injury severity that can be manipulated by the investigator by adjusting one or more independent variables. It is important that the dependent variables are clearly defined and quantifiable. Finally, an animal model must be amenable to replication by numerous other investigators and laboratories. An injury model that requires a unique skill set by the investigator or requires a device that cannot be replicated has little to no utility.

The weight drop, fluid percussion, and cortical contusion injury techniques are all models of closed head injury regardless of whether or not a craniotomy is performed. These models mimic a contusiontothe cortical surface without truly penetrating it and should not breach the dura. In most cases, when a craniotomy is performed, it is resealed immediately after the injury. The idea behind the different models is to control the location, magnitude, and histopathology associated with the injury, so that future pharmacologic studies can have a baseline from which to judge the utility of treatment.

Weight drop

One of the simplest forms of injury is the weight drop (WD) model in which a free falling weight of a known mass is guided onto a specific area of the animal's head. One of the earliest reports using this model was in adult dogs by Pilcher in 1937 [100]. This study is important since it described the fact that the animal's head was immobilized during the impact resulting in a failure to detect any significant edema. Subsequent experimentation by Denny-Brown and Russell [101] demonstrated that the brain needs to have significant acceleration-deceleration for significant edema. An early study by Walker and colleagues [102], injured cats with a weight falling through a glass tube onto a column of water that was in contact with the exposed dura. Injury severity depended

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upon the height and mass of the weight. A similar WD model using rats came from the Feeney laboratory [103, 104]. In this model, a craniotomy was performed, and a stainless steel circular "footplate" at the end of a guide tube rested on the exposed dura. A weight falling through the tube then struck the footplate contusing the brain. The result was a focal lesion resulting in a necrotic cavity.

More current models have used a variety of techniques in both rats and mice. In rodents, the WD can be used without any prior surgery or with the skull exposed. Both of these variations can create a diffuse type of injury. There are remarkably few models that have injured rats and mice without exposing the skull. While the rat studies used somewhat elaborate instrumentation [105, 106], the mouse models were very simple and resulted in considerable variability [107–110]. The primary advantage of this model is the simplicity of the animal preparation and anesthesia requirements, which can be quite minimal. In one case, animals were injured without anesthesia [107]. There are two different techniques that have been used when the skull is exposed prior to injury. These two preparations differ in the type of neuropathology that is elicited. Shohami and colleagues use a rather simple technique in which a calibrated long vertical metal rod is guided by the aid of two horizontal platforms towards the animal's exposed skull. The tip is flat and small and can be used to deliver an impact onto one side of the skull. This model has been characterized for both rats [111] and mice [112, 113]. In this model, the animals are anesthetized and placed prone onto a hard surface without any restraints. The result is a somewhat focused injury. The best characterized WD model for the rat is called the Marmarou model [114, 115]. This model results in a more diffuse injury than the Shohami model. With the Marmarou model, a metal disc is cemented onto the exposed rat skull vertex prior to injury. When the predetermined brass weight falls through a Plexiglas guide tube and hits the disc, which absorbs the force of the impact, the injury is spread more evenly over the skull of the animal. In most cases this prevents the skull from fracturing. The special foam pad that the anesthetized animal lies upon is also extremely important to the model because it produces the effects of an acceleration/deceleration that appear to make this model more translational. Currently a mouse version of this model is under development. A common criticism of the WD model is the possible repetitive injury as a result of the impacting weight "bouncing". Both the Marmarou and Shohami models result in considerable neuropathology including hemorrhage, neuronal cell loss, glial activation, and DAI.

Recently there have appeared reports, for both rats [116, 117] and mice [118], using a pneumatic impactor to hit a disc cemented on the exposed skull. This modification to the Marmarou model would eliminate the possible rebound effect. One limitation is the fact that experimental animals need to be constrained when using an impactor to strike a metal disc. Three rather novel variations using a pneumatic impactor hit the skull without any surgical preparation [119–121] and may be applicable for repetitive injuries. As with all WD models, there appears to be high variability in the degree of injury severity and histopathology.

Fluid percussion

The fluid percussion injury (FPI) has been extensively used in animal models of TBI and currently is one of the most common. A rudimentary FPI model was first described by Groat in the cat [122] and then later in a more elegant form in the rabbit by Lindgren and colleagues [123–125] and the cat by Sullivan [126]. Typically, the animal is anesthetized and a craniotomy is performed either over the midline suture or lateral to this cranial landmark. A plastic interface is attached to the skull and the experimental subject is then tethered to the FP device. This custom device consists of a fluid filled reservoir with a compressible fitting at one end. A pendulum falling from a predetermined distance strikes the fitting and creates a pressure pulse in the tube that results in a small amount of sterile saline applied to the intact dural surface via the plastic interface. The injected fluid then moves in the epidural space concentrically from the injection region. The height of the pendulum at the point of release determines the magnitude of the injury. A transducer at the end of the reservoir monitors the pressure (atm) used to inject the fluid. A rather interesting alternative to the pendulum was reported by Dixon et al. [127] in which a gas-accelerated piston was used to drive the saline into the intracranial cavity.

The placement of the craniotomy and plastic interface plays a significant role in the type of injury. Originally, the model was set up using a midline location, and many of the early cat [128–130] and rat [131, 132] studies used this placement. Subsequent modifications placed the craniotomy and interface laterally [133]. This placement is currently one of the most popular and well characterized [134]. There

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has been some discussion as to the lateral placement of the initial craniotomy position and the subsequent differences in the magnitude of cortical damage and hippocampal cell loss [135, 136]. Although the FPI technique was originally designed for the rabbit and cat, it has been modified/adapted for multiple different other species besides the rat. There are multiple reports of FPI used in dogs, sheep, and piglets [137–139]. Importantly, the lateral FP technique was also adapted for mice [140–142], which makes it a useful tool for studying changes in transgenic animals.

The midline FP model produces a subdural hematoma and significant breaching of the blood-brain barrier (BBB). Because of the effects on the brainstem there is usually a relatively high mortality rate. The lateral injury placement produces a cortical lesion along with significant cell loss in the hippocampal formation and thalamus. There is also considerable axonal injury in both the corpus callosum and internal capsule. Accompanying these changes is a disruption of the BBB and considerable gliosis [98].

Cortical contusion injury

In the late 1980s, Lighthall and colleagues working at the General Motors Research Laboratories developed a controlled cortical impact model of mechanical brain injury in the ferret [143]. In this particular injury model, a craniotomy is made over the brain region of interest leaving the underlying dura intact. A rod fitted with an impactor tip powered by compressed air cylinder then strikes the dura and compresses the underlying brain tissue. The investigator can vary the velocity and depth of the compression, along with the duration of the insult thus manipulating the injury severity. A major advantage to this model is this ease in altering the injury severity from extremely mild to severe [144]. This model was quickly scaled down for use in both rats and mice and has been used in miniature swine [145, 146]. The early studies involving rats used a midline contusion with the craniotomy centered over the parietal region [127, 147–149]. Subsequent studies impacted only one hemisphere but used a bilateral craniotomy that produced a bilateral injury [150–152]. The most common technique now is to use a single craniotomy placed laterally over the parietal region [144, 153–156]. The first mouse study [157] to use the cortical contusion injury (CCI) model used the lateral craniotomy site and reported significant pathophysiology including hippocampal cell loss. This injury also produces considerable cognitive dysfunction in mice [157, 158]. While most groups now perform unilateral contusions centered over the parietal area, Stein and colleagues developed a bilateral frontal cortical contusion injury [159].

The CCI model results in focal neuronal cell loss in both cortex and hippocampus, along with gliosis, activation of neuroinflammatory cascades, breach of the BBB, and behavioral/memory dysfunction. Unlike the FP and some WD models, the CCI model does not result in any significant brainstem damage. Consequently, there are minimal deaths with this type of injury. However, this TBI model can result in epilepsy similar to that in humans [160, 161]. One major disadvantage is the fact that, unlike the FP model where there are primarily only two manufacturers of the injury device, multiple different renditions of the CCI are available. Consequently, it is extremely difficult to compare results between laboratories. Unless the pneumatic cylinder is very rigidly mounted, there can be considerable "over shoot" of the impacting tip depending upon the amount of velocity used. Determining the actual depth of a compression can also be problematic because there does not appear to be any standard in determining cortical surface as a zero point prior to the injury. In most cases the craniotomy is only slightly larger than the diameter of the impacting tip making visual determination virtually impossible. Two firms make devices that have a mechanism for determining dural surface to use as a reference depth prior to impact making the outcome more consistent [162, 163]. Methodologically it is important that the craniotomy site be resealed so that subsequent brain swelling can contribute to increased intracranial pressure, which more closely mimics the human condition.

Blast injury

In the last three to four years, there has been a surge in the development of blast injury models to study TBI in response to the increased number of fatalities and medical issues associated with wartime conflicts. When a blast occurs, there is an immediate increase in atmospheric pressure (peak overpressure) followed by an exponential decay and a negative pressure, with an eventual return to normal atmospheric pressure. One of the earliest models investigating cognitive dysfunction in rats [164] used a device that was originally characterized in 1998 [165] using a wide

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variety of different species. Nakagawa and colleagues [166] studied cortical hemorrhages and microscopic changes in cortical neurons in rats following a blast injury and described mild to moderate brain injury using a micro-explosion device. There are basically two different types of animal blast models: chemical explosion and gas-driven membrane rupture. The chemical explosion model has been primarily used on adult swine [167–169] and non-human primates [170], while the membrane rupture techniques has been used exclusively in rodents. The techniques used to apply the blast differ considerably between laboratories and there does not appear to be a commercially available device, thus there are no real standards. This makes interpretation and comparison of data between laboratories extremely difficult. To further complicate this field of neurotrauma, sometimes the animals are exposed to blasts with [171–176] or without [164, 177–183] protective covering of the torso and/or limbs.

Almost all of the rat studies have concentrated on histopathological changes [171, 173–176, 180, 181, 184] and only two studies reporting cognitive deficits [164, 182]. All of the mouse studies report changes in both histopathology and cognition [172, 177, 179, 183]. The degree of histopathology varies greatly between studies although DAI and edema are common findings. Several studies have reported cerebral contusions, BBB breakdown, and gliosis. There does not appear to be very pronounced gross pathological change. The histopathology variability may be a consequence of differences as to the location of the animal inside or outside of the blast tubes at the time of injury.

Repetitive injuries

There are several reports in the literature using various TBI animal modelstoinflict repetitive mild injuries in order to test for possible effects of preconditioning or exacerbation of histopathology. Most of the studies use mice and employed either a WD model without exposing the skull [110, 185, 186] or a CCI device with [187, 188] or without a modified tip [189]. Rat studies have used both a pneumatic impactor without surgical intervention [119, 190] or the FP model [191]. Numerous histopathological and cognitive changes have been noted, but because of the unique characteristics of the different injury models it is extremely difficult to make any type of generalizations.

TAU AND TBI

Tau neuropathology after TBI

In the TBI papers that are discussed herein, the authorsperformapathological assessmentofthe brains to detect unique tau features that change as a result of the traumatic event or animal model. A common approach to identifying tau-based effects is to discern changes in the levels and distribution of different types of tau species. Research groups use immunohis-tochemical staining, ELISA, and western blotting with antibodies that detect many forms of tau (Tables 1 and 2), including full-length, cleaved, and phosphorylated tau variants identified with antibodies that recognize pS396/S404 (PHF1), dephosphorylated S199 (Tau-1), the carboxy-terminus (Alz5), and the amino-terminus (Alz50), among others [12, 14, 192–196]. Yet, these antibodies are not concertedly used in all studies, thereby adding potential inconsistencies between the conclusions of each study.

The most commonly used antibody is PHF1, which recognizes the pS396/S404 tau species that is prevalent in late-stage NFT of AD [10, 197, 198]. Phospho-S396 tau is abundant in the hippocampus, entorhinal cortex, neocortex, brain stem, and spinal cord of two well-characterized CTE brains [14]. Similar to other tauopathies like AD, PHF1-tau accumulates in soma and axons of neurons throughout the brain; however, unlike AD, glial cells show PHF1-tau immunoreac-tivity [14, 193, 198]. These glial inclusions are not the *bona fide* tangle pathology as observed in AD, and they vary from a diffuse to focal distribution. Moreover, neuronal staining is stronger than glial, which is not surprising since tau is primarily expressed in neurons [199]. In fact, PHF1 is prevalent in distrophic axons. These signs could be used for differential diagnoses to rule out AD.

Another interesting finding is that tau pathology appears in perivascular neurons, suggesting an intrinsic relationship of susceptibility to TBI between neurons, glia, and vasculature [193]. This emerging concept of "trophic uncoupling" in the brain is also considered to be at play in the pathogenesis of vascular dementia. It suggests that the proximity of these multicellular interfaces creates a microenvironment of trophic communication, and dislocation of one element leads to instability and death of the others.

PHF1 staining is less consistent in mouse models (Table 1). Unlike in rats, changes in mouse tau are less pronounced in the CCI model of TBI. For instance, CCI does not induce tau pathology in wild-type mice,

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Table 1
Studies in animal models of brain injury studying tau

Finding	Species	Model	Sample (size and age)	Severity	Brain tissue	CSF	Serum	Method	Antibody	Ref.
Tau is increased in CSF post injury	Rat	CCI	>90 adult rats	varied	N/A	Increased	N/A	ELISA	Innotest hTau	[17]
Calpain-cleaved tau increases in CSF	Rat	FP /transient forebrain ischemia	$N = 3$ per time point per severity;	mild and moderate	N/A	Increased	N/A	WB	tau-1 chemicon	[18]
cTau is a biomarker of TBI; increases with severity and over time	Rat	CCI	adult $n = 120$; adult	varied	cTau increased over time and with severity except in contralateral cortex	N/A	cTau was highest and only significant at 6 hours	ELISA, WB, IHC	cTau 7, 8, 12	[19]
Mild repetitive TBI does not induce NFT in non-Tg mice, and it only increased NFT in 1of 18 mice	Mice (T44, which express the smallest human tau isoform)	Mild repetitive TBI	18 T44 versus 24 non; 12 months old	mild	Extensive telencephalic NFT in only 1 mouse after 9 months, which also displayed cognitive deficits	N/A	N/A	IHC, WB	T1, PHF1, PHF6, AT270, 12E8, AT8	[212]
Serum tau increases over time and with severity of injury	Rat	Feeney model of mild and severe TBI	22 rats (6 sham, 8 mild, and 8 severe); adult	mild and severe	N/A	N/A	Tau increased and peaked at 1 day was more abundant with severity	ELISA and WB	Biosource kit and Santacruz hTau	[21]
pTau species increase in TBI brain	Rat	Bead implantation/compression	79 at 1.5 m/o	N/A	pT231 and pS262 increased; total tau did not change	N/A	N/A	IHC and WB	Tau SC, pS262 SC, and pT231 calbiochem	[209]
Cleaved tau increases with TBI	Rat	CCI	$n = 6$; adult	N/A	Increased cleaved tau (35 and 15 kDa fragments	N/A	N/A	WB	Tau-1 Cedarlane (total tau) and custom calpain-cleaved and caspase-3-cleaved tau	[22]

(*Continued*)

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Table 1
(*Continued*)

Finding	Species	Model	Sample (size and age)	Severity	Brain tissue	CSF	Serum	Method	Antibody	Ref.
Tau pathology increases in brain	Mice (tau findings determined in Tg P301L tau)	CCI	$n = 6$; 6 m/o	N/A	Severe pathology which changed vastly in different regions over time	N/A	N/A	IHC	total tau (Pierce), PHF1 and pS199 (invitrogen)	[25]
Tau pathology increases after severe TBI	Mice (3xTg)	CCI	48 mice between 5–7 months	varied	Pathology with multiple antibodies	N/A	N/A	IHC	pS199 and PHF1	[24]
Total tau increases in serum	Rat	Scalable TBI by rotational acceleration	75 adult rats	mild, moderate, severe	N/A	N/A	Significant increases at all time points	RPPM	hTau Santacruz	[20]
Blast injury increases tau	Mice	Blast injury	8–10 per group; 2.5 m/o	N/A	Filaments in perivascular neurons and glia	N/A	N/A	IHC	CP13, AT8, PHF1	[193]
Tau accumulates in blebbed axons	*in vitro* rat cortical neuron stretch model	Axonal stretch model	N/A	N/A	N/A	N/A	N/A	IF/TEM	less than 30% of microtubules in an axon rupture	[202]

CCI, controlled cortical impact; CSF, cerebrospinal spinal fluid; ELISA, enzyme-linked immunosorbent assay; FP, fluid percussion; IHC, immunohistochemistry; N/A, not applicable; NFT, neurofibrillary tangles; TBI, traumatic brain injury; Tg, transgenic; RPPM, reverse-phase protein microarray; WB, western blot.

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Table 2
Human TBI studies focusing on tau

Finding	Conditions	Sample size	GCS	Source of CSF	Brain tissue	CSF	Serum	Method	Antibody	Notes	Ref.
cTau is elevated in CSF of TBI patients	Severe closed head injury	15 (v-CSF) versus <5 control groups with 5–15 samples in each group	≤8	TBI and <5 hydrceph: v-CSF; all other controls l-CSF	Tau proteins increase: full-length and cleaved as seen by WB with PHF1, tau 1, Alz5, and Alz50	cTau and PHF1 in CSF	N/A	ELISA and WB	C7, C8, and C12, PHF1, tau 1, Alz5, and Alz50	CSF was ventricular for head injury and non-hydro controls	[192]
CTE pathology contains tau aggregates in glia	Repetitive TBI	2 CTE, 3 AD, and 1 LBD	N/A	N/A	pTau pathology is similar to AD, but unique due to glial tau aggregates and broader distribution	N/A	N/A	WB and IHC	Ntau, Tau12, T14, AT8, PHF6, T3P, and tau 46		[14]
cTau levels in serum correlate with injury and outcome	Closed head injury	17 CHI versus 11 controls	≤10	N/A	N/A	N/A	cTau increased in serum	ELISA	cTau12	Serum collected within 10 hours post injury	[208]
Total and cTau levels are increased post severe TBI	CSF collected every 24 hours; severe head injury	28 with severe head injury, 87 neurologic controls, 67 non-neurologic controls	3 to 8 after resucitation	l-CSF	Increased levels of total tau and cTau fragments	Increased levels of cTau; total tau levels were not evaluated	N/A	WB and ELISA	BYA and cTau 7, 8, and 12	cTau levels inversely correlated with positive clinical outcome	[12]
CSF tau increases in post TBI as a biomarker	Frank TBI	13 TBI and 13 controls	≤8	v-CSF	N/A	Significantly increased	N/A	ELISA	Innogenetics	CSF was assayed every day for 5 days	[11]
CSF Tau is a good marker of TBI	Frank TBI	29 (TBI), 19 (demented controls), 12 (non-demented with various clinical issues)	≤8	CSF was collected by ventricular pump ($n = 15$) or LP ($n = 14$)	N/A	Tau was significantly increased in more than 80% of TBI samples	N/A	ELISA	Innotest hTau	Tau levels peaked early and normalized completely after 43 days	[13]

(*Continued*)

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Table 2
(*Continued*)

Finding	Conditions	Sample size	GCS	Source of CSF	Brain tissue	CSF	Serum	Method	Antibody	Notes	Ref.
Tau pathology increases with severity	TBI with varying grades if severity	45 TBI brains (15 for each age group) and 15 controls (5 for each age group)	N/A	N/A	Pathology varied with severity and age	N/A	N/A	IHC	Dako	Evaluated sensitivity with age (<20, 20–50,>50 y/o); $n = 5$ and 1 control for each group	[15]
Tau is increased in brain	Severe closed head injury	18 (8 plaque and 10 no plaque)	N/A	N/A	Increased pathology; mixed intensity	N/A	N/A	IHC	17026 for pan-tau and PHF1	TBI brains showed distrophic PHF1 positive axons/somatic tau staining was only present in 2 TBI brains (older).	[194]
CSF tau increased in severe TBI and correlated with clinical outcome (GOSE) score	Severe TBI	39 severe TBI and 20 controls	≤8	v-CSF	N/A	1 year outcome correlated with pTau levels at 2–3 days post TBI.	Not detectable	ELISA	Innogenetics hTau for total tau		[207]
Serum cTau is a poor predictor of clinical outcome post TBI	Mild TBI	35 mild TBI	≥13	N/A	N/A	N/A	cTau levels in serum at 6 hr post injury did not correlate with ouctome Rivermed Post-concussive questionnaire	ELISA	cTau	Hard to control whether cTau was increased at all per patient.	[210]
Normal and abnormal CT head injuries cannot be distinguished by changes in serum tau	Mild TBI	55 normal CT and 33 abnormal CT	>13	N/A	N/A	N/A	No difference between the 2 groups	ELISA	Biosource hTau	The two groups had head injury, but only one group had abnormal CT.	[211]

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Tau accumulates in axons and soma post TBI	Catastrophic head injury	18 and 6 controls	Death after 4h-5 weeks	N/A	2 cases were tau positive with neuronal (stronger) and glial (astrocytes, weaker) signal; mostly PHF1.	N/A	N/A	IHC	17026 for pan-tau, PHF1, and PHF6	APP, BACE, PS1 tau and α-syn together model axonal degeneration. May all be related and comorbidities. Tau pathology can still ensue after inh γ-sec	[198]
Tau is increased in interstitial fluid after severe TBI	Collected interstitial fluid with microdialysis after TBI for 5 days.	8 (3 DAI and 5 mixed)	4 to 8	N/A	N/A	N/A	N/A	ELISA from microdialysis of brain interstitial fluid	Monoclonal antibodies from [213]	Collected interstitial fluid with microdialysis after TBI for 5 days.	[214]
Serum tau is a good predictor of outcome	Severe TBI	35 severe TBI and 10 controls	$\leq$8	N/A	N/A	N/A	Total tau levels increase	ELISA	Biosource hTau	Blood collected within 6 hours after TBI	[21]
NFT develop in 30% of patients with at least 1 TBI event	Single moderate to severe TBI	39 TBI versus 47 controls	N/A	N/A	NFT	N/A	N/A	IHC	Dako tau and thio-S	Effect was only present in patients <60 y/o. NFT persist in people for at least 1 year and throughout 47 years	[16]
Tau pathology appears in TBI brains	Varied types of injury	12 (vets), 4 (athletes), and 4 controls	N/A	N/A	Tau aggregates	N/A	N/A	IHC	CP13, AT8, PHF1	Tau filaments in perivascular neurons and glia	[193]
Tau is increased in CTE	Repetitive TBI	3 boxers and 3 controls	N/A	N/A	Increased levels	N/A	N/A	ELISA, WB (1D and 2D)	HT7	Multiple tau conformers are different from controls and unique within CTE	[196]

AD, Alzheimer's disease; CHI, closed head injury; CT, computed tomography; CTE, chronic traumatic encephalopathy; CSF, cerebrospinal fluid; DAI, diffuse axonal injury; ELISA, enzyme-linked immunosorbent assay; GCS, Glasgow Coma Scale; GOSE, Glasgow outcome score extended; IHC, immunohistochemistry; l-CSF, lumbar CSF; LBD, Lewy body dementia; LP, lumbar puncture; N/A, not applicable; NFT, neurofibrillary tangle; TBI, traumatic brain injury, v-CSF, ventricular CSF; WB, western blot.

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and it only does so in transgenic models where wild type or mutant tau (P301L) is expressed [24, 25, 200]. Even so, not every transgenic model shows significant tau pathology. However, a recent study reported that wild-type mice exposed to blast head injury develop significant tau pathology with abundant PHF1-positive inclusions and pS199/S202/T205 [193]. Therefore, the form of injury determines the type of alteration to tau homeostasis. Another inconsistency is that one study reports that total tau levels (identified by an antibody that detects the first 150 residues of the human tau sequence) did not change after TBI [23]. This example was a unique model where plastic beads were implanted in the epidural space over the right sensorimotor cortex (2 mm caudal to bregma and 2.5 mm lateral to the midline). The data showed that tau was hyperphosphorylated at pT231 and pS262. Interestingly, in AD, these two sites become hyperphosphorylated in pre-NFTs. The explanation for these inconsistencies is not clear, but it is possibly rooted in the severity of the injury and the time at which assays are performed after the damage.

Another consideration is that the amount of tau that is susceptible to changes after TBI may change with age. One study that assessed tau pathology in brain after TBI showed no correlation between brains of injured and control individuals [16]. Interestingly, control brains showed NFT pathology that was not significantly different from TBI samples. However, there was a statistically significant effect when the samples from patients older than 60 were excluded. This shows that the NFT present in controls can confound the amount of pathology, which in turn is not furthered after TBI.

TBI alters tau-modifying proteins

As described earlier, tau undergoes many modifications that include cleavage and phosphorylation. These changes could be the result of changes to the enzymes that process tau due to disease. Therefore, it is important to identify what tau-acting proteins, such as kinases and cleavage enzymes, are modified after a head injury event. Alternatively, tau misfolding, which is inherent to mutated tau in frontotemporal dementia, could be the pathogenic event that facilitates different post-translational modifications that become evidentin TBI.

In the case of TBI, Chen et al. recently showed that using the bead implantation model of brain compression transiently modified the activity of kinases and phosphatases [23]. Specifically, the authors showed activation of tau-acting kinases and abrogation of protein phosphatase 2A activity, a tau phosphatase. The end result was enrichment of phospho-tau species. However, changes were transitory, and it affected enzymes differently. For instance, p38 only became active between 10 and 30 minutes post-compression, while ERK1/2 was active between 10 minutes and 3 hours. This also means that changes in tau may be transitory since many of these enzymes return to normal after one day. Whether these enzymatic changes are sufficient to generate long-lasting effects on tau, mainly by enriching phospho-tau species, has not been assessed.

Similarly, Tran et al. show that tau-modifying kinases are enriched in axons after CCI [201]. One of the most TBI-dependent activated kinases in this study was Jun N-terminal kinase (JNK). The authors inhibited JNK and observed a remarkable reduction of intraneuronal tau aggregation. These data suggest that altering the activation status of tau-modifying enzymes could lead to better outcome for TBI patients.

Another set of studies assessed changes in the levels of cleaved tau. The authors used the antibodies C-tau-7, -8, and -12, which bind to cleaved tau species that range in size between 30–50 kDa. Multiple studies concertedly show that tau is cleaved after brain injury in patients and in murine models [19, 192]. Calpains and caspases are responsible for tau cleavage under these conditions [18, 22], but the steps leading to these phenomena are not clear.

It is possible that the impact of DAI on brain cell health may play a role in TBI-mediated tau pathology. The effect of axonal shearing during DAI ruptures 30% of microtubules. Consequently, tau, an intrinsically disordered protein, becomes exposed in the compromised intracellular environment disturbing proteostasis and initiating cytotoxic cascades [202]. It is possible that tau cleavage is a cellular response to more easily degrade the pool of free tau [203]. More severe cellular damage would activate the caspase cascade leading to apoptosis, and tau could simply become a bystander substrate of caspase-3 [60].

The paper by Chen et al. also highlights the many short-lived changes in tau-processing enzymes after the injury. It is possible that compensatory pathways aiming to reduce the expression and activity of kinases become active over time thereby normalizing the effects on tau. This substantiates the importance of time post-injury on TBI-dependent tau process-

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ing. Therefore, any attempt to alter tau-modifying enzymes would likely have to be done very quickly after the traumatic event.

Tau versus amyloid in TBI

In a study by Tran et al. amyloid and tau pathology accelerated after CCI in a 3xTg transgenic mouse model of AD [24]. Interestingly, tau pathology still ensued after CCI despite the treatment with a gamma secretase inhibitor as a means of abrogating Aβ production and amyloid pathology. This is an important finding as it shows independence of the mechanisms leading to tau and amyloid pathologies in an *in vivo* model. This particular model generates both amyloid and tau pathologies, and abrogating Aβ production with Compound E, a gamma secretase inhibitor, suppressed amyloid pathology. These findings do not challenge the amyloid cascade hypothesis in AD, which contends that Aβ abnormalities precede tau pathology; instead, they present an alternative mechanism of tau deposition that could be unique to TBI and distinct from that of AD. Moreover, this alternate, Aβ-independent deposition may be commonly shared with other tauopathies that are Aβ-independent disorders.

It is also important to consider that both amyloid and tau pathologies are at play in a complex manner, and simply establishing that one precedes the other may be unidimensional. One possibility is that tau and amyloid pathological processes potentiate one another (one preceding the other as it has been postulated for AD). Alternatively, tau and Aβ pathologies are independent of each other; this is partly established in the study by Tran et al. and it would be interesting to test even further using microtubule stabilizers or tau-abrogating compounds. Tilting the balance of pathology, by abrogating tau pathogenesis or attenuating Aβ production, would show whether amyloid pathology is contingent or independent of tau.

Importantly, in the Tran et al. study, head injury was necessary for both pathological processes to occur, which implies that these pathogenic pathways are unique to head injury in this model. Moreover, the model is limited to mimic individuals who have genetic predispositions, as the authors discuss in the manuscript. Nonetheless, a major strength of these findings is that the actual form of axonal injury is at the center of the mechanism of CCI-induced tau pathology.

Tau in CSF after TBI

A major drive to detect tau in CSF is its suitability as a biomarker. In humans, full-length, cleaved, and PHF1-positive tau are found in the CSF of TBI patients [11–13, 192]. In fact, some studies show that full-length tau is significantly increased in 80% of TBI patients. Similarly, cleaved tau is detectable in CSF of two rat models of TBI: CCI and FP models [17, 18]. In these studies, the assays used antibodies that detect calpain-cleaved tau and two tau monoclonal antibodies, namely HT7 (human tau residues 159–163, which corresponds to the sequence PPGQK) and BT2 (which detects the full length bovine tau sequence). In addition, one study identified PHF1 tau in CSF via western blot [192]. Taken together, full-length, cleaved, and modified tau species are released in the CSF after TBI.

There are, however, important caveats to these findings. First, the basal levels of CSF tau may already be increased by other pathological circumstances. One study used 50 control subjects, two of which showed detectable tau levels in CSF: a patient with multiple sclerosis and another with Guillain-Barre syndrome [192]. It is now known that tau levels are increased in the CSF of multiple sclerosis patients, which would explain why this sample had higher tau levels compared to other controls [204]. The same could be of Guillain-Barre patients, but this is not known. Therefore, other confounding events may alter basal tau levels in CSF. This is a critical parameter to consider when studying head trauma in the aging population, which is most susceptible to falls. The aging population is more likely confounded with diagnoses of mixed dementias and other neurodegenerative processes [205]. In addition, further research categorizing these effects could help determine a correlation between tau changes and progression of particular disorders and clinical outcome.

A second caveat is that the source of CSF may alter the amount of tau that becomes available. According to a recent report, tau is more abundant in ventricular than lumbar CSF after injury [206]. One study found that tau levels were virtually undetectable in CSF and brain of 50 controls regardless of age and source of CSF (ventricular or lumbar) [192]. The authors state that the source of CSF in at least 46 of the controls was lumbar instead of ventricular. Therefore, the control sample, lumbar CSF, should inherently have less tau than the ventricular TBI CSF. Despite this potential difference, the <5 samples obtained from the ventricle still had significantly lower amounts of tau, thereby

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supporting the finding that TBI causes tau to leak into the CSF. Albeit, it is important to consider that tau protein variants may permeate into the CSF under various circumstances and be more than "undetectable", but TBI induces a significant amount of tau to migrate into the CSF.

Tau in serum after TBI

Serum tau levels increase over time and with TBI severity in most studies [11, 19–21, 207–209]. Experiments using various models of TBI in rats assessed whether tau levels in serum were proportional to the severity of damage and time post-injury. Although the common theme of tau increasing with TBI prevails, there are significant oscillations in tau levels that are based on the model, the type of tau, severity of injury, and time. One such study shows that tau serum levels were increased in rats subjected to TBI in the Feeney model of mild and severe TBI, and the levels were higher in severe compared to mild injury [21]. Tau levels in both TBI groups increased significantly between one and 48 hours and declined to baseline thereafter. Interestingly, tau levels in both mild and severe TBI were significantly increased after 7 days, and the mild TBI group had more tau than its TBI counterpart.

In another study, the CCI method was used to identify increased levels of cleaved tau in serum in mild and severe TBI [19]. Only the severe TBI group showed significantly increased levels of cleaved tau, and this occurred at 6 hours but decreased again at 24 hours. Neither of these studies looked at uncleaved or cleaved tau, so it is difficult to ascertain whether the peak detection of either tau species would be different based on the model. Also, more time points would help determine oscillations in the levels of different tau species, as it seems that there is sufficient variability over time. This suggests that there are mechanisms of BBB leakage that are still at play and regulate permeability.

A third and unique approach subjected rats to a scalable rotational acceleration model of TBI and measured total tau levels via reverse-phase protein microarray [20]. The results were more consistent and the assays were more sensitive than in other studies. Serum levels were collected at 1, 3 and 14 days after the injury, and tau levels were significantly increased throughout this period. Yet again, this study shows that the model dictates variability in the collection of biomarkers.

Some studies showed that total tau and cleaved tau increases significantly in serum after TBI, yet others contended it does not [207–210]. Another study presents data suggesting that serum tau is not sensitive enough to differentiate between head injury groups with normal and abnormal computed tomography (CT) scans [211]. The major difference between these reports is the severity of the injury according to the Glasgow Coma Scale (GCS), which measures verbal performance, motor response, and eye opening on a 3–15 scale (low score depicts poor performance in the test). If the patients scored less than 8 in the GCS at the time of admission to the emergency room after head injury, then differences of total tau levels in serum would be significant [209]. Conversely, studies that included patients with GCS greater than 13 did not detect tau in serum or did not find a difference between the groups [211].

This finding has two important considerations. First, since tau positively correlates with GCS, then there might be a direct relationship between cell structure and cognitive function that obviates a more macroscopic, CT-detectable compromise and makes microscopic integrity more relevant to diagnosis. This tau-GCS relationship is more reliably predictive than CT imaging results. A study that used CT as a means to compare two groups of injured patients showed no difference [211]. In fact, it suggests that CT evaluation is not sufficiently sensitive to predict outcome, while GCS and tau correlate more strongly [209].

Second, these studies redefine severity of head injury. Despite the ability to see large pathological signs of damage via CT and magnetic resonance imaging, attempts to visualize DAI are marred by the lack of resolution of these technologies. Axonal injury is the cellular basis of brain injury, and it must be of sufficient extent for it to be detectable. We conjecture that microscopic axonal injury may be more predictive of tau release and clinical outcome (Fig. 1). Proportionally, the larger the extent of damage, the deeper the cognitive decline, and the greater the amount of tau that will be liberated from cells to the BBB.

There is no definitive answer as to whether tau is an accurate serum marker of TBI in humans. This is partly due to the vast amount of tau modifications. Another difficulty is that different experimental models may yield different consequences for tau. For example, if the type of trauma does not compromise the BBB, it is unlikely that proteins will leak into the CNS or the serum. Nonetheless, each one of the varied experimental models assists in address-

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ing unique questions, the answers to which must be interpreted with trepidation. Often, generalizations are made from these studies, which lead to the inconsistencies.

The salient feature between the biomarker studies conducted to date is the difference in the severity of damage post-injury [208–210]. These studies use the GCS and the Glasgow Outcome Scale, which measures functional outcome by rating patients into 5 categories: Dead (1), Vegetative State (2), Severe Disability (3), Moderate Disability (4), or Good Recovery (5). When the groups are distributed based on the GCS measures, there is a positive correlation between the amount of serum tau and cleaved tau and clinical outcome. This correlation is strongest between the groups that suffered extensive damage. However, it was difficult to discriminate between the moderate and mild TBI groups [210].

The degree of injury that must compromise the BBB seems to dictate whether head injury markers like tau can leak into the vasculature. To confirm this, a more comprehensive study of tau migration would provide better insight. However, there are not many studies assessing changes in brain pathology, CSF, and serum tau simultaneously. One study that addressed this in humans found that tau was elevated in CSF but undetectable in serum [207]. This may suggest that the conditions of the injury restricted tau from exiting the site of damageor that tau was cleared from serum much faster and the CSF levels accrued over time. CSF and serum were collected at day 1, 2–3, 4–6, and 8–14, and tau peaked significantly in the 2-3-day range. Therefore, it is possible that the injury was not severe enough to compromise the BBB. This point is substantiated in scalable murine models of TBI that show increased tau and cleaved tau levels in serum when the injury was more severe [19, 20].

CONCLUSIONS

Studies agree that TBI changes tau proteostasis in a variety of ways. Tau and its multiple post-translationally modified variations (Fig. 2) are differentially enriched in brain, CSF, and serum after at least one traumatic brain incident. Therefore, tau could serve as a biomarker for TBI. As a surrogate marker for trauma, tau could help assess potential clinical outcome and determine efficient course of treatment. However, based on inconsistencies between studies, tau is currently an unreliable marker. The main reasons for the discrepancies can be explained by (1) type of tau assessed, (2) severity of damage between mild, moderate, and severe TBI, and (3) means of CSF collection.

Despite the variability, valuable information about the consequences of TBI on tau is harnessed from the variety of experimental studies. For example, in severe and repetitive models of TBI, sensitive assays detect tau in CSF, and to a lesser extent, in serum. Moreover, phospho-tau immuno-reactivity appears in perivascular glia and neurons early post trauma. Tau pathology is sensitive to TBI in tau-overexpressing transgenic mice and wild type rats, but not wild type mice (except in blast injury). However, more sensitive assays may show that all murine models are subject to tau pathology after TBI. Similarly, TBI studies in humans show that the levels of tau and particularly the levels of some of its post-translationally modified species increase in CSF and serum in the early stages following TBI. Yet, these abnormal changes in tau levels are subject to severity of the injury, age of the patient, confounding diseases, and time post-injury.

The data show that TBI induces changes in tau that lead it to aggregate or be released. This type of tau can enter new cells, to aggregate, and can leak into the CSF and even the vasculature. Moreover, TBI-affected tau is pathogenic, and inhibiting tau kinases after injury may attenuate damage. Therefore, altering tau processing is a therapeutic strategy that is currently being explored. Nonetheless, further insight into the mechanisms leading to TBI-initiated, tau-mediated pathogenesis could reveal many more therapeutic targets.

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Neuropathology in Consecutive Forensic Consultation Cases with a History of Remote Traumatic Brain Injury

Rudy J. Castellani[a,b,*], Margaret Smith[b], Kristi Bailey[b], George Perry[c] and Joyce L. deJong[b]
[a]*Department of Pathology, Anatomy, and Laboratory Medicine, Rockefeller Neuroscience Institute, West Virginia University, Morgantown, WV, USA*
[b]*Department of Pathology, Western Michigan University Homer Stryker MD School of Medicine, Kalamazoo, MI, USA*
[c]*College of Sciences, University of Texas, San Antonio, San Antonio, TX, USA*

Abstract. Traumatic brain injury (TBI) is widely assumed to be causal in neurodegenerative disease, based on epidemiological surveys demonstrating an increased risk of Alzheimer disease (AD) following TBI, and on recent theories surrounding repetitive head movement. We tested this assumption by evaluating 30 consecutive forensic examinations in which neuropathology consultation was sought, and in which a history of remote TBI was uncovered during the course of the investigation. In this series, there was a high frequency of psychiatric co-morbidities (100%), remote contusion (90%), and seizures (63%). Extent of proteinopathy showed no differences with age-matched controls. A subset of the cases showed focal geographic tauopathy that correlated with older age at autopsy, but had no correlation with clinical signs, and was minimal in comparison with the encephalomalacia secondary to trauma. The results suggest that cerebral contusion and post-traumatic epilepsy may be over-represented in civilian TBI, while structural brain damage from trauma is the predominant cause of morbidity following TBI. We found no evidence that TBI initiates a progressive proteinopathy.

Keywords: Alzheimer's disease, amyloid-β, contusion, epilepsy, neurodegenerative disease, phosphorylated tau, traumatic brain injury

INTRODUCTION

Traumatic brain injury (TBI) is a major cause of morbidity and mortality in the civilian population. In 2013, there were approximately 2.5 million TBI-related ED visits, approximately 282,000 TBI-related hospitalizations, and 56,000 TBI-related deaths in the United States [1]. One population based study indicated that 37% of people hospitalized with TBI required assistance of another person in physical and/or cognitive activities of daily living [2]. In another study, among 288,009 hospitalized TBI survivors in the United States in 2003, an estimated 124,626 developed a long term disability, with an estimated cost of $2 billion for rehabilitative and supportive services in the year following discharge [3]. Falls and motor vehicle accidents predominated among the TBI mechanisms.

Although sport-related TBI is matter of intensive investigation, morbidity and mortality in this setting is comparatively small [4, 5]. Factors such as subconcussion, repetition, and delayed onset changes are often raised in terms of changes in brain tissue immunohistochemistry [6–8], but are difficult to

*Correspondence to: Rudy J. Castellani, MD, 64 Medical Center Drive, 2289 HSC-S, MS 9203, Morgantown, WV 26506-9203, USA. Tel.: +1 304 293 1625; Fax: +1 304 293 1627; E-mail: Rudolph.castellani@hsc.wvu.edu.

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extrapolate to the civilian TBI population outside of sport. Evidence of TBI is generally inferred from the sport history [9, 10], rather than by a distinct neurological injury or pathological evidence of trauma. Given these and other uncertainties [11–14], geographic p-tau aggregates are in need of further study not only in the sport construct but in the setting of civilian TBI.

Decedents presenting to medical examiner's offices for post-mortem examination may be informative with respect to civilian TBI. Evidence of TBI history may be uncovered by death scene investigators or medical record review, which while potentially limited in terms of details of medical evaluation, would not suffer from selection bias inherent in case material sought by researchers or families. Further studies on this population may therefore be warranted. In this study, we examine neuropathological findings in consecutive decedents with a history of TBI who presented for forensic autopsy and were subsequently referred for neuropathological consultation. Included in the analyses is assessment for proteinopathy.

MATERIALS AND METHODS

Case selection

This study was presented to the Institutional Review Board at Western Michigan University Homer Stryker MD School of Medicine (WMed), and was exempted from full board review. All control samples were obtained with informed consent of next-of-kin. Protocol number 20111080. We searched forensic autopsy cases with accompanying neuropathology consultation from the Office of the Medical Examiner at WMed from 2016 to 2018, for any cases with a TBI history that was uncovered as part of the death scene investigation and/or by the forensic pathologist assigned to the case. For cases in which a history of remote TBI was uncovered, further data was collected including autopsy reports, any available medical records, death certificates, and when available, time between TBI and death in years. Cases of pediatric TBI were excluded, as were cases of acute TBI resulting in death.

Gross brain examination

All brains were fixed in 10% neutral buffered formalin and examined by a neuropathologist following fixation (RJC). Following fixation, external brain and dura were examined, the Circle of Willis and proximal branches were dissected, the cerebral hemispheres were sectioned in the coronal plane, the brainstem was sectioned in the axial plane, and the cerebellar hemispheres were sectioned in the sagittal/parasagittal plane. External and sectioned brain were photographed.

Histology

Representative sections of neocortex from all cerebral lobes, medial temporal allocortex, deep gray matter, midbrain, pons, rostral medulla, caudal medulla, cerebellar hemisphere, dura mater, and pituitary gland were processed through graded ethanol and xylene solutions, embedded in paraffin, sectioned at 5 microns, and stained with hematoxylin and eosin.

Immunohistochemistry

Antibodies to p-tau (AT8), amyloid-β, and TPD-43 were used for immunohistochemistry following standard antigen retrieval procedures (see Table 1), and immunostained using a Thermo Scientific 480S IHC Autostainer. Following H&E interpretation, immunohistochemical assessments generally followed the 2016 NINDS/NIBIB recommendations for sport-implicated geographic tauopathy [15], and modified NIA-AA 2012 consensus guidelines for assessment of AD neuropathologic change [16]. The CERAD component of the NIA-AA 2012 consensus guidelines was modified with the use primarily of Aβ immunohistochemistry to assess Aβ burden and plaque frequency, otherwise semiquantitated into sparse, moderate, and frequent per schematic illustrations in the original CERAD publication [17]. We

Table 1
Antibodies

Antibody	Clone	Vendor	Dilution	Antigen retrieval
Phospho-tau	AT8	Thermo Scientific	1:250	ThermoScientific Dewax and HIER L Buffer 20 min 98°C
Amyloid-β	BAM01	Thermo Scientific	1:50	95% Formic Acid 30 min
TDP-43	TARDBP	ThermoFisher Scientific	1:50	95% Formic Acid 30 min

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chose this approach as we were interested in proteinopathy, and because the "neuritic plaque" is not precisely defined. Modified CERAD plaque scores were obtained in this fashion by examination of the four neocortical areas, with the score reflecting the most abundant areas. The Braak stage [18] was obtained by examination of hippocampal formation, subiculum, entorhinal cortex, and inferior temporal neocortex bilaterally, along with four neocortical areas (superior/middle frontal gyrus, superior/middle temporal gyrus, inferior parietal lobule, calcarine cortex). Paraffin sections from all paraffin blocks were immunostained for p-tau (AT8) to approximate the preliminary NINDS/NIBIB recommendations. Sections from selected paraffin blocks were additionally immunostained for TPD-43. In order to compare proteinopathy burden with the general population, Braak and modified CERAD plaque scores obtained using similar methodology and consent criteria were compared to control donors with no TBI history.

For control samples, p-tau and Aβ burdens were assessed by immunohistochemistry similar to the remote TBI cases, with immunohistochemistry for Aβ performed on the four neocortical areas noted above, and p-tau immunohistochemistry performed on the above four neocortical areas plus hippocampal formation and adjacent medial temporal lobe.

RESULTS

Demographic and clinical information

30 decedents were identified in the neuropathology consultation files with "traumatic brain injury" indicated in the available case information. History of contact sport was not considered a surrogate for TBI history. 26 (87%) were men and 4 (13%) were women. The average age was 52.3 ± 15.6 years, with a range of 21 to 92 years. Where information was available, 13 out of 21 (62%) had a history of drug and/or alcohol abuse, 17 out of 27 had a history of "seizures" (one of which had seizures since birth), 4 out of 22 had a history of cognitive impairment or dementia not otherwise specified, 15 out of 15 had a history of psychiatric problems (including depression and major depressive disorder, suicidality, bipolar disorder, general anxiety disorder, post-traumatic stress disorder, and schizophrenia; see Table 2). The interval between TBI and death ranged from 9 months to 41 years (average: 11 ± 11 years; data available in 15 cases). One decedent likely sustained head injury from sport (motocross). One decedent sustained TBI from a blast injury while on active duty in the military and a subsequent fall with epidural hematoma during civilian life. The remaining subjects sustained accidental TBI in civilian life outside of sport.

Neuropathology

The most remarkable gross pathological finding in this series was the high frequency of remote contusion (Fig. 1), noted by gross examination in 27 out of 30 decedents (90%). Aggregate diameter of damaged brain in individual cases ranged from 0.5 cm to 17.6 cm. Subjects described as having "dementia" had contusions (2 cases, 6 cm aggregate diameter and 8.8 cm aggregate diameter), and penetrating shotgun injury (published previously as a case report [19]). One subject with cognitive impairment including memory loss did not have a contusion, but did have chronic subdural hematoma, atrophic fornices, atrophic mamillary bodies, remote microhemorrhages in the substantia nigra, and a fenestrated septum pellucidum.

Immunohistochemistry

Immunohistochemistry was performed on cases in which paraffin blocks were available ($n = 19$) (Figs. 2–4). Assigning a value of 1 to 6 for Braak scores ranging from I to VI indicated an average Braak score of 1.26 overall. Assigning a value of 1, 2, and 3 to sparse, moderate, and frequent respectively, the average modified CERAD score across the group was 0.42. The age range for those cases with immunohistochemistry ranged from 21 to 78 years, with a mean of 52 years, ±15 years (standard deviation). Braak and modified CERAD data from control samples age 50 and above (immunostains are not obtained in control samples under the age of 50 per protocol) showed an average Braak/modified CERAD of 0.97/0.22, 2.14/0.58, and 2.87/0.84, for decedents in their 50 s ($n = 90$), 60 s ($n = 78$), and 70 s ($n = 38$), respectively. Average Braak and modified CERAD for TBI samples involving decedents age 50 and older (mean age 63.5 ± 8.1 years) was 2.18 and 0.73, respectively. These averages are not significantly different from an age-matched control sample of decedent's in their seventh decade (mean age 64.1 ± 3.1 years, $n = 78$) (unpaired student t-test; $p = 0.93$ for Braak; $p = 0.63$ for modified CERAD). The Braak and modified CERAD data are therefore, not surprisingly, age-related rather than trauma-related.

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Table 2
Demographic, clinical, and pathological information

	Age	Sex	Abuse	Sz	Psych	Other neuro	Age at neurotrauma exposure*	CTE path	Braak	CERAD	Remote Contusion(s)	Diameter (cm)
Case 1	42	F	No	Y	?		21	No	0	0	N	
Case 2	47	M	A	Y	Anx, BPD		?	NA	NA	NA	Y	1.5
Case 3	33	M	D	N	?	s/p EDH	32	NA	NA	NA	Y	2.5
Case 4	69	M	Unk	N	Suicidal		?	NA	NA	NA	Y	6.3
Case 5	56	M	A/D	Y	?		36	NA	NA	NA	Y	6.5
Case 6	37	M	No	N	?		?	NA	NA	NA	Y	9.3
Case 7	51	M	A	Y	Anx, Dep		45	NA	NA	NA	Y	2
Case 8	56	M	A/D	Y	Schiz		51	NA	NA	NA	Y	5
Case 9	54	M	A	N	?		52	No	2	Mod	Y	7
Case 10	62	M	Unk	?	?		?	Yes	2	0	Y	3.2
Case 11	40	M	D	Y	PTSD, Dep	s/p crani	31–34	No	0	0	Y	2.5
Case 12	69	M	Unk	?	?	ALS	?	Yes	1	0	Y	5.4
Case 13	59	M	Unk	Y	?	Dementia	?	Yes	3	0	Y	6
Case 14	36	M	D	N	?		?	No	0	0	Y	9.8
Case 15	49	F	No	Y	?	Aneurysm clip	33	Yes	1	0	Y	0.9
Case 16	41	M	Unk	Y	Dep		?	No	0	0	Y	17.6
Case 17	66	M	A	Y	Dep		?	Yes	3	Sp	Y	0.5
Case 18	54	M	A	Y	MDD, GAD		?	NA	NA	NA	Y	?
Case 19	55	M	Unk	Y	?		?	NA	NA	NA	Y	11
Case 20	60	M	No	N	Dep		56	Yes	0	0	Y	11.5
Case 21	70	M	No	Y	?	Dementia	60	Yes	4	Sp	Y	8.8
Case 22	92	M	Unk	Y	?		52	NA	NA	NA	Y	?
Case 23	78	M	No	N	Dep, Del, suicidal, GAD	Dementia	?	NA	NA	NA	Y	?
Case 24	69	M	Unk	?	Schiz, OCD, GAD		?	Yes	3	Sp	N	
Case 25	40	F	No	Y	BPD		38	No	0	0	Y	7
Case 26	31	M	A	Y	?		21–23	No	0	0	Y	3.5
Case 27	63	M	No	Y	?	Dementia	22	Yes	1	0	Y	?
Case 28	21	F	D	N	Suicidal, GAD, Dep		?	No	0	0	N	
Case 29	37	M	D	N	BPD, Schiz		36	No	0	0	Y	5
Case 30	60	M	Unk	N	Dep		?	?	?	?	N	

A, alcohol; ALS, amyotrophic lateral sclerosis; anx, anxiety; BPD, bipolar disorder; dep, depression; D, drugs; EDH, epidural hematoma; F, female; GAD, general anxiety disorder; M, male; MDD, major depressive disorder; mod, moderate; N, no; NA, not available; PTSD, post-traumatic stress disorder; schiz, schizophrenia; sp, sparse; unk, unknown; Y, yes.

With respect to NIA-AA consensus guidelines for AD assessment, none of the 19 cases had changes corresponding to "high" AD neuropathologic change. Three of 19 cases had changes corresponding to "intermediate" AD neuropathologic change, including the presence of neuritic plaques as defined by p-tau AT8 immunohistochemistry. Two of 19 cases had changes corresponding to "low" AD neuropathologic change. The remaining fourteen cases had changes corresponding to "no" AD neuropathologic change. Two decedents with "dementia" in the clinical history had no AD neuropathologic change, while one decedent with dementia had intermediate AD neuropathologic change along with abundant cerebral contusions. One 78-year-old decedent with memory impairment and heterogeneous neuropathology (chronic subdural hematoma, septal fenestration, atrophy the fornices and mamillary bodies, remote microhemorrhages in the substantia nigra, and lacunar infarct) also had intermediate AD pathology. This decedent was living independently shortly before death.

All paraffin blocks were examined for geographic p-tau aggregates according to NINDS-NIBIB consensus recommendations. Although these recommendations are "preliminary" and may overlap with aging-related pathology depending on how strict criteria are applied [14] we interpreted cases according to the specific wording of the consensus recommendations for the required criterion and supportive criteria. These criteria were applied blindly with respect to all clinical data.

10 of 19 decedents were thus interpreted as having geographic t-tau aggregates consistent with the required criterion described. The pathology was generally limited in extent, in comparison with the structural brain damage. The mean age of decedents with geographic p-tau aggregates was 64.5 ± 7.8 years (standard deviation). The average Braak stage was 2.2 and the average modified CERAD score was

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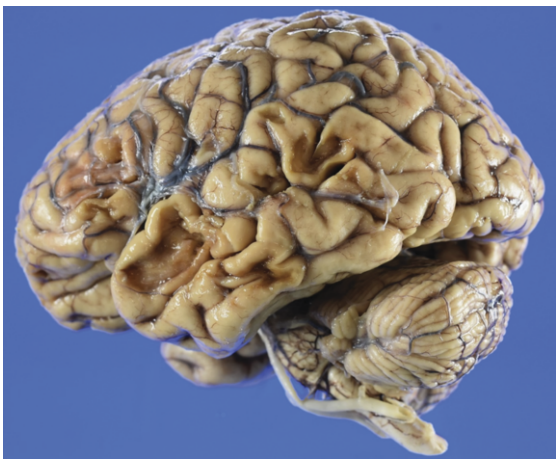

Fig. 1. Multiple remote contusions are present in the left temporal lobe and left frontal lobe. The decedent was a 70-year-old man with a history of "dementia" after a fall approximately 10 years prior to death. The same subject had Braak stage IV neurofibrillary change, and CERAD sparse neuritic plaque pathology, which by itself is an insufficient explanation for dementia. The temporal relationship between the head trauma and the dementia instead indicates that brain damage from trauma is the relevant pathological substrate.

0.6 in this group. These averages are similar to an average Braak of 2.14 and average modified CERAD of 0.58 in our control sample without TBI of decedents in their 60 s ($n = 78$, average age of 64.1 ± 3.1 years). The time period between trauma and autopsy examination ranged from 4 years to 41 years (data available in three cases).

Of the nine decedents without geographic p-tau aggregates, the average age was 38 ± 8.9 years. The average Braak score was 0.22 and the average CERAD score was 0.22, although 8 of the 9 decedents were essentially devoid of p-tau and Aβ (Braak 0, modified CERAD 0), while one decedent had Braak stage II p-tau and modified CERAD moderate Aβ. Time from trauma to autopsy examination ranged from 1 year to 21 years (data available in 6 out of 9 cases). The only significant different between the group with geographic p-tau aggregates and the group without geographic p-tau aggregates was age, with the geographic p-tau aggregate group being significantly older ($p < 0.0001$; two-tailed student t test).

DISCUSSION

The frequency of contusions at autopsy in our civilian population with a history of "TBI" uncovered by death scene investigation was high. This suggests that structural brain damage is a common feature of civilian TBI. Although contusion is not a feature of most definitions of TBI, it has a tendency to occur in the aftermath of moderate or severe injury [20], and is associated with poor outcome in the setting of mild TBI [21]. The high frequency in this study is in line with the morbidity of civilian TBI in general.

Of note were cases in which the decedent suffered from antemortem "dementia" solely on the basis of mechanical damage to brain tissue. Two decedents with "dementia" had no AD neuropathologic change, while one decedent with dementia had intermediate AD pathology along with abundant cerebral contusions. A fourth subject with memory impairment and heterogeneous neuropathology also had intermediate AD pathology. Given the strong genetic influence of *APOE* genotype, it would be important to know whether subjects with intermediate AD pathology had one or two ε4 alleles before inferring a role for trauma in the occasional older subjects with moderate Aβ plaque and p-tau pathology.

Geographic p-tau aggregates were present in some decedents but were sparse and microscopic in comparison to the gross brain tissue destruction from trauma. Since there is at present no lower limit to sport-implicated geographic p-tau aggregates, the significance of isolated or patchy microscopic foci of geographic p-tau aggregates is unclear, as any putative clinical correlation may be obscured by the much more abundant tissue destruction from contusion. Importantly, contusion is a traumatic lesion by definition. Geographic p-tau deposition as a traumatic lesion instead has to be inferred from the sport history, and does not appear to be an absolute indicator of neurotrauma [22–29].

In this series, overall p-tau burden as assessed by Braak staging, and amyloid burden assessed by modified CERAD plaque score, were not significantly different from age-matched controls, indicating no discernible relationship between TBI and AD pathology in this series. Taken together, our data suggest that dementia due to "TBI *in situ*" or mechanical damage to brain tissue may be under-appreciated. Large scale epidemiological studies examining dementia risk in the absence of neuropathological characterization may over-estimate AD dementia or otherwise dementia due to a supervening progressive proteinopathy as hypothesized. Our results instead indicate that neurodegenerative disease *per se* appears to have a marginal, if any, role in the long-term morbidity and mortality from civilian TBI. As further evidence of the predominant role of age over trauma in the appearance of geographic p-tau aggregates, the only detectable difference between those cases with such

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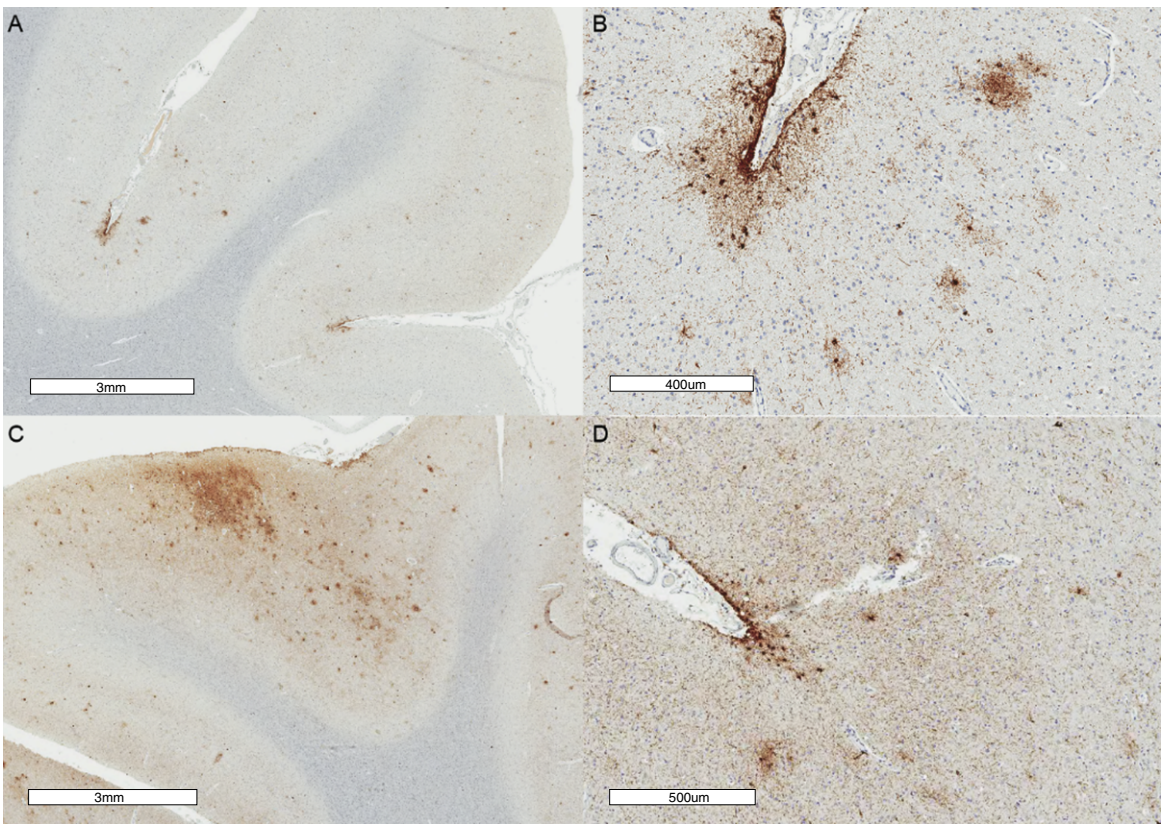

Fig. 2. Tau immunostains in A and B show irregular p-tau aggregates, predominantly but not solely astrocytic. Irregular neocortical p-tau along with neuritic plaque pathology is present in C, and an additional irregular collection of p-tau aggregates in neurons, astrocytes, and cell processes around small blood vessels and accentuated at sulcal depths is present in D. These features are consistent with a preliminary required criterion for sport-implicated geographic tauopathy according to a recent consensus effort [15]. This case was interesting in that the decedent was a 78-year-old man with memory impairment who additionally had remote microhemorrhages in the substantia nigra, chronic subdural hematoma, septal fenestrations, dilated third ventricle with convex thalami, and atrophic mammillary bodies. There was no sport history although he did have a history of frequent falls, falls off a horse, and motor vehicle accidents. Overall, this case demonstrated many features of dementia pugilistica described in the classical study by Corsellis et al. [32]. There was also Braak stage IV neurofibrillary change, and CERAD moderate plaque pathology. Although the extent of proteinopathy in this case may be deemed sufficient explanation for dementia, the multiple additional brain lesions most likely played a role in the decedent's neurological status. The fact that he was living independently also indicates a level of reserve even in the face of extensive pathological lesions.

aggregates and those without, was the significantly younger mean age of the latter group.

The frequency of seizure disorders in this case material was striking. Although medical records are limited, the available data suggest post-traumatic epilepsy is prevalent in the civilian TBI population, and may be particularly common with temporal lobe contusion [30]. Given that the risk of sudden death is substantial in subjects with epilepsy [31], TBI history with an accompanying contusion, especially temporal lobe contusion, may be a common sequence in civilian TBI leading to reduced life expectancy. More studies are needed to fully elucidate the relationship between TBI-related contusion, post-traumatic epilepsy, and sudden death. Vigilance in diagnosis and monitoring of seizures in the aftermath of civilian TBI nevertheless seems warranted.

At present, three overall conclusions remain plausible: 1) mechanical damage to the brain from the acute event is responsible for the long-term morbidity and mortality of TBI; 2) proteinopathy as identified by immunohistochemistry is either absent or inconsequential as a long-term effect of TBI; and 3) genuine traumatic encephalopathy appears to be a static process over the long term, rather than neurodegenerative disease-like, either clinically or pathologically.

This study has clear limitations. The case numbers are overall small and heterogeneous, as are the numbers of cases with dementia due to TBI *in situ*. The TBI histories, while empirically evident in the

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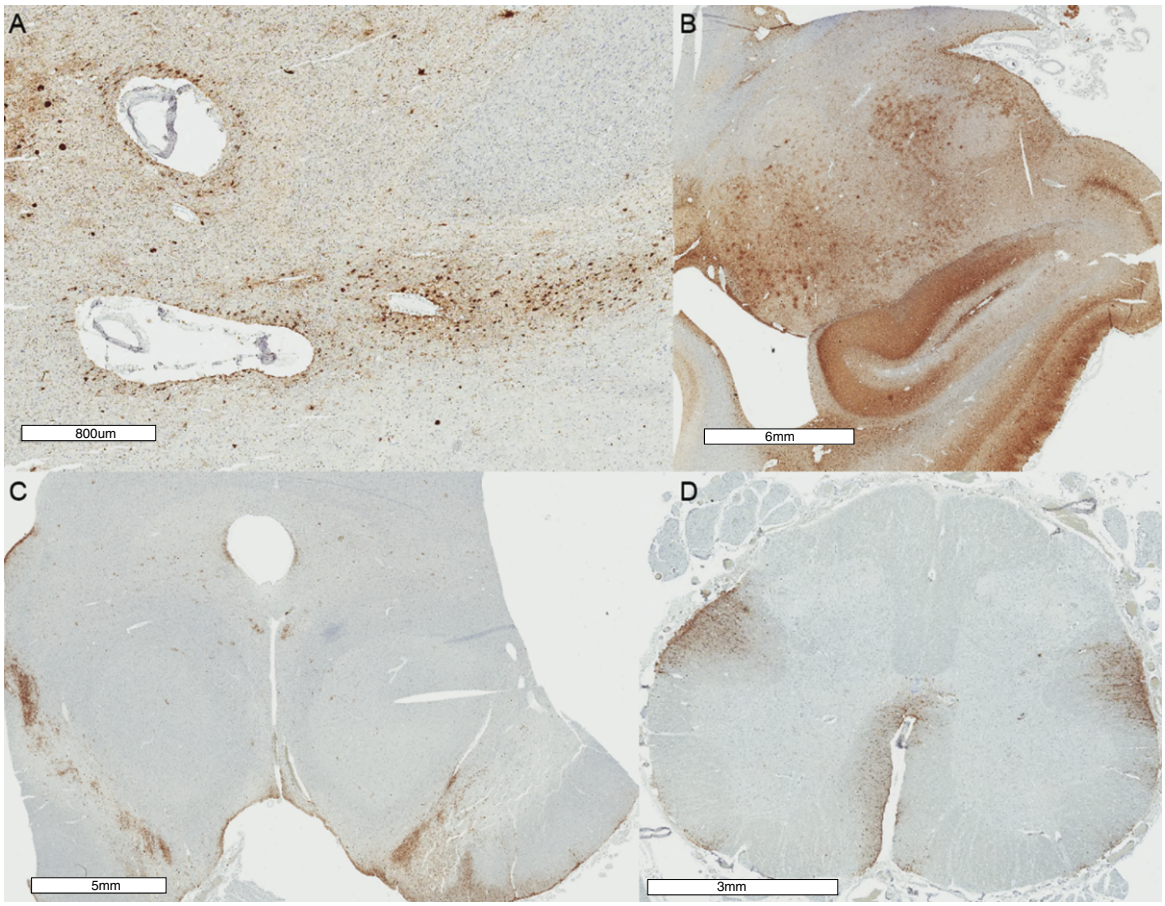

Fig. 3. The same subject as in Fig. 2 showed abundant perivascular, astrocytic p-tau in areas of temporal subcortical white matter (A), and abundant p-tau in the medial temporal lobe including the amygdala, anterior hippocampal formation, and entorhinal cortex (B). Patchy p-tau aggregates in the midbrain substantia nigra, cerebral peduncles, cranial nerve nuclei, subependymal glia, and subpial areas indicate an abundance of subcortical p-tau (C). Although there is substantial overlap between sport-implicated geographic p-tau aggregates and that of age-related tau astrogliopathy, such an extent and irregular distribution may be interpreted as more advanced pathology and repetitive head trauma-related. On the other head, p-tau pathology in the lumbar spinal cord (D), while geographic or sport-implicated p-tau-like in its irregularity or patchiness, is difficult to ascribe to head trauma by virtue of its presence in the lower spinal cord.

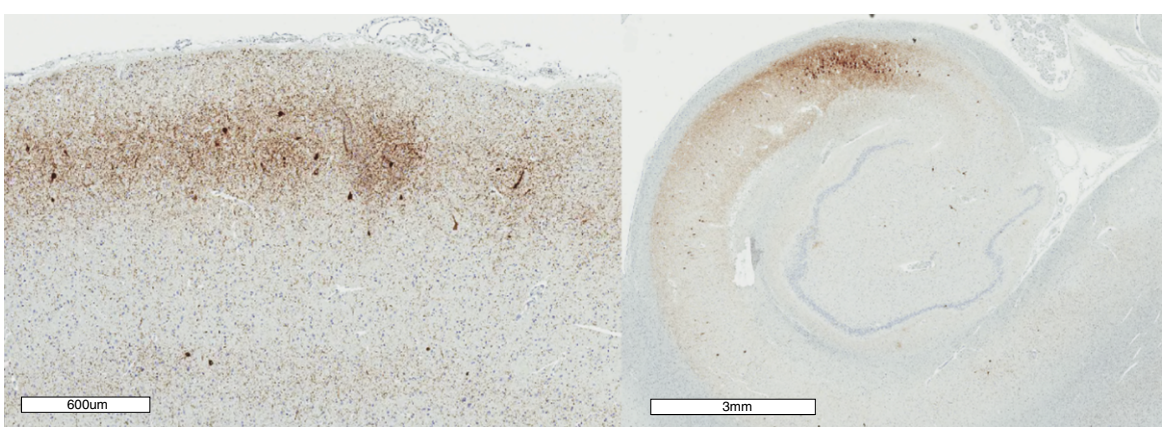

Fig. 4. Superficial cortical lamina p-tau, and relatively abundant CA-2 p-tau with focal CA-4 p-tau within dendritic processes, in this 70-year-old man also depicted in Fig. 1, are among the supportive criteria for sport-implicated geographic p-tau aggregates. As noted above, this decedent's dementia is more clearly related to structural brain damage or extensive frontotemporal contusion.

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high frequency of contusion, generally lack detail in terms of severity of the acute event. Detailed neuropsychiatric evaluation is not available in this case series, making it difficult to comment on the presence or absence of progressive clinical deterioration with certainty. On the other hand, given the lack of proteinopathy in this series, the mechanism of any identifiable disease progression would remain unclear. Finally, as noted above, epilepsy was common in this series. The series may therefore be skewed toward sudden-unexpected death in epilepsy, which likely differs from the spectrum of morbidities associated with civilian TBI in general.

In summary, we studied neuropathological details, including immunohistochemistry for proteinopathy, in consecutive decedents presenting for forensic autopsy and neuropathology consultation who also had a remote history of TBI. Cerebral contusions and post-traumatic epilepsy were present in the majority of study subjects, suggesting a sequence of TBI-related mortality that may be underappreciated. Our findings further suggest that mechanical brain tissue injury and a resulting static encephalopathy determines the long-term TBI morbidity, while neurodegenerative proteinopathy is specifically absent, notwithstanding clinically inconsequential, microscopic geography tauopathy in a subset of cases.

DISCLOSURE STATEMENT

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/19-0782r1).

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Brain Injury and Later-Life Cognitive Impairment and Neuropathology: The Honolulu-Asia Aging Study

E. Julia Chosy[a,*], Noele Gross[a], Marnie Meyer[a], Catherine Y. Liu[a], Steven D. Edland[b], Lenore J. Launer[c] and Lon R. White[a]
[a]*Pacific Health Research and Education Institute, Honolulu, HI, USA*
[b]*University of California at San Diego, San Diego, CA, USA*
[c]*National Institute on Aging, National Institutes of Health, Bethesda, MD, USA*

Abstract.
Background: Findings are inconsistent regarding the role of traumatic head injury in the subsequent development of neurologic outcomes.
Objective: Examine the relationship between head injury and later cognitive impairment.
Methods: A sample of 3,123 Japanese-American men was assessed for history of head injury and evaluated for cognitive impairment using the Cognitive Abilities Screening Instrument (CASI). For a subsample of 676 respondents, neuropathologic results from those with and without head injury were compared.
Results: Although the crude model showed an association between history of head injury and later severe cognitive impairment, the relationship lost significance in the adjusted model (OR = 1.320, CI: 0.90–1.93), regardless of time between injury and impairment. Similar to cognitive impairment, hippocampal sclerosis was observed significantly more in the brains of respondents with a history of head injury in the crude model, but the relationship weakened in the adjusted model (OR = 1.462, CI: 0.68–3.12). After adjustment, decedents with a head injury demonstrated marginally higher brain weight (OR = 1.003, CI: 1.00–1.01).
Conclusion: We did not find a relationship between head injury and subsequent cognitive decline in this cohort. The neuropathology results also displayed no strong association between history of head injury and specific brain lesions and characteristics. These results support other findings in prospective cohorts. However, they could be influenced by the demographic make-up of the sample (male Japanese-Americans) or by the observation that the majority reported only a single head injury.

Keywords: Alzheimer's disease, brain lesions, cognitive dysfunction, dementia, Honolulu-Asia Aging Study, neuropathology, traumatic brain injury

INTRODUCTION

There is a long-standing question of whether traumatic brain injury (TBI) affects later-life cognitive function. In the early 1900 s, a condition called *dementia pugilistica* was used to describe progressive neurodegeneration in boxers resulting in a 'punch drunk' state [1]. More recently, a condition observed among athletes with repeat head injuries has been termed chronic traumatic encephalopathy (CTE) and has been shown to be associated with a variety of neurologic outcomes [2, 3]. Although the recognition of CTE has renewed interest in the association between head injury and neurologic outcomes, researchers have been addressing this question for decades, with mixed results. Even the findings from several meta-

*Correspondence to: E. Julia Chosy, PhD, 3375 Koapaka Street, Suite I-540, Honolulu, HI 96819, USA. Tel.: +1 808 564 5458; Fax: +1 808 524 5559; E-mail: ejchosy@phrei.org.

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analyses have not been entirely consistent, as Table 1 summarizes.

The pathological mechanism by which a TBI may increase the risk for cognitive decline has not been established. Evidence suggests TBIs increase the accumulation of multiple abnormal proteins commonly associated with neurodegeneration, include tau, amyloid-β, α-synuclein, and tar DNA binding protein-43 [4, 5]. TBIs may also cause substantial metabolic disturbances resulting in excitotoxicity, neuroinflammation, and oxidative stress [5, 6]. Some researchers suggest TBIs can trigger cerebrovascular pathology, predisposing individuals to neurodegenerative diseases [7, 8]. Others suggest the neuropathologic changes resulting from TBI may represent a distinct disease process rather than the initiation of development of a specific condition, such as Alzheimer's disease (AD) [9–14].

With the current study, we examined the relationship between traumatic head injury and subsequent cognitive function in the Honolulu-Asia Aging Study (HAAS), a population-based prospective cohort of Japanese-American men living in Hawaii stemming from the Honolulu Heart Program (HHP) [15]. Additionally, brain autopsies conducted on a subset of the sample allowed for the comparison of neuropathologic changes in individuals with a history of head trauma. In the HAAS, head injury data were self-reported and cognitive impairment was measured using the Cognitive Abilities Screening Instrument (CASI) [16]. The pathology data were obtained from a thorough assessment of brain tissue by clinical neuropathologists. Given the combination of cognitive and neuropathologic outcomes in a well-defined cohort, our results add uniquely to an expanding collection of research to understand the possible effects of head injury on subsequent neurologic outcomes.

METHODS

Study population

At the fourth exam (1991–1993), a subset of the cohort with no dementia was identified. Those who agreed to participate were placed in the HAAS, which was designed to study cognitive function and diseases of aging [17]. This group of 3,734 men, representing almost 80% of the surviving HHP cohort, was examined approximately every two to three years through 2012. The average length of follow-up was 10.6 years. In addition to self-reported information on general health status, respondents were assessed for cognitive function with the CASI at each follow-up exam. Excluding respondents with missing data for head injury, the final sample for the TBI analysis was 3,123 participants. A subset of respondents agreed to a brain autopsy upon death, allowing for neuropathology assessment of 676 participants.

Definition of cognitive impairment

The CASI is a comprehensive screening instrument specifically designed for the cross-cultural assessment of cognitive impairment and dementia [16]. It combines measures of attention, concentration, orientation, memory, language, visual construction, abstraction, and judgement to create a composite score ranging from 1 to 100. It includes items identical or similar to ones used in three validated cognitive assessments: the Hasegawa Dementia Screening Scale (HDSS), the Mini-Mental State Examination (MMSE), and the Modified Mini-Mental State (3MS). As such, estimated scores for the HDSS, MMSE, and 3MS can be calculated from the CASI. They are only estimates because some items had to be modified to allow for larger cross-cultural reach.

For this analysis, a CASI score of less than 60 was used to indicate severe cognitive impairment, while a score from 60 to 74 was considered to reflect mild to moderate impairment, and a score of 74 or greater indicated negligible or no cognitive impairment. The HAAS sample was restricted to respondents whose baseline (HHP exam 4) CASI score was 60 or greater. During subsequent exams, if a respondent's CASI score fell below 74 they underwent additional evaluation including a neurological exam, the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) Neuropsychological Assessment Battery, the Behavior Pathology in Alzheimer's Disease Rating Scale (BEHAVE-AD), and Clinical Dementia Rating (CDR). More than 90% of the included participants had died before the final examination cycle. The last recorded CASI score available for each respondent was used to determine the presence ($n = 515$) or absence ($n = 2{,}608$) of severe cognitive impairment. For the majority of respondents, once their CASI score fell below 60, it did not rebound. Of the 538 respondents who ever had a CASI score less than 60, 217 (40.3%) had additional CASI scores recorded after dipping below this threshold. Of these, 183 (84.3%) stayed below 60, while 34 (15.7%) had at least one subsequent CASI score at or above 60.

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Table 1
Summary of results from five meta-analyses examining brain injury and neurologic outcomes

Meta-analysis	# of studies*	Pooled results	
Mortimer et al. [45]	8	RR for head trauma and AD	**1.82 (1.26–2.67)**
	8	• Among men	**2.67 (1.64–4.41)**
	7	• Among women	0.85 (0.43–1.70)
	5	• TBI ≤10 years before dementia onset	**5.33 (1.55–18.3)**
	5	• TBI >10 years before dementia onset	**1.63 (1.04–2.57)**
	5	• Age of AD onset <70 years	**1.95 (1.12–3.48)**
	5	• Age of AD onset 70+ years	**1.81 (1.03–3.25)**
Launer et al. [46]	4	RR for head trauma and dementia	1.14 (0.74–1.56)
	4	RR for head trauma and AD	1.02 (0.68–1.51)
	4	• Among men	1.66 (0.94–2.95)
Fleminger et al. [47]	15	OR for head injury and AD	**1.58 (1.21–2.06)**
	8	• Among men	**2.29 (1.47–3.58)**
	7	• Among women	0.91 (0.56–1.47)
	7	• Studies conducted after 1991	1.35 (0.94–1.94)
Perry et al. [48]	7	OR for TBI and dementia	1.36 (0.84–2.19)
	19	OR for TBI and AD	**1.40 (1.02–1.90)**
	15	OR for TBI and PD	**1.45 (1.18–1.78)**
	2	OR for TBI and MCI	**2.69 (1.51–4.77)**
Li et al. [49]	11	RR for head injury and dementia	**1.63 (1.34–1.99)**
	5	• Among men	**1.81 (1.38–2.38)**
	1	• Among women	1.30 (0.60–2.81)
	28	RR for head injury and AD	**1.51 (1.26–1.80)**
	11	• Among men	**1.70 (1.35–2.13)**
	5	• Among women	1.17 (0.89–1.53)
	2	RR for head injury with LOC and dementia	0.92 (0.67–1.27)
	8	RR for head injury with LOC and AD	1.49 (0.91–2.43)

AD, Alzheimer's disease; LOC, loss of consciousness; MCI, mild cognitive impairment; OR, odds ratios; PD, Parkinson's disease; RR, relative risk; TBI, traumatic brain injury. NOTE: Bolding identifies significant results. *Length of follow-up ranged from 0 to 43 years across all studies.

Definition of traumatic brain injury

Head injury data were obtained by self-report at baseline (HHP exam 4) of any head injuries 'severe enough to have lost consciousness.' Additionally, respondents were asked about 'serious head injuries with more than a momentary loss of consciousness' at exams 8 through 12 (2002–2012). Respondents who reported a head injury at exam 4 or exams 8–12 were coded as having a history of TBI ($n = 216$). Only 6% of these respondents had more than one TBI and none had more than 3. As such, we included these respondents with those reporting a single injury. Those who had no head injury at exam 4 and reported no follow-up injuries were coded as negative for TBI ($n = 2{,}907$). The respondents were asked about when the injury occurred. This information was used in addition to the date of the respondent's last CASI test to estimate the age of the injury at final CASI assessment.

Neuropathologic assessment

Details on the neuropathologic assessment have been published previously [15]. Briefly, brain tissue was examined for the presence of AD lesions using Braak staging [18] and Lewy bodies using the McKeith score [19]. Microvascular lesions and lacunar infarcts were counted from multiple areas of the brain and the presence of unilateral or bilateral hippocampal sclerosis was noted. Counts of neuritic and diffuse plaques were performed and the presence of evidence of trauma or subdural hematoma was observed. Brain weight was obtained prior to dissection. A combined index was calculated using the measures for AD lesions, Lewy bodies, microvascular lesions, hippocampal sclerosis, and brain weight. The index had a range from 0 (no pathology) to 5 (moderate to severe presence of all five characteristics).

Covariates

Covariates were calculated using a range of demographic and health data obtained at baseline (HHP exam 4). Health status measures included presence or absence of diabetes, hypertension, hearing impairment, and a history of depression. Stroke history was monitored until the final CASI. Lifestyle measures included smoking and drinking status (ever versus

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never) at baseline as well as educational attainment in years. Additional risk factors included apolipoprotein ε4 allele positivity (*APOE* ε4) and family history of AD. Military status was also examined, using a sample limited to respondents born in 1910 or later as the majority of military service in this cohort was during World War II. Age at death, follow-up time, and CASI score at baseline were also used.

Statistical analyses

Pearson's chi-square and *t* tests (Pooled or Satterthwaite) were used to examine bivariate differences between respondents with and without a history of TBI. Multivariate logistic regression was used to compare respondents with and without severe cognitive impairment at their final CASI test. To account for variable follow-up times, the number of days between baseline and final CASI was controlled for in the analysis. Cox Proportional Hazard regression models using follow-up time were also run, but the main results did not change. As such, the results reported here are from logistic regression models for ease of interpretation. Multivariate regression analyses were also adjusted for age at baseline, CASI score at baseline, educational attainment, *APOE* ε4 status, and history of stroke. For the neuropathology analyses, multivariate logistic regression models controlled for age at death, final CASI score, and time between final CASI and death. An alpha value of 0.05 was used for all analyses. All analyses were conducted using SAS 9.4 statistical package (Cary, NC).

RESULTS

Table 2 shows the results of the comparison of respondents with and without a history of TBI. The two groups showed significant differences between their final CASI score ($p = 0.0110$), CASI score at baseline ($p < 0.0001$), and follow-up time ($p < 0.0001$). Multivariate logistic analyses showed that, after controlling for CASI score at baseline and follow-up time, respondents with a history of TBI were no more likely to have a final CASI score below 60 than those without a TBI (OR = 1.422, CI: 0.99–2.05; data not shown).

Table 3 displays the differences between respondents with and without severe cognitive impairment (CASI score <60) at the final CASI assessment. Crude estimates and those controlling for CASI score at baseline, follow-up time, age at baseline, education, *APOE* ε4 status, and history of stroke are shown. Although a history of hearing problems at baseline was marginally associated with cognitive impairment (OR = 1.277, CI: 1.02–1.60, $p = 0.0306$), it was not included in the adjusted model because the relationship lost significance when controlling for the other covariates. In the crude model, having a history of TBI increased the risk for severe cognitive impairment by more than 50% (OR = 1.537, CI: 1.10–2.15, $p = 0.0115$). However, the relationship did not retain significance after adjusting for relevant covariates (OR = 1.320, CI: 0.90–1.93, $p = 0.1534$). No association was observed when limiting the sample to old injuries (5+ years before the final CASI) or new injuries (<5 years from the final CASI; data not shown). The same results were obtained when respondents who were lost to follow-up during the study were excluded (data not shown).

Looking at the subsample of respondents with a history of TBI, those with severe cognitive impairment at the final assessment were significantly older at the time of their first injury (71.7 years versus 60.5 years; Table 3). Although the magnitude of effect was not large, it remained marginally significant after controlling for covariates (OR = 1.017, CI: 1.00–1.04).

Table 4 displays the neuropathology results for those with and without a history of TBI. Crude estimates and those controlling for age at death, final CASI score, and time between final CASI and death are shown. In the crude model, respondents with a history of TBI were almost 130% more likely to show hippocampal sclerosis (OR = 2.298, CI: 1.17–4.50, $p = 0.0152$). However, the relationship weakened after controlling for covariates (OR = 1.462, CI: 0.68–3.12, $p = 0.3266$). Conversely, brain weight showed no difference in the crude model, but displayed marginal significance in the adjusted model (OR = 1.003, CI: 1.00–1.01, $p = 0.0174$). In this sample, respondents with a history of TBI had slightly heavier brains at death than those without. Results excluding respondents who were lost to follow-up were the same (data not shown).

DISCUSSION

The results from this study suggest no significant association between history of TBI and development of severe cognitive impairment or neuropathologic changes. Although a small, but statistically significant association with TBI was observed in the crude model, this relationship did not hold once the model was adjusted for relevant covariates. The results did

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Table 2
Comparison of characteristics among respondents with and without a history of head injury

Parameter	TBI+ ($n=216$) % (#), Mean (SD)	TBI−($n=2907$) % (#), Mean (SD)	p*	OR (95% CI)‡
Final CASI score <60	**22.7% (49)**	**16.0% (466)**	**0.0110**	**1.537 (1.10–2.15)**
CASI score at baseline	**88.1 (6.9)**	**86.0 (8.2)**	**<0.0001**	**1.038 (1.02–1.06)**
Follow-up time (y)	**10.6 (6.3)**	**8.0 (6.0)**	**<0.0001**	**1.073 (1.05–1.10)**
Age at baseline (y)	77.2 (4.1)	77.7 (4.3)	0.0779	0.970 (0.94–1.00)
Education (y)	11.1 (3.4)	10.7 (3.1)	0.0862	1.042 (1.00–1.09)
APOE ε4 positive	23.0% (49)	18.3% (523)	0.0903	1.333 (0.96–1.86)
Parent(s) with AD	8.7% (17)	6.3% (165)	0.1935	1.411 (0.84–2.38)
Relative with AD	9.1% (19)	7.8% (219)	0.5193	1.175 (0.72–1.92)
Ever had depression	2.3% (5)	1.0% (28)	0.0604	2.442 (0.93–6.39)
Ever had hearing problem	25.8% (55)	20.9% (606)	0.0929	1.304 (0.96–1.81)
Ever had a stroke	18.5% (40)	17.5% (507)	0.6944	1.074 (0.75–1.53)
Hypertension at baseline	18.5% (40)	18.0% (524)	0.8594	1.033 (0.72–1.48)
Diabetes at baseline	15.0% (32)	17.4% (501)	0.3590	0.834 (0.57–1.23)
Ever smoked at baseline	59.8% (128)	62.8% (1824)	0.3854	0.882 (0.66–1.17)
Ever drank at baseline	62.7% (133)	58.8% (1704)	0.2654	1.178 (0.88–1.57)
Served in military§	31.1% (61)	30.0% (736)	0.7343	1.056 (0.77–1.45)

AD, Alzheimer's disease; APOE e4, apolipoprotein allele e4; CASI, Cognitive Abilities Screening Instrument; SD, standard deviation; TBI, traumatic brain injury. NOTE: Dichotomous measures are shown as percentage and sample size while continuous measures are displayed as mean and standard deviation (SD). Bolding identifies significant results. *p value for Pearson chi-square or Satterthwaite t value. ‡Odds ratio (OR) and 95% confidence interval (95% CI) for parameter. §Limited to respondents born in 1910 or after.

Table 3
Comparison of characteristics among respondents with and without severe cognitive impairment (final CASI <60) at their final CASI assessment

Parameter	SCI+($n=515$) % (#), Mean (SD)	SCI−($n=2608$) % (#), Mean (SD)	p*	Unadjusted OR (95% CI)‡	Adjusted† OR (95% CI)‡
History of TBI	**9.5% (49)**	**6.4% (167)**	**0.0115**	**1.537 (1.10–2.15)**	1.320 (0.90–1.93)
CASI score at baseline	**82.1 (8.7)**	**86.9 (7.7)**	**<0.0001**	**0.935 (0.93–0.95)**	**0.908 (0.89–0.92)**
Follow-up time (y)	**11.7 (5.0)**	**7.5 (6.0)**	**<0.0001**	**1.126 (1.11–1.15)**	**1.217 (1.19–1.25)**
Age at baseline (y)	**78.5 (4.5)**	**77.6 (4.2)**	**<0.0001**	**1.049 (1.03–1.07)**	**1.105 (1.08–1.14)**
Education (y)	**9.9 (3.1)**	**10.9 (3.1)**	**<0.0001**	**0.902 (0.87–0.93)**	0.964 (0.93–1.00)
***APOE* ε4 positive**	**25.3% (129)**	**17.3% (443)**	**<0.0001**	**1.616 (1.29–2.02)**	**1.778 (1.38–2.30)**
Ever had a stroke	**26.6% (137)**	**15.8% (410)**	**<0.0001**	**1.939 (1.55–2.42)**	**2.008 (1.56–2.59)**
Ever had hearing problem	**24.9% (127)**	**20.6% (534)**	**0.0306**	**1.277 (1.02–1.60)**	1.198 (0.93–1.54)
Parent(s) with AD	7.1% (31)	6.4% (151)	0.5931	1.115 (075–1.67)	1.091 (0.69–1.73)
Relative with AD	8.4% (42)	7.8% (196)	0.6336	1.088 (0.77–1.54)	1.001 (0.67–1.49)
Ever had depression	1.4% (7)	1.0% (26)	0.4545	1.377 (0.59–3.19)	1.749 (0.62–4.97)
Hypertension at baseline	18.1% (93)	18.1% (471)	0.9934	0.999 (0.78–1.23)	0.895 (0.68–1.18)
Diabetes at baseline	16.3% (83)	17.4% (450)	0.5284	0.921 (0.71–1.19)	1.237 (0.93–1.65)
Ever smoked at baseline	59.0% (302)	63.3% (1650)	0.0656	0.834 (0.69–1.01)	1.052 (0.84–1.31)
Ever drank at baseline	56.1% (286)	59.7% (1551)	0.1283	0.862 (0.71–1.04)	0.958 (0.77–1.19)
Served in military§	26.7% (111)	30.7% (686)	0.1025	0.822 (0.65–1.04)	1.047 (0.79–1.39)
Among those with a TBI¶	($n=48$)	($n=162$)			
Age at first TBI (y)	**71.7 (20.1)**	**60.5 (17.0)**	**.0002**	**1.033 (1.02–1.05)**	**1.017 (1.00–1.04)**

AD, Alzheimer's disease; *APOE* ε4, apolipoprotein allele e4; CASI, Cognitive Abilities Screening Instrument; CI, confidence interval; OR, odds ratio; SCI, severe cognitive impairment; SD, standard deviation; TBI, traumatic brain injury. NOTE: Dichotomous measures are shown as percentage and sample size while continuous measures are displayed as mean and standard deviation (SD). Bolding identifies significant results. *p value for Pearson chi-square or Satterthwaite t value. †Adjusted for CASI score at baseline, age at baseline, follow-up time, education, *APOE* ε4 status, and history of stroke. ‡Odds ratio (OR) and 95% confidence interval (95% CI) for parameter. §Limited to those born in 1910 or after. ¶Unable to calculate age at TBI for six respondents due to missing data.

not change when examining old or new injuries (5+ years and <5 years from final CASI, respectively) or when respondents lost to follow-up were excluded. Among respondents who reported a TBI, those with severe cognitive impairment were older at the time of injury than those who did not develop impairment. This calls into question the possibility of reverse causality. That is, people in the early stages of cognitive impairment may be more likely to fall and incur a brain injury. Previous studies have shown that a diag-

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Table 4
Neuropathologic characteristics among respondents with and without a history of head injury

Parameter	TBI+($n=51$)	TBI−($n=625$)	p^*	Unadjusted	Adjusted†
	% (#), Mean (SD)			OR (95% CI)‡	
AD lesions	45.1% (23)	40.6% (254)	0.5336	1.200 (0.68–2.13)	1.103 (0.60–2.04)
Lewy bodies	9.8% (5)	12.7% (79)	0.5524	0.750 (0.29–1.94)	0.704 (0.24–2.07)
Microvascular lesions	39.2% (20)	31.9% (199)	0.2827	1.378 (0.77–2.48)	1.395 (0.76–2.57)
Hippocampal sclerosis	**25.5% (13)**	**13.0% (81)**	**0.0129**	**2.298 (1.17–4.50)**	1.462 (0.68–3.12)
Brain weight (grams)	**1224 (100)**	**1213 (123)**	0.5287	1.001 (0.10–1.00)	**1.003 (1.00–1.01)**
Neuritic plaques	68.6% (35)	61.9% (387)	0.3416	1.345 (0.73–2.48)	0.954 (0.50–1.82)
Diffuse plaques	64.7% (33)	62.4% (390)	0.7435	1.105 (0.60–2.00)	0.835 (0.45–1.56)
Trauma§	19.6% (10)	11.7% (73)	0.0972	1.844 (0.89–3.84)	1.923 (0.90–4.11)
Subdural hematoma§	25.5% (13)	15.7% (98)	0.0690	1.840 (0.95–3.58)	1.831 (0.92–3.65)
Combined index	1.05 (0.94)	1.03 (0.83)	0.7566	1.055 (0.75–1.47)	0.765 (0.49–1.19)
Total # of microinfarcts	2.53 (4.0)	2.42 (4.3)	0.8558	1.006 (0.95–1.07)	1.006 (0.94–1.08)
Total # of lacunar infarcts	2.12 (3.9)	1.75 (3.0)	0.5220	1.034 (0.95–1.12)	1.033 (0.95–1.12)
Total # of infarcts	4.65 (5.7)	4.17 (6.0)	0.5863	1.012 (0.97–1.06)	1.013 (0.97–1.06)

AD, Alzheimer's disease; CI, confidence interval; OR, odds ratio; SD, standard deviation; TBI, traumatic brain injury. NOTE: Dichotomous measures are shown as percentage and sample size while continuous measures are displayed as mean and standard deviation (SD). Bolding identifies significant results. *p value for Pearson chi-square or Satterthwaite t value. †Adjusted for age at death, final CASI score, and time between final CASI score and death. ‡Odds ratio (OR) and 95% confidence interval (95%CI) for parameter estimate. §Remote or acute.

nosis of dementia increases the chances of one or more falls [20]. However, there was a greater number of recent injuries (<5 years before final CASI) among those without severe cognitive impairment than those with, suggesting reverse causation is not playing a role.

The current findings support the results of several studies looking at TBI and cognitive impairment using large, prospective cohorts. Crane et al. [11] found no association between TBI with loss of consciousness (LOC) and incident dementia in three large cohorts: Adult Changes in Thought (ACT), Religious Orders Study (ROS), and Memory and Aging Project (MAP). Dams-O'Connor et al. [12] also found no relationship between TBI with LOC and dementia or AD in the ACT cohort. No association was found in the Rotterdam Study [21] or in the Canadian Study of Health and Ageing (CSHA) [22]. Within the National Alzheimer's Coordinated Center (NACC) Uniform Data Set, there are conflicting results about the importance of TBI in subsequent cognitive decline or dementia [14, 23–25]. However, several studies using the NACC consistently found a relationship between earlier onset of cognitive decline and history of TBI [23, 26, 27].

Some studies have examined age at head injury as a factor for subsequent cognitive impairment. Gardner et al. [28] found the association between TBI and risk of dementia was stronger among older adults. In our sample, among respondents with a history of TBI, those with severe cognitive impairment at their final CASI assessment were significantly older at the time of their first injury. This may reflect increased vulnerability to TBI with age as a consequence of age-related decline in resilience or increase in frailty. Alternatively, it could be a result of older respondents in the early stages of dementia being more likely to fall.

Our neuropathology results partially align with the findings from three large prospective cohorts in which an increase in Lewy bodies and microinfarcts was observed among individuals with a history of TBI, but no difference was seen in neuritic plaques or neurofibrillary tangles [11]. Other studies have also shown no difference in AD pathology between people with and without a history of TBI [27, 29], while some have found an increase in amyloid beta precursor protein or amyloid plaques [13, 30]. Although neuronal loss from the hippocampus has been demonstrated as a result of TBI [31, 32], other studies have found no difference in hippocampal volume [10]. We observed more hippocampal sclerosis in respondents with a history of TBI, but the relationship was lost after adjusting for covariates. The pathology of TBI is complex and dependent on a number of variables, including age at injury, severity of injury, and time to death. Variations in these and other variables may explain the inconsistent neuropathological results observed across studies.

Although our study results suggest no association between TBI and cognitive impairment or neuropathology, it is possible the absence of correlation is an artifact of the demographic make-up of the cohort (Japanese-American men). However, Kondo et al. [33] found an association among their cohort of elderly Japanese. Alternatively, the results may be

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influenced by the fact the majority of our respondents reported only a single head injury. Although evidence exists that even a single injury can increase the risk of dementia and cause neurodegenerative pathology [34], the risk may be negligible until multiple injuries have occurred.

One issue in this body of research that is often cited as part of the explanation for the inconsistent results is the lack of a standardized definition for TBI and the use of varied criteria for diagnosis of neurodegenerative disease [12, 35]. Beyond the lack of standardized definition, injury data are obtained from different data sources across the studies. Self-reported head injury data may suffer from recall bias, but may also capture more mild events. Hospital data are generally accurate, but exclude those who did not seek medical treatment as a result of their injury. Additionally, there is no agreed upon scale for injury severity, though many researchers used length of time spent unconscious as a metric. There is evidence to suggest that increased frequency or severity of the injury may increase the risk of dementia [36]. Only 6% of our cohort reported more than one head injury and severity in terms of time of LOC was only collected at baseline. At subsequent exams, respondents were asked if they had a head injury with more than momentary loss of consciousness. As such, we were not able to make comparisons based on the severity or frequency of injury.

It is interesting to note our results demonstrate no association between a history of TBI and service in the military. There is much concern about the effects of combat on the future health of service members and several studies have found an increased risk for neurodegenerative diseases among veterans with a history of TBI [36–42]. The cohort for this study may be slightly different in regard to their military experience. The vast majority of the veterans in the group served during World War II and may have been exposed to qualitatively different injury risks than those serving in more recent combat.

As with other research of similar design, there are limitations to be considered when interpreting the results of this study. Any information collected via self-report is subject to recall bias that may affect the accuracy of the results. The longitudinal study design allowed for the collection of injury data prior to onset of cognitive impairment, which should reduce this bias. However, we still relied on the respondent's memory, the accuracy of which may vary depending on cognitive function. To measure cognitive impairment, we used the CASI screening instrument, which has been validated as an effective tool for measuring cognitive impairment and progression of dementing disorders across cultures [16]. However, it has limitations in its use as a proxy for determining the presence of dementia. Ideally, multiple metrics should be used in this determination.

As a result of data collection methodology, the study was also limited in what could be examined. In the sample, only 6% of respondents reported multiple head injuries, and data on injury-related amnesia LOC were only collected at baseline. As such, we were unable to test for differences by injury frequency or severity. Given the composition of the cohort, we were also unable to examine the relationship by gender or race. The sample definition also brings generalizability of the results into question, given the cohort consists only of men with Japanese ancestry. However, previous studies using the HAAS data have shown similar findings as other cohorts, including the Nun Study, the ROS, and the MAP [17, 43, 44].

The current study has many strengths, including the longitudinal study design, large cohort size, and high participation rates. Data were collected prospectively, reducing bias, and there was a long follow-up period for monitoring cognitive function, up to 21 years. With exams every two to three years, the HAAS obtained considerable data on the changing health and behavior of the cohort as it aged. The availability of autopsy data for a subset of the sample allowed for neuropathologic assessment, another strength of the study.

Conclusion

In this prospective cohort, there was no association between a history of TBI and subsequent severe cognitive impairment. This is in accordance with studies looking at other prospective cohorts [11, 12, 21, 22]. Additionally, we found no strong neuropathologic differences with a history of TBI. However, the results However could be influenced by the demographic make-up of the sample or by the observation that the majority of respondents reported only a single head injury.

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Head Trauma and Alzheimer's Disease: A Case Report and Review of the Literature

Rishi D.S. Nandoe[a], Philip Scheltens[a,*] and Piet Eikelenboom[b]
[a]*Department of Neurology, Vrije Universiteit Medical Centre, Amsterdam, the Netherlands*
[b]*Department of Psychiatry, Vrije Universiteit Medical Centre, Amsterdam, the Netherlands*

Abstract. The authors describe a case of a 55-year-old woman who was diagnosed with Alzheimer's disease 1.5 years after a car accident in which she experienced a mild concussion. Extensive history taking disclosed no cognitive changes prior to the car accident. The case is discussed in view of the inflammation hypothesis regarding Alzheimer's disease and the role of the apolipoprotein E4 genotype of the patient.

Keywords: Alzheimer's disease, amyloid, ApoE, atrophy, cholinesterase inhibitors, head trauma, inflammation

INTRODUCTION

Dementia is an important cause of disability, particularly in the elderly. Alzheimer's disease (AD) is the predominant cause of dementia, accounting for at least half of all cases. One of the theories regarding the pathogenesis of AD is an "ageing hypothesis" that regards senile dementia of the Alzheimer type as a manifestation of accumulated ageing changes, an inevitable result of old age [1]. Although the exact aetiology of the disease is still unknown, there are other factors known to be associated with an increased risk of early and late onset AD. Case-control studies have evaluated parental age, female sex, birth order, family history of dementia or AD, some occupational and environmental exposures and a variety of medical conditions as possible risk factors for developing AD [2–6]. Traumatic brain injury (TBI) is also a well-known risk factor of AD that may accelerate neuronal loss by reducing the safety factor provided by the brain [7–9]. As a result of this AD will clinically express itself earlier in life. The apolipoprotein E (ApoE) protein, which is engaged in growth and repair of the nervous system, may influence the outcome after TBI. Despite the observed effect of head trauma on AD in epidemiological studies, in clinical practise one rarely finds a clear temporal relationship between TBI and the occurrence of AD. In the following we describe a previously healthy patient who developed AD after a minor head trauma.

*Correspondence to: Philip Scheltens, Department of Neurology and Alzheimer Center Amsterdam, Amsterdam Neuroscience, Vrije Universiteit Amsterdam, Amsterdam UMC, Amsterdam, the Netherlands; E-mail: p.scheltens@amsterdamumc.nl.

CASE REPORT

A 55-year-old woman was admitted to the hospital after a traffic accident in which she bumped her head against the front window, when driving in her car without safety belts. She experienced a brief period of loss of consciousness and was brought to the emergency department, in which she was diagnosed with a minor concussion and admitted for observation. A skull Xray revealed no fractures. She recovered completely and was discharged the next day. Her medical history revealed head traumas of similar severity at the age of 9, followed by one at the age of 32. After discharge from the hospital she suffered from pain in the upper neck region and head and she received physiotherapy for a brief period, until complete recovery. Almost 1.5 years after the accident the patient was seen on our neurological outpatient clinic because of 'progressive forgetfulness'. Upon further question-

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ing she also disclosed problems concerning writing and mathematics, as well as word finding difficulties. Her spouse and two children were convinced that she had displayed no signs of cognitive impairment before the head trauma at all and blamed the accident for the current complaints. She had had a demanding job in a television studio and had performed to full satisfaction of her employer, until the accident.

Physical examination showed no abnormalities. Pulse and blood pressure were within normal range. Neurological examination was normal. The Mini-Mental State Examination (MMSE) score was 17/30. Neuropsychological examination showed global impairment in all cognitive domains, including executive dysfunction. In addition, there were difficulties in simple arithmetic, reading aloud and word finding and she displayed perseverations and omissions. On the Visual Association Test, after one attempted trial, she was unable to remember any associations' figures. There were clear deficits in drawing simple figures and writing a sentence. Although no earlier neuropsychological examinations had been performed, these results were out of proportion to the accident she had experienced and a dementia syndrome was diagnosed clinically. Family history revealed that her mother was the only relative who had suffered from dementia but had died at the age of 93. Routine laboratory examination was normal. ApoE genotyping disclosed $\varepsilon 4$ homozygosity. Routine brain MRI showed mild cortical and moderate hippocampal atrophy (Fig. 1), but no traumatic abnormalities or vascular changes. A diagnosis of probable AD according to the NINCDS-ADRDA criteria [10] was made and the patient was treated with Donezepil 10 mg daily, which she tolerated well. However, during a follow up period of 3 years she deteriorated further and started having vivid delusions and anxiety. She had to be treated with neuroleptics and was admitted to a nursing home 3.5 years after the accident.

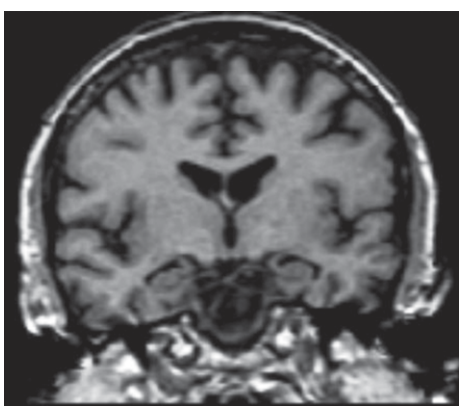

Fig. 1. Coronal T1 weighted MRI scan showing moderate medial temporal and mild global brain atrophy.

DISCUSSION

We present a case of AD in a young woman shortly after she had experienced a minor head trauma. Since formal neuropsychological examination was first done 1.5 years after the accident, it remains unclear in what state the subject was exactly at the time of the accident. Thus, we cannot rule out that the patient was in fact already suffering from early onset AD at the time of the accident. However, extensive history taking from family, friends and employer, disclosed no signs of cognitive impairment at all before the head trauma. Appropriate laboratory tests and imaging procedures were done to rule out other contributing factors, so the diagnosis of AD was made in line with current criteria. Below we will discuss the possible role of the preceding head trauma.

The most common type of posttraumatic dementia is dementia pugilistica as shown in boxers who have experienced repeated head injuries [11, 12]. For a long time this type of dementia was described as the 'punch drunk' syndrome. In the last decade dementia pugilistica received increasing attention, whereas the association with other types of dementia was considered. After the isolation of the main protein of the AD deposits [13] antibodies against this protein were produced [14], which turned out to be more sensitive than the routine histological techniques [15]. As a result of this, large numbers of diffuse plaques could also be discovered in the boxer's brain [16], that were indistinguishable from those seen in AD.

Epidemiological studies clearly show that head trauma also is a risk factor for the development of dementia of the Alzheimer type [8, 9, 17, 18]. However, in clinical practice one rarely finds such a clear temporal relationship between TBI and AD as seen in this case.

It is suggested that in the pathogenesis of AD an inflammation process and genetic factors play an important role. Until recently, the brain was considered to be an immunological privileged area, with limited mechanisms to induce an immunological defence. Studies suggested that AD with head trauma might cause an inflammation process

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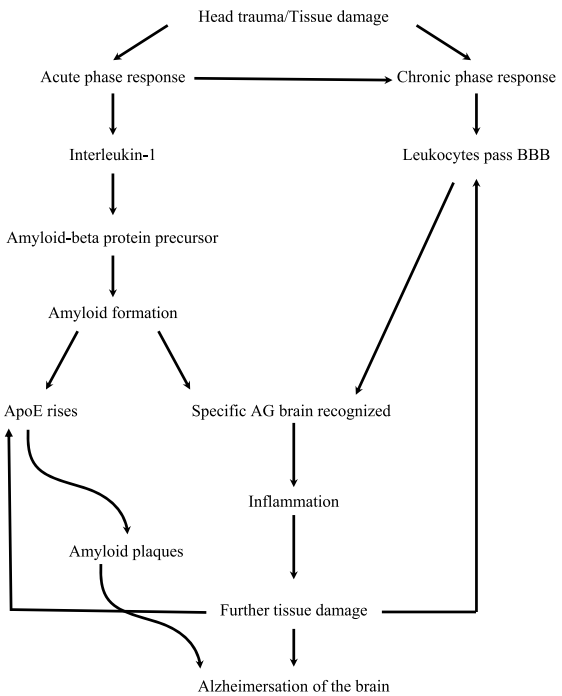

Fig. 2.

by damage of the blood-brain barrier (BBB), permitting extravasation of serum proteins into brain parenchyma. As a result of the extravasation, contact of serum leukocytes with specific brain antigens in the brain parenchyma may sensitise the immune system, permitting an increased secondary response after exposure to the same antigens many years later [19]. There is considerable evidence that deposition of amyloid-β in AD is one of those triggers that might lead to a local brain inflammatory response. However, a local brain inflammatory response rather than inflammatory mediators passing the BBB was considered to play an essential role. Such an 'acute phase response', occurring within days after the TBI, may initiate the process of inflammation and plaque formation in the brain. It is suggested that interleukin-1 (Il-1) present in the brain induces this acute phase response leading to increased synthesis of amyloid-β protein precursor [20–22]. As a result of the overexpression of both these proteins one finds both astroglial activation and amyloid deposits [23]. Perhaps the local influence of Il-1 plays an important role in the acute phase, whereas the periphery may contribute, through passing the BBB, to a chronic inflammatory state in longer-time survivors or repetitive TBI patients, especially when the initiating factors persist (see Fig. 2). Fibrillar amyloid-β peptide itself can induce a chronic inflammatory response in the brain [24]. Remote head injury, as also seen in our patient, may therefore be considered as an extra risk factor [19, 25]. The repeated acute phase responses and the chronic inflammatory state may attribute to "alzheimerisation" of the brain. The idea that inflammation is involved in the pathogenesis of AD is supported by epidemiological studies indicating that the use of non-steroid anti-inflammatory drugs (NSAIDs) may prevent or retard the process of AD [26]. An unresolved issue however, is the pathogenetic link between amyloid deposition and neurofibrillary pathology, the second neuropathological hallmark of AD. The neurofibrillary pathology consists of intraneuronal accumulation of paired helical filaments with abnormal phosphorylated tau as major component. Evidence has been presented that both phosphorylation and modification by oxidative stress are required for tau filament formation [27]. This finding is in line with the view that abnormal phosphorylation of proteins in AD may be a consequence of oxidative stress [28]. Activated microglia, such as those that surround most senile plaques, are a source of reactive oxygen species [29]. If oxidative stress is required for the development of abnormal tau deposits, the inflammation related production of reactive oxygen species could contribute to the neurofibrillary pathology.

Due to the inflammation and the amyloid plaques, tissue damage will occur, with as result an increase of neuronal loss, a process that also appears in the normal human brain with aging. It appears that a threshold, a critical number and/or location of lesions, must be reached before cognitive loss become evident [30]. In our case one has to assume that, in spite of the lack of a history of progressive cognitive decline before the accident, some cerebral degeneration must have been pre-existent, which was accelerated by the accident, passing the threshold for clinical detection of dementia. A similar process seems to be present in the occurrence of post-stroke dementia [31].

A variety of inflammatory mediators can be found in association with the pathogenesis and presence of amyloid plaques. Although the precise role of these proteins in the pathogenesis of senile plaques is yet unknown, it has become clear that the balance between amyloid-β fibrillogenesis factors and disaggregation factors may ultimately determine the initial deposition of amyloid-β in the plaques. ApoE is a known amyloid-β fibrillogenesis factor and therefore likely to contribute to the initiation of the process of plaque formation [24].

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Other than its role in the inflammatory response by contributing to the formation of amyloid plaques, the ApoE protein also influences the development of AD through a genetic pathway.

The ApoE protein is engaged in the redistribution of cholesterol from cells, neuronal extension, but more important in growth and repair of the nervous system. The ApoE gene, located on chromosome 19, is highly polymorphic. The protein encoded by ApoE ε4 allele binds best to the amyloid-β peptide in comparison with the other alleles. Patients who are ApoE ε4 positive, such as our patient, have a disturbed cell repair system. The role of the ApoE is to transport the produced amyloid-β peptide from the brain. In the ApoE ε4/ε4 variant this important function is disturbed. As a result of this the plaques cannot be transported and will stay in the brain, hence leading to a process of "alzheimerisation" of the brain [32–34]. In addition to this, studies show that patients with ApoE ε4 are more than twice as likely as those without the ApoE ε4 to have a poor outcome after TBI [33, 35–38].

In sum, after the acute phase response the inflammation may become chronic because the initiating factors persist. As tissue damage leads to a (i) raise of ApoE with its role in plaque formation and (ii) passing of serum leukocytes through the BBB, further inflammation and ongoing tissue damage will be the result, thus ultimately resulting in a chronic inflammatory state of the brain. On the other hand a mutation of the ApoE protein, as seen in our patient, will make it unable to repair damaged neuronal cells by transporting the formed amyloid plaques. This second pathway also leads to a chronic state of inflammation. Finally the role of ApoE, as β-fibrillogenesis factor, in the plaque formation may lead to a typically known "alzheimerisation" of the brain. A combination of both may well be the final common pathway in starting a viscous circle of progressing neurodegenerative disease (see Fig. 2).

In this case the outcome of TBI was different than expected. In the absence of pathological confirmation, we hypothesize that a failure of the repair mechanism after TBI, due to mutation of the ApoE protein, may lead to a cascade leading to "alzheimerisation" of the brain and causing symptoms characteristic of AD.

In spite of recent studies showing that mild head trauma is not significantly contributing to the pathogenesis of AD, at least in the elderly [7], epidemiological studies clearly show TBI and ApoE polymorphism as risk factors for AD, but in clinical practice one rarely finds such an evident temporal relation like in our case, although preclinical AD at the time of the accident cannot be ruled out completely.

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Handbook of Traumatic Brain Injury and Neurodegeneration
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Traumatic Brain Injury and Risk of Long-Term Brain Changes, Accumulation of Pathological Markers, and Developing Dementia: A Review

Christian LoBue[a,b,*], Catherine Munro[a], Jeffrey Schaffert[a], Nyaz Didehbani[a], John Hart, Jr.[a,c,d], Hunt Batjer[b] and C. Munro Cullum[a,b,d]
[a]*Department of Psychiatry, University of Texas Southwestern Medical Center, Dallas, TX, USA*
[b]*Department of Neurological Surgery, University of Texas Southwestern Medical Center, Dallas, TX, USA*
[c]*School of Behavioral and Brain Sciences, University of Texas at Dallas, Dallas, TX, USA*
[d]*Department of Neurology and Neurotherapeutics, University of Texas Southwestern Medical Center, Dallas, TX, USA*

Abstract. Traumatic brain injuries (TBI) have received widespread media attention in recent years as being a risk factor for the development of dementia and chronic traumatic encephalopathy (CTE). This has sparked fears about the potential long-term effects of TBI of any severity on cognitive aging, leading to a public health concern. This article reviews the evidence surrounding TBI as a risk factor for the later development of changes in brain structure and function, and an increased risk of neurodegenerative disorders. A number of studies have shown evidence of long-term brain changes and accumulation of pathological biomarkers (e.g., amyloid and tau proteins) related to a history of moderate-to-severe TBI, and research has also demonstrated that individuals with moderate-to-severe injuries have an increased risk of dementia. While milder injuries have been found to be associated with an increased risk for dementia in some recent studies, reports on long-term brain changes have been mixed and often are complicated by factors related to injury exposure (i.e., number of injuries) and severity/complications, psychiatric conditions, and opioid use disorder. CTE, although often described as a neurodegenerative disorder, remains a neuropathological condition that is poorly understood. Future research is needed to clarify the significance of CTE pathology and determine whether that can explain any clinical symptoms. Overall, it is clear that most individuals who sustain a TBI (particularly milder injuries) do not experience worse outcomes with aging, as the incidence for dementia is found to be less than 7% across the literature.

Keywords: Alzheimer's disease, brain imaging, chronic traumatic encephalopathy, cognition, concussion, dementia, frontotemporal dementia, Lewy body dementia, Parkinson's disease, traumatic brain injury

INTRODUCTION

Traumatic brain injuries (TBI) are common, resulting in an estimated 10 million hospitalizations worldwide, but the actual prevalence of TBI is

*Correspondence to: Christian LoBue, PhD, 5323 Harry Hines Blvd, Dallas, TX: 75208 8564, USA. Tel.: +1 214 648 4604; Fax: +1 214 648 4660; E-mail: christian.lobue@utsouthwestern.edu.

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difficult to estimate because many milder injuries do not come to medical attention [1]. The Centers for Disease Control and Prevention reported that rates of TBI have increased more than 50% in the United States over the past decade, with falls, being struck by an object, and vehicle crashes being the most common causes of injury [2]. However, it is likely that the dramatic increase in rates of TBI reflects a greater awareness for milder injuries among the general population and a much more conservative approach to diagnosing mild TBI than has existed in the past. Diffuse axonal injury, involving damage to cerebral white matter tracts, occurs in nearly all severities of TBI, acutely disrupting biochemical and cytoskeletal functions to produce physical, emotional, and/or cognitive symptoms. Diffuse axonal injury can be induced during a TBI through several mechanisms, including axonal stretching or shearing, swelling, or secondary physiological changes downstream from neuronal damage (e.g., Wallerian degeneration) [3]. Although many individuals fully recover from brain trauma, especially milder injuries, there is a growing public health concern about the potential long-term consequences from TBI. Widespread media coverage following the suicide of several high-profile professional football athletes with a history of head impacts and reported cognitive and emotional changes in middle-age has contributed to fears of developing neurodegenerative disorders many years after TBI among not only athletes, but also military personnel and members of the general community. However, there is currently a debate in the scientific literature relating to whether TBI is a static injury or possibly a dynamic injury process, in which TBI leads to chronic neuroinflammation or progressive neurodegeneration linked to later-in-life functional changes. This article provides a critical review of the evidence to date surrounding TBI as a risk factor for long-term brain changes and cognitive decline, in addition to an increased risk of neurodegenerative conditions.

CLASSIFICATION OF TBI

TBI is often graded on the severity of symptoms resulting from a blow to the head or body that affects the brain. Alteration in mental status, such as disorientation and cognitive difficulties, loss of consciousness (LOC), a loss of memory for events occurring before or after the injury (i.e., posttraumatic amnesia), and the Glasgow Coma Scale score, when available, have historically been used to characterize whether an injury is mild, moderate, or severe. While there is no universally agreed upon system for classifying TBI severity, in general, cutoffs for the duration or severity of each of these elements are often used in clinical practice (See Table 1) [4, 5]. However, not all individuals fit neatly into these categories, and as a result, such classification systems are waning in popularity. For example, individuals who meet criteria for mild TBI generally do not show intracranial abnormalities (e.g., cerebral hemorrhage or contusion) on standard neuroimaging techniques. However, there is a subset of mild TBI individuals that do show one or more brain abnormalities, and this is often associated with poorer recovery outcomes relative to individuals with mild TBI without such complications [6]. Thus, a further classification widely used is *uncomplicated* mild TBI when less severe injuries do not result in intracranial abnormalities and *complicated* mild TBI when these are present.

A methodological challenge that has limited research on TBI as a risk factor for long-term brain changes and cognitive decline relates to TBI classification. Despite some studies having TBI criteria similar to those used in clinical practice, many have relied on broad definitions of TBI that are based on presence/absence of LOC, intracranial abnormalities, or reported number of cumulative head impacts. Duration of LOC, when available, has often times been divided into arbitrary intervals (i.e., <5 and ≥ 5 minutes or <1 and ≥ 1 hour) that do not correspond well to common rubrics used to reflect mild, moderate, or severe injuries. This is because research on the long-term effects of TBI frequently rely on retrospective reports derived from medical records or data from longitudinal aging studies, where TBI-related questions were limited since that was not the purpose of the investigation. Heterogeneity in the classification of TBI in research studies has likely contributed to at least some of the mixed findings in the literature, as different associations may be possible depending on how TBI was defined (e.g., LOC >1 hour versus any head impact). Whereas several meta-analyses have examined the link between TBI and dementia [7–9], to date these also have relied on broad classifications of TBI and have the potential to mask possible associations that may be related to TBI severity, multiple injuries, and age at injury, among other factors. Because TBI is a heterogeneous condition, and neurodegenerative processes may develop over decades, it is important to consider how methodological factors and differences could influence study findings.

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Table 1
Common systems for grading traumatic brain injury severity

System	Classification	LOC	GCS	PTA	AMS	Imaging
VA/DOD System	Mild	0–30 min	13–15	0–1 day	<24 h	Normal
	Moderate	30 min–24 h	9–12	1–7 days	>24 h	Normal or abnormal
	Severe	>24 h	<9	>7 days	>24 h	Abnormal
Mayo System	Mild	<30 min	13–15 in 1st 24 h	<1 day	Necessary for a "Possible" injury if other Criteria are Absent	Depressed, basilar, or linear skull fracture (no hemorrhage, hematoma, contusion, or brain stem injury)
	Moderate-to-Severe	>30 min	<13 in 1st 24 h	>1 day	Severity Based on Other Criteria	Abnormal

AMS relates to blurred vision, confusion, dazed, dizziness, focal neurological symptoms, headache, and nausea.LOC, loss of consciousness; GCS, Glasgow Coma Scale; PTA, post-traumatic amnesia; AMS, altered mental status.

ARE THERE LONG-TERM BRAIN CHANGES FROM TBI?

Structural neuroanatomical imaging

Modern imaging techniques have evolved to allow *in vivo* evaluation of structural brain changes post-TBI (see Table 2). Imaging techniques, such as magnetic resonance imaging (MRI), can be performed longitudinally to evaluate whether some individuals continue to show structural brain changes years after acute-injury recovery. In fact, evidence of degeneration of white matter tracts (e.g., corpus callosum) and brain structures has been observed years after a moderate to severe TBI [10–12]. Longitudinal MRI scans have shown decreases in global brain volume 1 to 2.5 years after patients sustain a single moderate-to-severe TBI [13–17]. Although frontal and temporal regions are most susceptible to traumatic injury [18], longitudinal studies examining hippocampal volume [a key neuroanatomical region in neurodegenerative diseases such as Alzheimer's disease (AD)] at least 1-year post-injury have been mixed, with some reporting decreased volume up to 2.5 years after injury and others observing no hippocampal changes at all [10, 13, 19, 20]. However, it is difficult to determine when volume loss and white matter changes directly related to the acute effects of TBI develop and may plateau, as most studies only investigate one or two time points. For example, in the longest-follow up study to date, decreased volume of the corpus callosum, but not the hippocampus, was found between MRI scans at 1 and 8 years-post-injury [10]. It is unknown if volume loss stopped shortly after the first scan, plateaued after a few years, or continued up to 8-years post-injury given that additional serial scans were lacking. Interestingly, in one study that examined white matter integrity at 3 time points over several years (2 months, 1 year, and 4 years) after patients sustained a moderate-to-severe TBI in early adulthood (average age = 34.5), white matter degeneration was observed across all time points when compared to controls [12]. Because many individuals with moderate-to-severe TBI achieve maximum clinical recovery by 1-2 years post-injury, these data suggest that TBI can induce neurodegenerative changes in some individuals that can persist for at least several years following more severe injuries. Whether the neurodegenerative changes may advance beyond this time point has not been examined to determine whether TBI triggers a progressive process in the long-term that could directly lead to the development of a neurodegenerative condition in some individuals.

Degeneration of white matter tracts and brain structures after mild TBI is not as well established in humans, partly because injuries can vary in terms of diagnosis, mechanism, and severity. Blast-related mild TBI was shown to be associated with multiple regions of focal decreased white matter integrity 4–6 years after injury in 72 military veterans in comparison to veterans with no history of TBI, even after accounting for the presence of psychiatric symptoms (e.g., post-traumatic stress) [21]. Other studies have reported reductions in global brain volume by at least 1-year post-injury following a mild TBI, though uncomplicated as well as complicated injuries (i.e., brain hemorrhage, psychiatric disorders, and persistent cognitive impairment) were included in these samples [22–24]. When considering that the above studies included individuals with blast-related as well as complex injuries, it is unknown if the findings would generalize to the vast majority of individuals with mild TBI who do not experience these

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Table 2
Long-term brain changes from TBI: Structural neuroanatomical imaging

Study	Participants	Key Findings
Tomaiuolo et al., 2012 [10]	mTBI individuals ($n = 16$); Healthy controls ($n = 19$)	• Memory performance in mTBI subjects tested 8–10-years post-injury was significantly better compared to 3–13 months post-injury • Hippocampal and fornix volume in nmTBI subjects was significantly reduced compared to controls at both time points, but remained stable over time • Corpus callosum volume continued to decrease significantly at each time point
Dinkel et al., 2014 [11]	Severe TBI patients ($n = 13$); Healthy controls ($n = 8$)	• FA values in several sub-structures of the corpus callosum were significantly lower in patients with severe TBI compared to controls, indicating white matter damage (specifically to myelin) in these areas • FA values in the corpus callosum structures decreased significantly over 2-years post-injury, but afterward remained stable up to 5-years post-injury
Farbota et al., 2012 [12]	TBI patients ($n = 46$); Healthy controls ($n = 36$)	• FA values in the corpus callosum, the superior longitudinal fasciculus, and parts of the optic radiation in TBI patients continually declined at multiple time points over 4 years compared to controls • FA values in the corpus callosum and sagittal striatum were positively correlated with performance on finger tapping and visuomotor speed tasks in TBI patients • Longitudinal increases in regional FA values were correlated with improvements in visuomotor speed and cognitive control
Ng et al., 2008 [13]	Moderate-to-severe TBI patients ($n = 14$)	• Majority of patients (10 out of 14) showed significant increases in CSF and decreases in bilateral hippocampal volume, consistent with brain atrophy, 2.5 years after initial testing done 4.5 months post-injury
Gale et al., 1995 [14]	Moderate-to-severe TBI patients ($n = 2$); Mild TBI patient ($n = 1$); Age-matched control group ($n = 3$)	• Moderate-to-severe TBI patients showed ventricular changes consistent with brain atrophy in several cortical structures and the cerebellum 6–8 weeks post-injury compared to controls, but midbrain and brainstem regions remained intact • Mild TBI patient showed no significant anatomical changes
Blatter et al., 1997 [16]	TBI patients ($n = 123$)	• TBI patients showed increase in ventricular CSF volume and decreases in brain volume, consistent with brain atrophy • Increases in ventricular CSF preceded changes in total brain volume and subarachnoid CSF volume • Correlations were observed between performance IQ scores and volume of total brain and lateral ventricles 71–210 days post-injury, such that as atrophy increased, performance decreased
Sidaros et al., 2009 [17]	Severe TBI patients ($n = 24$); Healthy controls ($n = 14$)	• Brain volume in TBI patients was, on average, 8.4% lower compared to healthy controls at baseline (8 weeks to 12 months post-injury) • %BVC in TBI patients post-injury ranged from –0.6% to –9.4% over the 1-year scan interval • %BVC was significantly correlated with injury severity, functional status at baseline and follow-up, and outcome at 1-year post-baseline
Warner et al., 2010 [19]	TBI patients ($n = 25$); Age- & sex-matched healthy controls ($n = 22$)	• TBI patients on an inpatient unit showed whole-brain volume loss of 4.5% at 6–14 months post-injury compared to baseline (0.5–9 days post-injury) • Whole-brain volume loss and atrophy of certain brain regions (i.e., the inferior parietal cortex, pars orbitalis, pericalcarine cortex, and supramarginal gyrus) were predictive of long-term disability

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Brezova et al., 2014 [20]	Moderate-to-severe TBI patients ($n = 62$)	• TBI patients displayed significant loss of cortical gray matter by 3-months post-injury, which was predictive of 12-month outcome • Hippocampal and lenticular nucleus volume loss was present by 12-months post-injury • Rates of cortical and subcortical volume change were significantly different and were related to factors such as length of post-traumatic amnesia, 12-month outcome, and regional differences
Jorge et al., 2012 [21]	Veterans with blast-related mTBI ($n = 72$); Veterans with no TBI ($n = 21$)	• No significant differences in DTI parameters between veterans with mTBI and those with no TBI were found • Veterans with mild TBI had more spatially heterogeneous areas of decreased FA ("potholes") than those with no TBI • The number of "potholes" seen in the brain was correlated with TBI severity and executive functioning abilities
Zhou et al., 2013 [22]	Patients with mTBI ($n = 19$); Matched controls ($n = 22$)	• Patients with a single mTBI who also experienced postconcussive symptoms had significantly more global brain atrophy than control subjects 1-year post-injury • Certain white matter regions (i.e., anterior cingulate and left cingulate gyrus isthmus tracts) in addition to the right precuneal gray matter had significantly less volume in these patients, even 1-year post-injury • Left cingulate gyrus isthmus white matter volume was significantly correlated with postconcussive symptoms and anxiety symptoms at 1-year post-injury
MacKenzie et al., 2002 [23]	Patients with mTBI or moderate TBI ($n = 14$); Controls ($n = 10$)	• At baseline, brain volume and CSF volume in TBI patients did not differ from controls, even when corrected for %VBP • Rate of decline in %VPB and change in %VPB between first and second time points was significantly greater in TBI patients compared to controls, particularly for those who lost consciousness
Ross et al., 2012 [24]	Patients with mTBI ($n = 16$); Normal controls ($n = 20$)	• When imaged several years after injury (mean 2.44-years post-injury), mTBI patients showed greater atrophy in the whole brain volume, forebrain volume, cerebral white matter, and cerebellum compared to baseline scans and when compared to controls using NeuroQuant • Increased atrophy in these patients was predictive of vocational outcome (i.e., inability to return to work)
Jarrett et al., 2016 [25]	Hockey players ($n = 45$)	• Post-season imaging showed that all athletes showed a 0.32% reduction in brain volume, with the sub-set of concussed players showing a 0.26% reduction in brain volume • Concussed players had a –0.08% change in brain volume 2 weeks post-injury and a –0.23% change in brain volume 2-months post-injury • No significant changes in total number of white matter hyperintensities found across all time points
Davenport et al., 2014 [26]	High school varsity football players without concussions ($n = 24$)	• Combined (linear and rotational) components of impact, as measured by Head Impact Telemetry sensors in helmets, were predictive of FA values (i.e., white matter damage) post-season

(*Continued*)

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Table 2
(*Continued*)

Study	Participants	Key Findings
		• Combined components and linear components of impact were predictive of all DTI values (i.e., integrity of white matter tracts) • DTI values were correlated with change in verbal memory performance, such that decreased white matter integrity was associated with worse performance
Terry & Miller, 2018 [27]	mTBI group (history of 2 + mTBI) ($n = 20$); Control ($n = 20$)	• All participants were former high-school football players (adult males, aged 40–65) • No statistically significant difference in regional brain volumes found between mTBI group and control group, when matched on age, education, premorbid IQ, and current concussive symptomatology
Bazarian et al., 2014 [28]	College football players ($n = 10$; Non-athlete controls ($n = 5$)	• Using helmet-mounted accelerometers, total head impacts measured for the season ranged from 431–1,850, with no clinically evident concussions • Athletes demonstrated greater change in FA and mean diffusivity values (i.e., integrity of white matter) compared to controls at both the post-season scan and after 6 months post-season with no-contact rest • Decreases in FA seen post-season (as compared to pre-season scan) were associated with several head impact measures • Although white matter changes were associated with changes in two autoantibodies, these changes were not consistently associated with cognitive performance or balance
Hart et al., 2013 [29]	Retired football players ($n = 34$); Controls ($n = 26$)	• Significant differences in white matter abnormalities in cognitively impaired and depressed players compared to controls were observed • In players with cognitive impairment, regional blood flow differences were seen that corresponded to regions associated with impaired neuropsychological performance
Strain et al., 2015 [30]	Retired football players ($n = 28$); Controls ($n = 27$)	• Cognitively normal, retired athletes with history of concussion had significantly lower (but not impaired) scores on a verbal memory measure compared to controls • Retired athletes with cognitive impairment and a history of concussion performed worse on this measure than both players without cognitive impairment and controls • Retired athletes with at least one grade 3 concussion showed reduced bilateral hippocampal volumes compared to controls and reduced right hippocampal volume compared to athletes with no history of grade 3 concussion • Athletes with history of grade 3 concussion were more likely to have MCI compared to those without and showed reduced left hippocampal volume compared to control participants with MCI
Solomon et al., 2016 [31]	Retired football players ($n = 45$)	• In retired athletes, years of exposure to pre-high school football were not significantly associated with any neurological, neuroradiological, or neuropsychological outcome measures

%BVC, percent brain volume change; %VBP, cranial volume; CSF, cerebrospinal fluid; DTI, diffusion tensor imaging; FA, fractional anisotropy; MCI, mild cognitive impairment; mTBI, mild traumatic brain injury; nmTBI, non-missile traumatic brain injury; TBI, traumatic brain injury.

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injury mechanisms or complications. Furthermore, imaging studies on the later-in-life effects of sports-related mild TBI have largely been mixed. MRI scans pre- and post-season in a small sample of collegiate hockey players showed slight, but significant, reductions in whole brain volumes in both non-concussed ($n = 34$) and concussed ($n = 11$) players when compared to those in non-contact sports ($n = 15$) [25]. Another investigation examined non-concussed high school football players ($n = 24$) pre- and post-season, and showed significant MRI changes following a single season of sport [26]. However, it is unclear whether the brain alterations observed in that study may be separate from normal developmental changes given a control group was not included. While these results may suggest that even non-concussive head impacts may be associated with structural neuroanatomical changes in some cases, it is unknown if the changes are chronic given scans were limited to only two time points (pre- and post-season). For example, a recent study compared 20 middle aged men who experienced repetitive mild TBIs during their high school football careers to age-matched controls, and no significant volumetric or brain structural differences were found between the groups on MRI [27]. However, at the professional level of sport, some differences later-in-life have been shown in small groups of former National Football League players. For example, smaller hippocampal and thalamic volumes on MRI and greater disruption of cerebral white matter integrity on diffusion tensor imaging have been reported in a few studies of aging former professional athletes when compared to controls [28–30]. However, other investigators have found no association whatsoever between exposure to head impacts and structural brain changes in former professional athletes [31].

Functional imaging

Functional connectivity MRI (fcMRI), positron emission tomography (PET), and arterial spin labeling are imaging techniques that can be performed to evaluate additional aspects of altered brain status long after acute-injury effects (see Table 3). For example, resting state fcMRI scans in comatose/minimally conscious patients admitted to a hospital for acute severe TBI ($n = 17$) were compared to 16 healthy control subjects [32]. Altered synchronicity of communication between connected brain regions in the default mode network (DMN), a system involved in performing goal-oriented tasks that has also been implicated in AD [33, 34], was observed in severe TBI patients relative to controls. However, DMN connectivity normalized for all TBI patients ($n = 8$) imaged 6 months later who fully recovered consciousness, though structural brain changes and cognitive/behavioral impairments remained [32]. While some research has suggested high school football athletes with multiple subclinical head impacts ($n = 22$) demonstrated changes in functional connectivity in the DMN during and after a single season of play when compared to high school non-contact sport athletes ($n = 10$) [35], similar to studies of structural brain changes in collegiate athletes, it is unknown if the changes may be chronic, normalize with time, or reflect developmental changes, since longitudinal studies beyond a single athletic season with appropriate control subjects are lacking. Interestingly, the football athletes in the study by Abbas et al., (2015) demonstrated hyperconnectivity of the DMN, whereas patients with AD typically show reduced connectivity in the DMN compared to controls.

Altered brain metabolism and cerebral blood flow have also been found in former athletes of high-contact sports who experienced multiple mild TBIs and have cognitive impairment later-in-life. Increased levels of metabolites in the brain involved in the metabolism of phospholipids have been reported in former athletes ($n = 15$) having their last concussion 30 years prior when compared to healthy controls ($n = 15$) [36]. In that study, former athletes also demonstrated more cortical thinning, ventricular enlargement, and worse cognitive performance, raising some question about the cognitive status of the athletes and how many may have suffered from either mild cognitive impairment (MCI) or early-stage dementia. Decreased blood perfusion in the left temporal and right parietal regions and increased blood flow in the left inferior parietal and superior temporal regions have been shown in a small sample of former professional football athletes with cognitive impairment ($n = 8$ with MCI, $n = 2$ with dementia) compared to controls [29]. Hypometabolism of glucose in the medial temporal lobes, cerebellum, vermis, and pons has also been found in individuals with multiple remote mild TBIs, though this was in Iraq war veterans with a history of numerous reported blast exposures (average of 13) [37].

In sum, moderate-to-severe TBI appears to result in neuronal and white matter degenerative changes that can persist for at least several years after injury, but the time point at which such changes may stabilize

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Table 3
Long-term brain changes from TBI: Functional neuroanatomical imaging

Study	Participants	Key Findings
Threlkeld et al., 2018 [32]	Acute severe TBI patients ($n = 17$); Healthy control patients ($n = 16$)	• Patients with acute disorder of consciousness (e.g., coma, unresponsive wakefulness, etc.) and who remained in a coma or unresponsive wakefulness state showed no anticorrelations or correlations in the DMN • Patients who recovered to a minimally conscious or confusional state showed partially preserved DMN correlations • All patients who recovered beyond the confusional state (around 6-months post-injury) showed normal DMN correlations and anticorrelations that were not significantly different from controls
Johnson et al., 2014 [34]	Collegiate rugby players ($n = 24$)	• Post-game scans in rugby players showed increased connectivity from the left supramarginal gyrus to bilateral orbitofrontal cortex and decreased connectivity from the retrosplenial cortex and dorsal posterior cingulate cortex compared to pre-game scans • In a sub-group of players with history of concussions showed decreased functional connectivity post-game, while players with no history of concussions only showed increased functional connectivity
Abbas et al., 2015 [35]	High-school football players ($n = 22$); Non-collision athlete controls ($n = 10$)	• Football players had significantly different functional connectivity patterns than controls, even at pre-season scans • Compared to controls, football players showed hyperconnectivity in the DMN across most scans over the full season, despite no evidence of clinical concussive symptoms
Tremblay et al., 2013 [36]	Former college athletes; mTBI history ($n = 15$); No mTBI history ($n = 15$)	• When compared to athletes with no history of mTBI, athletes with a remote history of mTBI (>3 decades prior to testing) demonstrated abnormal enlargement of lateral ventricles, increased cortical thinning in regions vulnerable to the aging process, and several regional neurometabolic anomalies • Athletes with remote history of mTBI also showed decline in episodic memory and verbal fluency performance when compared to controls
Peskind et al., 2011 [37]	Veterans with blast-related mTBI ($n = 12$); Non-veteran controls ($n = 12$)	• Decreased rate of brain glucose metabolism was observed across several brain regions (i.e., cerebellum, vermis, pons, and medial temporal lobe) in veterans with mTBI compared to controls • Veterans with a history of mTBI also showed subtle impairments in performance on verbal fluency, cognitive processing speed, attention, and working memory tasks

DMN, default mode network; mTBI, mild traumatic brain injury; TBI, traumatic brain injury.

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or whether these progress to influence the development of a neurodegenerative condition years or decades later is currently unknown. Findings for milder injuries are generally mixed and/or influenced by complicating factors such as injury severity, brain bleeding, post-traumatic stress disorder, cognitive impairment, and blast exposure. Longitudinal investigations with both structural and functional imaging are still needed in order to better understand the effects for both mild TBI and moderate-to-severe injuries on the brain long-term to clarify how TBI may contribute to the development of neurodegenerative dementias in some individuals.

DOES TBI CAUSE AN ACCUMULATION OF NEUROBIOLOGICAL MARKERS RELATED TO NEURODEGENERATIVE DISEASES?

TBI has been associated with a buildup of neurobiological markers that are also found in brains of patients with AD, Lewy body disease, and other neurodegenerative disorders (see Table 4). Amyloid-β (Aβ) plaques, one of the pathognomonic markers of AD, were reported in 60% of patients ($n = 39$) surviving a moderate-to-severe TBI for at least a year prior to autopsy [38]. Greater Aβ accumulation on PET neuroimaging has also been seen in the posterior cingulate region in individuals surviving a moderate-to-severe TBI within the past 1–17 years compared to healthy controls, with greater accumulation of amyloid found to be associated with more extensive white matter injury [39]. Hyperphosphorylated tau (*p*Tau) is the other primary pathognomonic biomarker for AD and is found in other tau-related neurodegenerative diseases called tauopathies (e.g., frontotemporal dementia). pTau has also been observed at autopsy in a small sample of moderate-to-severe TBI patients surviving for at least 1-year post-injury ($n = 39$), with tau deposits being more widespread in comparison to a healthy control group [38]. Transactive response DNA protein (TDP–43) is a pathognomonic marker for many cases with frontotemporal lobar degeneration and amyotrophic lateral sclerosis and has been observed in multiple brain regions of small samples of retired professional athletes from contact sports [40]. Greater cytoplasmic TDP–43 has been seen at autopsy in patients sustaining a moderate-to-severe TBI in comparison to a healthy control group [41]. However, only 4% of moderate-to-severe TBI patients ($N = 62$) had phosphorylated TDP–43, which was equivalent to the proportion of healthy controls, indicating that non-phosphorylated TDP–43 may increase after TBI and be a general marker of neuronal injury. Furthermore, alpha-synuclein, a protein associated with Parkinson's disease and Lewy body dementia, has been found at autopsy in 20% of patients with a prior history of TBI who had LOC of 1 hour or less and 16% of patients who had LOC for more than 1 hour, though interestingly, only the less severe injuries (LOC 1 hour or less) were associated with the presence of alpha-synuclein pathology [42].

While these studies suggest a relationship between TBI and presence of pathological processes in neurodegenerative conditions, particularly in moderate-to-severe injuries, the scientific community has not established whether the accumulation of these proteins is progressive after TBI. Moreover, the cascade of events responsible for a buildup of pathological markers in AD and related dementias in some individuals with a TBI history is unknown. There has been some indication that inflammation may play a role in the abnormal accumulation of these proteins. For example, microglial cells can be activated by TBI. In a preliminary PET imaging study of 10 patients with a moderate-to-severe TBI between 1–17 years post-injury, increased microglial activation was seen in subcortical brain regions (e.g., thalamus, basal ganglia, internal capsule), but not around the area of primary injury [43]. No relationship was observed with time since injury or extent of neuronal injury, suggesting that TBI may activate a chronic inflammatory response that can persist years after injury (at least in moderate-to-severe TBIs) that does not correlate with a "progressive-like process." Alternatively, it has been posited that TBI may alter the brain's ability to integrate neuronal networks when performing cognitive demands, reducing "cognitive/neuronal reserve" [44], which could interact with neurodegenerative changes to increase the risk for cognitive decline rather than contributing to pathological burden per se.

Despite several studies having documented an association between moderate-to-severe TBI and accumulation of pathological markers in AD and related conditions, there is a lack of research examining whether similar changes occur with mild TBI or subclinical head impacts. Clinicians and researchers would be misguided to assume that mild TBI simply carries the same amount of risk as moderate-to-severe TBI. This is in part because a buildup of the proteins that are precursors to AD and related conditions (e.g., Aβ and *p*Tau) have been shown to peak within weeks after mild injuries and rapidly clear thereafter. For

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Table 4
Relation between TBI and accumulation of neurobiological markers related to neurodegenerative disease processes

Study	Participants	Key Findings
Johnson et al., 2012 [38]	Moderate-to-severe TBI patients surviving at least 1 year ($n = 39$) and age matched controls ($n = 47$)	• 46% of the TBI group showed pathological evidence of NFT postmortem, compared to 34% of controls. • 34% of TBI cases under the age of 60 showed NFT compared to 9% of control cases under the age of 60. • 28% of both TBI and control groups showed amyloid-β plaques, but TBI cases showed greater adjusted plaque density compared to controls.
Scott et al., 2016 [39]	Moderate-to-severe TBI patients ($n = 9$), healthy controls ($n = 9$), and clinical AD patients ($n = 10$)	• TBI group showed increased PiB binding in the posterior cingulate cortex and cerebellum compared to controls, indicative of greater amyloid-β pathology. • Increased binding of PiB in posterior cingulate cortex was associated with decreased FA of relevant white matter tracts and increased time since injury. • Binding of PiB in TBI individuals was increased in the cerebellum compared to AD patients, but lower in neocortical regions.
McKee et al., 2010 [40]	Athletes with CTE ($n = 12$); individuals with sporadic ALS ($n = 12$); controls ($n = 12$)	• 10 of 12 CTE cases demonstrated widespread TAR-DNA binding of approximately 43 kd (TDP-43) proteinopathy across the frontal cortex, temporal cortex, medial temporal lobe, basal ganglia, diencephalon, and brainstem. • In 3 cases of comorbid CTE and MND, there was also TDP-43 pathology in the spinal cord, in addition to the presence of NFT, motor neuron loss, and degeneration of the corticospinal tract, • TDP and MND is also associated with other neurodegenerative disorders (e.g., frontotemporal lobar degeneration).
Johnson et al., 2011 [41]	Moderate-to-severe TBI patients ($n = 62$) and age matched controls ($n = 47$)	• Multiple brain regions were examined at autopsy, and only 3 TBI patients were found to have phosphorylated TDP-43 pathological changes versus 2 control subjects. • Cytoplasmic TDP–43 was significantly greater in TBI patients autopsied during the acute (surviving <2 weeks; $n = 23$) and more chronic (surviving >1 year; $n = 39$) stages relative to healthy control subjects.
Crane et al., 2016 [42]	Individuals aged 50 years and older with a history of TBI ($n = 865$) and without ($n = 6{,}265$)	• In this prospective cohort study, there was no significant relationship between history of TBI with LOC and incident dementia, AD, NFTs or neuritic plaques. • A history of TBI with LOC was associated with development and progression of Parkinsonian symptoms. • 1,652 subjects underwent a brain autopsy and a history of TBI with LOC $\leq$1 hour was found to be associated with the presence of Lewy bodies in frontal and temporal areas, while a TBI history with LOC >1 hour was not. • A reverse association was seen when only subjects with injuries occurring before the age 25 were examined; there were only 4 cases with more severe injuries and presence of any Lewy body pathology, however.
Ramlackhansingh et al., 2011 [43]	Moderate-to-severe TBI patients ($n = 11$) and control subjects ($n = 15$)	• Using PET imaging, microglial activation was seen in patients months to years after TBI in the thalami, putamen, occipital cortices, and posterior limb of internal capsules. • No microglial activation was seen at the original site of focal brain injury in these patients. • Increased microglial activation in the thalamus was associated with more severe cognitive impairment. • Binding was not associated with time since injury or extent of white matter damage measured by fractional anisotropy on DTI.

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Neselius et al., 2012 [45]	Olympic boxers ($n = 30$) and a matched controls ($n = 25$)	• CSF concentrations of total tau, NF-L, glial fibrillary acidic protein, and S-100B were significantly increased in boxers 1–6 days following a bout in comparison to controls. • 14 days after the bout, total tau and S-100B levels normalized, but NF-L and glial fibrillary acidic protein remained elevated.
Shahim et al., 2017 [46]	Amateur boxers ($n = 14$), professional hockey players ($n = 30$), and healthy controls ($n = 26$)	• Compared to controls, serum NF-L levels significantly increased for boxers 7–10 days following a bout. • Boxers who received more than 15 punches or felt groggy during/after the bout had significantly higher NF-L levels than boxers receiving fewer punches. • At 3 months rest, boxers NF-L levels decreased, but as a group remained significantly greater in comparison to controls. • Professional hockey players who sustained a concussion had higher serum NF-L levels compared to controls, with the maximum concentration measured 144-hours post-injury.
Bernick et al., 2018 [47]	Active ($n = 117$) and retired ($n = 52$) boxers, mixed martial artists ($n = 169$), and controls ($n = 79$)	• Active boxers had a greater number of sparring matches 2 weeks before measuring baseline levels and higher NF-L levels at baseline compared to active MMA fighters and controls. • Plasma tau levels at baseline were not significantly different among the groups. • Across 2 years, the annual change in tau but not NF-L levels increased for MMA fighters relative to controls, controlling for age, race, education, and baseline levels. • NF-L and tau levels remained constant for active and retired boxers over 2 years.
Oliver et al., 2018 [48]	College football athletes ($n = 35$)	• Pre- and post-season levels of plasma tau and serum NF-L were examined. • Plasma tau levels decreased over the course of a season in starting and non-starting football players. • Serum NF-L levels increased over the course of a season in the athletes, and while this did not reach statistical significance for non-starters, levels were significantly elevated post-season for starting players.
Rubin et al., 2019 [49]	College football athletes ($n = 18$)	• Pre- and post-practice levels of serum NF-L were examined across a single season of sport along with the number of impacts measured with accelerometer devices. • Serum NF-L levels increased following practice sessions and was correlated with the number of impacts and magnitude of impacts players sustained. • Years of football experience and concussion history were not associated with pre- and post-practice changes in NF-L.

AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; CSF, cerebrospinal fluid; CTE, chronic traumatic encephalopathy; DTI, diffusion tensor imaging; FA, fractional anisotropy; LOC, loss of consciousness; MMA, mixed martial arts; MND, motor neuron disease; NF-L, neurofilament light; NFT, neurofibrillary tangles; PET, positron emission tomography; PiB, Pittsburgh Compound B; TBI, traumatic brain injury.

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example, among 30 Olympic boxers, a large percentage (80%) showed elevated cerebrospinal fluid tau concentrations 1–6 days following a bout, but all boxers' tau levels returned to normal when re-assessed 14 days later [45]. It is also not known whether mild TBI can produce a persistent inflammatory response in individuals to confer a vulnerability to worse aging outcomes. However, there is a growing body of literature suggesting that the neurofilament light (NF-L) protein may be a biomarker of neuronal injury after TBI. NF-L has been found to be increased following a bout in boxers [46, 47], concussion in professional hockey players [46], and a single season in collegiate football athletes [48, 49]. However, similar to tau, NF-L levels appear to normalize at some point following injury. Retired professional boxers ($N = 471$) having their last bout 2 years prior did not differ significantly from healthy controls on NF-L concentrations nor show significant changes in NF-L over a 2-year period [47]. It is unclear at present how the reparative process may be overcome in some individuals with a mild TBI to potentially become associated with a greater accumulation of Aβ, pTau, alpha-synuclein, and/or markers of inflammation/neuronal injury years later. Therefore, potential mechanisms to explain how mild TBI may be a risk factor for AD or other neurodegenerative disorders remain unknown and is an area that will be important for future research to address. The reader is referred to recent publications by Wang et al. (2018) and Zetterberg, Smith, and Blennow (2013) for an in-depth review of neurobiological markers post-TBI [50, 51].

DOES TBI INCREASE THE RISK FOR DEMENTIA?

TBI has been found to be a risk factor for later development of dementia in several retrospective cohort studies using medical records (see Table 5). In a large Swedish sample, individuals with a single mild TBI, more severe TBI, and multiple brain injuries ($n = 164{,}334$) were found to have a 1.6, 2.0, and 2.8 fold higher risk, respectively, for developing dementia [52]. Risk for dementia following a TBI was still observed even among those who were matched to a sibling without any TBI history ($n = 46{,}970$). Risk was greatest 1-year post-injury (odds ratio [OR] = 3.5) but remained significant for up to 30 years later (OR = 1.6). A similar study of over 2.7 million individuals in Denmark found largely the same results, with both mild (hazard ratio [HR] = 1.1) and severe (HR = 1.3) TBI associated with an increased risk for dementia [53]. Risk was again highest within 1 year of injury (HR = 1.2) and greater with multiple injuries (i.e., more than 4 prior TBI, HR = 2.8). The prevalence of dementia in this study was 5.3% for individuals with a history of TBI versus 4.7% for those without. Another investigation examining the incidence of dementia in United States veterans with ($n = 178{,}779$) and without history of TBI ($n = 178{,}779$) in their medical records, found that individuals with a history of TBI had a higher incidence of dementia (6.1%) over an average of 4 years post-injury versus 2.6% for those without a TBI history [54]. All levels of injury severity, including mild TBI without LOC (HR = 2.3), mild TBI with LOC (HR = 2.5), and moderate-to-severe TBI (HR = 3.7), were associated with an increased likelihood of receiving a dementia diagnosis in that investigation. However, in a separate study of California health data for persons aged 55 years and older ($N = 164{,}661$), a history of moderate-to-severe TBI was associated with a higher likelihood of developing dementia over 5–7 years in all participants (HR = 1.7), whereas a single, mild TBI (HR = 1.3) was only associated with increased risk among those older than 64 years at the time of injury [55].

Whereas many of these studies suggest a relationship between TBI of any severity and risk for developing dementia, it is important to consider the methodological challenges of epidemiological studies when interpreting any findings. Dementia within a few years of injury would be expected to relate to cognitive impairment directly from an injury or due to an underlying neurodegenerative disorder that was already present, but perhaps not obvious at the time of injury. Data on TBI and dementia are often based on clinical diagnostic codes, but the actual classification of TBI and cognitive status and the criteria used to make these determinations are not standardized, can vary between clinicians, and may not necessarily be accurate, particularly in large hospital-based data sources that were not designed for research. Dementia is also a non-specific clinical classification, and can be associated with medical conditions that could potentially be reversed or stabilized with treatment, unlike progressive neurodegenerative diseases. Because retrospective cohort studies sometimes rely on medical records, it is difficult to know the proportion of individuals with a dementia diagnosis that actually reflect neurodegenerative disease versus those related to direct cognitive impairments from TBI and/or a medical condition unrelated to the

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Table 5
TBI-related risk for all-cause dementia

Study	Participants	Key Findings
Nordstromet al., 2018 [52]	Swedish individuals aged 50 years and older during follow-up ($N = 3,329,360$)	• Medical records of individuals were used to form three cohorts: 1) 164,334 individuals with a TBI matched up to 2 controls, 2) 136,233 individuals who developed a dementia during follow-up matched up to 2 controls, and 3) 46,970 individuals with a TBI matched to a sibling without a TBI. • Risk for all-cause dementia was highest for moderate-to-severe TBIs (HR: 2.06) and multiple injuries (HR: 2.81) and significant but weaker for mild TBI (HR: 1.63). • Risk for all-cause dementia was highest within 1 year of injury, and although the risk decreased gradually after this period of time, the risk remained significant beyond 30 years after a TBI in the study cohorts 1 and 2. • The risk for dementia by severity and time following a TBI was similar in the 3rd cohort with sibling pairs.
Fann et al., 2018 [53]	Danish individuals aged 50 years and older during follow-up ($N = 2,794,852$)	• Risk for all-cause dementia was increased for individuals with a history of a single mild TBI (1.17) and moderate-to-severe TBI (HR: 1.35). • The risk consistently increased with multiple injuries of any severity (2+) out to $\geq$5 TBIs (HR: 2.83). • Risk for all-cause dementia was highest within 1 year of injury and decreased gradually after thereafter, but the risk remained significant beyond 14 years after a TBI. • Mild TBI (HR: 1.17) and more severe injury (HR: 1.17) were associated with an equally increased risk for a diagnosis of AD. • Risk for AD related to number of injuries or timing since TBI was not examined.
Barnes et al., 2018 [54]	Veterans with a TBI ($N = 178,779$) matched 1 : 1 to a veteran control group	• TBI and non-TBI subjects were on average age 50 years at baseline (either time of TBI or initial index visit). • Average time from a TBI to a dementia diagnosis was 3.6 years versus 4.8 years from the initial visit for non-TBI subjects. • Mild TBI without LOC (HR: 2.36), mild TBI with LOC (HR: 2.51), mild TBI with unknown LOC (HR: 3.19), and moderate-to-severe TBI (HR: 3.77) were all associated with an increased risk for a diagnosis of dementia during follow-up.
Gardner et al., 2016 [55]	Individuals aged 55 years and older in California hospitalized with a TBI ($N = 51,799$) and non-brain trauma ($N = 112,862$)	• Excluded patients diagnosed with a dementia within 1 year of injury. • The median duration of follow-up was 6 years and the mean time from trauma to diagnosis of dementia was 3.3 years for TBI (3.1) and non-brain trauma (3.3) subjects. • A single TBI (HR: 1.26) and $\geq$2 TBIs (HR: 1.56) were associated with an increased risk for dementia diagnosis when compared to those with non-brain trauma. • A significant interaction between TBI severity and age was found for risk of dementia diagnosis. • Moderate-to-severe TBI was associated with an increased risk for dementia diagnosis among those aged 55–84 years and $\geq$85 years, with a gradual reduction in dementia risk with increasing age. • Mild TBI was only associated with an increased risk for dementia among those aged 75 years and older.
Lee et al., 2013 [102]	Taiwanese individuals aged at least 18 years ($N = 720,933$)	• Risk for all-cause dementia over a 5-year period was increased for individuals with a history of mild TBI relative to those with no TBI history (HR: 3.26). • The average duration from TBI to the diagnosis of dementia was 1 year. • More severe TBI and multiple injuries were not assessed.

AD, Alzheimer's disease; HR, hazards ratio; LOC, loss of consciousness; TBI, traumatic brain injury.

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injury (e.g., cerebrovascular accident, toxin exposure, metabolic imbalance). More importantly, when one considers the overall risk for cognitive changes, less than 7% of individuals with a history of TBI have been found to develop dementia for any reason [54, 55], including moderate-to-severe injuries that are typically associated with residual cognitive deficits. Regardless of study limitations and methodological differences, this demonstrates that *most* persons who sustain one or several injuries, despite level of severity, do not experience significant cognitive changes or dementia with aging.

Alzheimer's disease

AD is the most common type of dementia and is generally associated with changes in learning, memory, and aspects of language, followed by global cognitive deterioration as the disease spreads [56]. The pathological hallmarks of AD are Aβ plaques and neurofibrillary tangles comprised of pTau, and typically begins in the temporal lobes before coursing to the frontal, parietal, and occipital lobes of the brain. There have been several studies examining TBI as a risk factor for developing AD, with mixed results (see Table 6). In a study of more than 2,500 World War II veterans, where TBI was documented during military service as being mild, moderate, or severe, moderate-to-severe injuries were associated with a higher risk for AD when evaluated approximately 40 years later in a standardized clinical evaluation, while mild injuries were not [57]. Another investigation of veterans examined incidence of AD across 9 years by collecting TBI and clinical data from the medical records of over 180,000 individuals, and found that a history of TBI of any severity was associated with a higher risk for AD (HR = 1.6) [58]. An investigation of Denmark's health data examining over 2.7 million cases showed both mild TBI (HR = 1.2) and severe TBI (HR = 1.2) to be associated with an increased risk for being diagnosed with AD [53]. However, results across the literature have been mixed overall, as other large studies have not found TBI to be associated with an increased risk for AD. An investigation of 4,225 adults aged 65 and older examined incidence of AD every 2 years for an average of 7 years [59]. In this study, individuals with a history of TBI with LOC occurring in early- (aged < 25), mid- (age 25–54), and mid-to-late life (aged 55+) were found to have a similar risk for developing AD compared to persons without a history of TBI (HR = 1.0; CI = 0.8–1.4) [59]. Another study followed 6,645 individuals aged 55 years and older for 2 years and found that presence of a history of TBI with LOC, duration of LOC, age at injury, and history of multiple TBIs were *not* associated with a greater risk for developing AD [60]. Interestingly, more consistent evidence for TBI being a risk factor for earlier onset of AD symptoms has been found in recent years. A retrospective analysis of a large multicenter national database showed that a history of TBI with LOC ($n = 571$) occurring more than 1 year prior to dementia evaluation was associated with an approximately 2-3 years earlier onset of AD compared to those without a history of TBI with LOC ($n = 7{,}054$) [61]. These results were seen in both females and males and were independent of apolipoprotein ε4 status, an allele that is known to be associated with greater accumulation of AD-related proteins. A follow-up investigation using only autopsy-confirmed AD cases also found that individuals with a history of TBI with LOC ($n = 194$) had a 2-3 year earlier onset of dementia symptoms than those without any TBI history ($n = 1900$) [62]. Although the research on TBI as a risk factor for developing AD has been mixed, there is strong support that moderate-to-severe injuries are associated with a higher likelihood for AD, and recent evidence suggests that TBI is associated with an earlier onset of symptoms.

Frontotemporal dementia

Frontotemporal dementia (FTD) collectively refers to three related types of dementia: behavioral variant FTD (bvFTD), primary progressive aphasia, and progressive non-fluent aphasia. Generally, FTD involves changes in personality and emotional expression, language, or a loss of semantic knowledge [63, 64]. The pathological hallmarks for all subtypes of FTD are TAR DNA inclusions or tau proteins, and pathogenesis involves the frontal lobes and anterior temporal regions [65]. An investigation of the National Alzheimer's Coordinating Center dataset examined the risk associated with having a history of TBI with ≥5 minutes of LOC with no persistent deficits and a diagnosis of FTD (including all subtypes) [66]. Among 1,016 subjects with an FTD diagnosis, individuals with a history of TBI ($n = 43$) were found to have a 1.7 fold increased risk (CI = 1.0–2.8) for an FTD diagnosis when compared to a sample of healthy controls ($n = 2{,}015$). In a smaller investigation ($n = 80$) examining potential lifetime medical risk factors, a history of TBI of any severity was shown to be associated with a 3.3 times higher

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Table 6
TBI-related risk for dementia: Alzheimer's disease

Study	Participants	Key Findings
Plassman et al., 2000 [57]	Veterans hospitalized with TBI ($n = 565$); Veterans without TBI ($n = 1,202$)	• A documented history of moderate (HR: 2.32) and severe TBI (HR: 4.51) during military service about 40 years earlier in World War II veterans was associated with increased risk for AD. • Mild TBI did not reach statistical significance. • There was a trend toward a stronger association for AD in those with TBI and APOE4.
Barnes et al., 2014 [58]	Veterans age 55 and older with a TBI ($n = 1,229$) and without ($n = 187,535$)	• Most common TBIs were skull fractures (23%) and intracranial injury without skull fracture (43%) • During a mean length of follow-up of 7.4 years (max: 9.7), TBI was associated with increased risk for dementia (HR: 1.81). • 16% of veterans with TBI were diagnosed with a dementia compared to 10% of veterans without a TBI. • Dementia began 2.1 years earlier for veterans with TBI compared to those without.
Dams-O'Connor et al., 2013 [59]	Individuals age 65 and older having a history of TBI with LOC ($n = 672$) or no TBI ($n = 3,553$)	• In a mean follow-up of 7.4 years (max: 16), TBI with LOC before age 25 (HR: 0.99), from ages 25–54 (HR: 1.01), and after age 54 (HR: 1.15) was not associated with increased risk for AD. • Recent TBI was also not associated with an increased risk for AD (HR: 0.95). • No significant interaction between TBI with LOC and APOE4 on risk for AD (HR: 0.71).
Mehta et al., 1999 [60]	Individuals age 55 and older in Rotterdam, Netherlands ($N = 6,645$)	• 800 subjects had a history of TBI which were classified as: single injury or >1, occurring within or more than 10 years prior, and resulting in $\leq$15 or >15 minutes LOC. • In a mean follow-up of 2.1 years, history of TBI with LOC, duration of LOC, time since injury, and multiple TBIs did not reach statistical significance for risk of developing AD.
LoBue et al., 2017 [61]	Clinically diagnosed AD patients having a history of TBI with LOC ($n = 565$) and those without ($n = 7,054$)	• Age of symptom onset of AD as well as diagnosis was on average 2.6 and 2.3 years earlier for patients with a TBI history with LOC compared to patients without a TBI history, controlling for sex, race, and education. • For both females and males, a history of TBI with LOC and APOE 4 were each independently associated with significantly earlier age of symptom onset of AD and diagnosis. • No significant interaction between TBI history and APOE4. • TBI severity and/or age of injury was not available in the NACC database that was used in this study.
Schaffert et al., 2018 [62]	Autopsy-confirmed AD patients having a history of TBI with LOC ($n = 197$) and those without ($n = 1,956$)	• Age of symptom onset of dementia as well as diagnosis was 2.4 and 2.8 years earlier for patients with a TBI history with LOC compared to patients without a TBI history, controlling for sex, race, and education. • A history of TBI with LOC also showed a 3.6 year earlier onset and diagnosis of dementia among patients with the highest histopathologic criteria for AD (Braak stages V-VI and *frequent* CERAD scores). • TBI severity and/or age of injury was not available in the NACC database that was used in this study.

AD, Alzheimer's disease; HR, hazards ratio; LOC, loss of consciousness; NACC, National Alzheimer's Coordinating Center; TBI, traumatic brain injury.

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risk for bvFTD [67]. A follow-up study found that a history of TBI with LOC was found to be associated with a 4.4 times higher risk of bvFTD compared to all other non-FTD dementia diagnoses combined [68]. While this finding suggests that a history of TBI may be associated with a greater risk for developing an FTD syndrome versus other types of dementia, it is important to note that the study's sample was small and only contained 8 cases with a TBI history out of the 63 having a bvFTD diagnosis, necessitating replication of the findings before such a generalization can be made. More recently, the age of bvFTD onset has been linked to past TBI history. In a study comparing individuals with a history of TBI and LOC but no chronic deficits ($n = 75$) to individuals without a TBI history ($n = 603$), age of bvFTD symptom onset and diagnosis was shown to be 2-3 years earlier on average for those with a TBI history [69]. Overall, however, no study to date has examined the risk for FTD based on TBI severity, multiple injuries, age of onset, or time since injury (see Table 7).

Lewy body disease (Parkinson's disease dementia and dementia with Lewy bodies)

Lewy body disease is comprised of two related syndromes, Parkinson's disease (PD) with dementia and dementia with Lewy bodies (DLB). The core features of PD involve characteristic progressive motor abnormalities (e.g., bradykinesia and either resting tremor or rigidity) prior to onset of cognitive impairment, while DLB consists of two or more of the following symptoms: impaired and fluctuating cognition, recurrent visual hallucinations, REM sleep behavior disorder, and spontaneous motor changes similar to PD [70, 71]. The pathological hallmark for both conditions is an accumulation of alpha-synuclein proteins into clumps called Lewy bodies. In patients with PD without dementia, Lewy bodies are typically seen in the basal ganglia; however, in those with PD with dementia and DLB, Lewy bodies are spread diffusely within the cortex. A California statewide study of medical record data of individuals aged 55 years and older compared the incidence of PD over 6 years between those with a history of TBI ($n = 52{,}393$) and non-TBI physical traumas ($n = 113, 406$) [72]. A history of mild TBI (HR = 1.2) and moderate-to-severe TBI (HR = 1.5) was associated with a higher likelihood for developing PD, with the risk being doubled for those who sustained multiple injuries of any severity (1 TBI HR = 1.4 versus > 1 TBI HR = 1.8) [72]. In that study, the incidence rate for PD was 1.7% for persons with a TBI history compared to 1.1% for those with non-TBI physical trauma. Another investigation examined the incidence of PD over 12 years among 325,870 veterans with and without a TBI history by combining three nationwide Veterans Affairs Health datasets [73]. Because TBI classification varied across datasets, mild TBI was characterized as injuries involving LOC up to 59 minutes and moderate-to-severe TBI was characterized as having LOC $\geq$60 minutes, with both being associated with a higher risk for developing PD (mild TBI HR = 1.5, moderate-to-severe TBI HR = 1.8). Although the incidence of PD in the study was <1%, individuals with milder (LOC <60 minutes) and more severe TBIs (LOC $\geq$60 minutes) had slightly higher incidence rates (0.4% and 0.7%, respectively) compared to controls with no TBI history (0.3%). However, there have been several case-control studies that have found no link between a history of mild TBI and later development of PD [74]. Furthermore, other studies have maintained that the risk for PD following a TBI is highest within a few years of injury [74]. As in all-cause dementia, a higher risk within a few years of injury raises the question of whether a neurodegenerative process was already present at the time of injury, leading some to speculate whether the TBI might be a consequence of pre-clinical PD rather than a precipitating factor. When one considers that individuals are at an increased risk for falls from PD, and the fact that the disease typically develops over several years, it is difficult to challenge this hypothesis. Nonetheless, a recent longitudinal investigation that examined 7,130 individuals aged 50 years and older from three cohorts found that a history of TBI with LOC was associated with a progression of PD-like symptoms over time, providing some strong support for TBI being a risk factor for development of PD in some individuals [42]. A higher risk for earlier onset of PD has also been linked to a history of TBI. Among 203 sibling pairs with PD, those with a history of TBI of any severity had, on average, a 3.3 years earlier onset of PD [75].

While there have been several investigations examining TBI as a risk factor for PD, only one to our knowledge has assessed the risk associated with developing DLB (see Table 8). In that study, patients with a clinical diagnosis of probable DLB ($n = 147$) were compared to healthy controls ($n = 294$) and patients diagnosed with AD ($n = 236$), and TBI history was not found to be associated with an increased likelihood for DLB diagnosis [76]. A more recent

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Table 7
TBI-related risk for dementia: Frontotemporal dementia

Study	Participants	Key Findings
Deutsch et al., 2015 [66]	Individuals with FTD ($n = 1{,}016$) matched to healthy controls ($n = 2{,}015$)	• Participants were evaluated for different levels of TBI severity (any TBI, TBI without chronic deficit, remote TBI with chronic deficit, remote TBI with LOC, but without chronic deficit) and compared on neurocognitive tests and risk of FTD. • A lifetime history of TBI with $\geq$5 min LOC was associated with a 1.6 fold higher risk for FTD diagnosis. • Participants with prior TBI with LOC or chronic deficit/dysfunction displayed significantly more behavioral disturbances, but performed better on some cognitive measures.
Rosso et al., 2003 [67]	Sporadic FTD cases ($N = 80$); matched informant controls ($N = 124$)	• A number of medical and environmental risk factors were examined as risk factors for FTD. • Head injury presented a 3.3 fold increase risk of FTD • Thyroid disease was associated with a 2.5 fold increase • Smoking, alcohol, and chemical exposure were not associated with higher or lower risk for FTD
Kalkonde et al., 2012 [68]	Veterans with non-FTD dementia ($n = 554$) and those with bvFTD ($n = 63$)	• Medical records were reviewed from veterans with cognitive and/or behavioral complaints between 2003–2008. • TBI was associated with a 4.4 fold increase risk of an FTD diagnosis compared to other dementias • Cerebrovascular disease and heart disease was less prevalent in patients with FTD compared to other dementias.
LoBue et al., 2016 [69]	Clinically diagnosed bvFTD patients having a history of TBI with LOC ($n = 75$) and those without ($n = 603$)	• TBI was defined based on reported lifetime history of TBI with LOC at least 1 year prior to bvFTD diagnoses. • Age of symptom onset and diagnosis was approximately 3 years earlier in patient with bvFTD and TBI

bvFTD, behavior variant frontotemporal dementia; FTD, frontotemporal dementia; LOC, loss of consciousness; TBI, traumatic brain injury.

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Table 8
TBI-related risk for dementia: Lewy body dementia

Study	Participants	Key Findings
Gardner et al., 2015 [72]	TBI ($N = 52,393$) or non-TBI trauma ($N = 113,406$) from California emergency departments	• 1.7% of those with TBI were diagnosed with PD versus 1.1% of those with non-TBI trauma in the 5–7-year follow-up, with corresponded to a 44% increase in risk (OR: 1.44) after adjusting for covariates. • A dose-response relationship was found. Risk was higher in those with >1 TBI (OR: 1.87) and moderate-severe TBI (OR: 1.50) compared to mild TBI (OR: 1.24) • No differences were observed in PD risk by mechanism of injury (i.e., falls versus non-falls)
Gardner et al., 2018 [73]	Veterans with TBI ($N = 162,935$) were 1 : 1 age-matched to a random sample of those without TBI	• In a mean follow-up of approximately 5 years, those with TBI were more likely to be diagnosed with PD (HR: 1.71). • Mild TBI was associated with increased risk of PD (HR: 1.56), but mild TBI without LOC ($N = 27$) did not reach statistical significance. • Moderate-severe TBI was associated with an increased risk of PD (HR: 1.83)
Marras et al., 2014 [74]	Systematic review of the association between mTBI and risk of PD	• 65 studies were reviewed, only 5 of which were accepted as having a low-risk of bias and included in the systematic review. • The definition of mTBI was highly heterogenous across studies. • Only 1 study [102] found a significant association between mTBI and PD. However, those with a diagnosis within 3 months of injury drove this association, and findings were attenuated when injury preceded diagnosis >10 years, strongly suggesting reverse causation. • Long-term, high quality, prospective studies are needed in the future.
Maher et al., 2002 [75]	Sibling pairs ($N = 203$) with PD as part of the *Gene*PD study	• Those with any TBI had a 3.3-year younger age of PD onset than those without PD. • No sensitivity analyses, including analyses with covariates, or stratifying by TBI severity and/or age of injury were reported.
Boot et al., 2013 [76]	Case-control cohort study with controls ($N = 294$), DLB ($N = 147$, and AD ($N = 236$)	• No significant associations between risk of TBI and subsequent DLB were identified. • 46% of the data on TBI were missing in the DLB group, and 68% in the AD group, compared to just 6% of the controls. • No sensitivity analyses, including analyses with covariates, or stratifying by TBI severity and/or age of injury were reported.
Nguyen et al., 2018 [77]	Individuals with clinical DLB and a history of TBI with LOC ($N = 50$) versus no TBI with LOC ($N = 526$)	• Controlling for sex, individuals with history of TBI with LOC had a 1.49-year earlier onset of DLB than those with no TBI with LOC, but this was not statistically significant ($p = 0.35$). • No significant association was observed for age of DLB diagnosis ($p = 0.71$). • TBI severity and/or age of injury was not available in the NACC database that was used in this study.

DLB, dementia with Lewy bodies; HR, hazards ratio; LOC, loss of consciousness; mTBI, mild traumatic brain injury; NACC, National Alzheimer's Coordinating Center; OR, odds ratio; PD, Parkinson's disease; TBI, traumatic brain injury.

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investigation analyzed whether a history of TBI with LOC was associated with an earlier age of onset for DLB in the National Alzheimer's Coordinating Center (NACC) database. Interestingly, unlike the reports in AD, FTD, and even PD, individuals with DLB with ($n = 50$) and without a TBI history ($n = 526$) did not significantly differ in terms of the timing of symptom onset or age of diagnosis [77]. Therefore, although available studies suggest that TBI may be a risk factor for PD, TBI history may not confer risk for DLB in the same way. Further research is needed to determine the impact a history of TBI has on a person's likelihood of developing DLB later on.

IS TBI RELATED TO CHRONIC TRAUMATIC ENCEPHALOPATHY?

Multiple mild TBIs and repetitive head impacts have been posited to cause chronic traumatic encephalopathy (CTE), a neuropathological syndrome reported to involve a progressive decline in emotional and cognitive functioning [78]. Nonspecific factors such as impulsivity, irritability, depression, suicidality, substance misuse, gait instability, motor slowness, and attention and memory changes have been used to characterize cases with CTE pathology based on postmortem interviews with informants [79]. Early exposure to head trauma has also been described to be associated with onset of symptoms in cases with CTE pathology, with retrospective reports of emotional and cognitive changes said to start approximately 13 years earlier for individuals who began playing football before the age of 12 in one sample [80]. This is 10-11 years earlier than the association generally found between TBI resulting in LOC and onset of AD and FTD as reviewed above. It is important to note, however, that retrospective interviews are extremely vulnerable to recall bias, allowing for symptoms to be described that may or may not actually have been present or may reflect longer-standing proclivities. In fact, many studies have demonstrated that self- and informant-reported cognitive complaints have a weak association with performance on objective neuropsychological tests, and more strongly relate to mood symptoms [81–84]. This raises questions about whether symptoms reported in cases with CTE may be misattributed to CTE pathology. In fact, depression and substance abuse can develop for a variety of reasons such as genetic predisposition, adverse life events, and other health-related factors, and may be a source of cognitive difficulties or complaints. An investigation of Wisconsin high school graduates from 1957 examined whether playing football during that time was a risk factor for having the symptoms previously described in cases with CTE pathology 30–45 years later [85]. Depression, anxiety, anger, heavy alcohol consumption, and neuropsychological functioning were evaluated at several time points (ages 52, 65, and again at age 72), comparing individuals who played high school football ($n = 834$) with individuals who played a non-contact sport ($n = 800$) or did not play any sport ($n = 1{,}951$). Football players had similar patterns of cognitive performance, alcohol use, and mood symptoms to each of the comparison groups in that large study, and actually demonstrated lower depressive symptoms overall than the other groups. These data contrast reports that football is linked to worse cognitive and emotional outcomes later in life, as previously described in cases with CTE. The reader is referred to recent publications by Ling, Neal, and Revesz (2017), Iverson et al., (2017), and Smith et al., (2019) for in-depth reviews of the history of CTE, neuropathological characteristics, and controversies [86–88].

Because a progressive neurobehavioral decline in individuals diagnosed with CTE pathology has been reported in several studies, CTE is believed by some to be a neurodegenerative disease. Along these lines, a few studies have staged CTE pathology based on the amount and location of the characteristic tau patterns present [89], and have reported a gradual change in functioning for some cases with CTE pathology at all levels of pathological burden [79]. As defined by McKee and colleagues, Stage I requires 1-2 CTE lesions at the depths of cortical sulci. Stage II requires ≥ 3 lesions across multiple cortical regions. Stage III requires multiple lesions and widespread neurofibrillary degeneration, and Stage IV requires diffuse lesions and neurofibrillary degeneration across the cerebral cortex, thalamus, and brainstem. Although the descriptions of progressive clinical change are again limited by use of retrospective interviews only, as outlined above, the criteria for what has been considered CTE lesions have varied over time and may have resulted in some cases being misdiagnosed. In 2015, a consensus conference was convened by the NINDS/NIBIB to define the neuropathological criteria for CTE. Brain tissue from 10 cases with presumed CTE and 15 cases from various tau neurodegenerative disorders, all considered at least moderate in disease severity, were selected (though specific selection methods were not specified) and then sent for

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blind evaluation by a group of seven neuropathologists [90]. Proposed pathological criteria for CTE and well-established criteria for various other tauopathies including AD, progressive supranuclear palsy, corticobasal degeneration, argyrophilic grain disease, primary age-related tauopathy, and Parkinson dementia complex Guam were provided to the evaluators. For the brain slices presumed to be CTE, 91% of the neuropathologists' reviews classified CTE as the diagnosis. In the end, a consensus panel further refined the neuropathological criteria for CTE to establish criteria that did not clearly overlap processes from the other tau-related neurodegenerative conditions. CTE criteria were defined as pTau aggregation in an irregular pattern in neurons, astrocytes, or cell processes around small blood vessels at the depths of sulci within the brain [90]. However, the consensus group did not incorporate any staging system for CTE, which may have been at least in part due to restricting the selection of patients to the moderate-to-severe end of pathological burden (e.g., Stage III and IV). As a result, it is unknown if CTE lesions thought to represent Stage I/II can be consistently recognized by neuropathologists and distinguished from other conditions since there has only been one reliability study for CTE to date. Despite a previous staging system being available, it would seem possible that there could be alterations or modifications to staging CTE given that prior criteria for recognizing the syndrome consisted of many features that overlapped other neuropathological conditions as well as normal aging. This may have important implications, and it is unclear how many cases in the literature previously diagnosed with CTE would in fact continue to receive a neuropathological diagnosis if re-examined using the new criteria. Additionally, no threshold of pathological burden was incorporated into the CTE criteria to represent when CTE lesions may be considered "significant," akin to other well-established neurological conditions. For example, when pathologists are diagnosing AD, the criteria for diagnosing AD relies on staging the pathological changes to reflect a No, Low, Intermediate, or High likelihood for AD to be associated with clinical symptoms [87], as we can see some accumulation of these pathological markers of AD in individuals without cognitive symptoms or a dementia diagnosis. In an autopsy study of 2,232 individuals aged 1 to 100 years, only 10 cases were negative for tau pathological changes and these were not older than 24 years of age at death, which raises a question of whether some pathological changes are simply a part of "normal" aging [92]. As such, it would seem likely that the presence of a single or a few CTE/tau lesions might not be associated with any clinical symptoms, and other processes, either neurological, medical, or psychiatric, may instead be the source of symptoms.

To complicate matters further, many cases identified with CTE also show a significant number of neuropathological lesions from other conditions, including AD and related dementias, often leading to a co-morbid diagnosis of AD or other types of neurodegenerative diseases [87]. Although these conditions would be linked to clinical symptoms for patients without CTE, often times, cases with alternative neuropathological diagnoses and CTE pathology are primarily classified as a "CTE case" rather than having the other well-established condition as the primary diagnosis. Due to an absence of systematic clinicopathological studies, it is unknown if CTE is associated with clinical symptoms that are distinct from alternative neuropathological diagnoses or contributes to the clinical profile in a meaningful way to allow for CTE to explain cognitive/behavioral changes when co-occurring pathological processes are present. Furthermore, CTE pathology has been reported in numerous other conditions, including epilepsy and opioid use, in addition to cases with no known history of TBI [93]. Between 1954 and 2013, a total of 153 patients with CTE were reported, and 20% had a documented history of a substance use disorder [94]. It is unknown how substance use and other conditions may influence CTE pathology, but a greater aggregation of *p*Tau lesions has been observed in those with a history of opioid use when compared to healthy age-matched control subjects [95]. At the very least, these findings suggest that CTE pathology is not seen exclusively within the context of a history of TBI and may relate to other factors.

While the neuropathological features of CTE have been recently published, the clinical implications, if any, of CTE pathology remain unclear. Thus, future research is clearly needed to systematically correlate clinical symptoms during life (using standardized neurological and neuropsychological assessments) with CTE pathology at autopsy in order to better understand CTE and isolate risk factors associated with its development.

ARE THERE LONG-TERM COGNITIVE CHANGES FROM TBI?

Potential long-term consequences on cognitive functioning related to a history of TBI have gained

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increased attention in recent years, even aside from the issue of CTE. In a study that examined neuropsychological performances of 61 patients with a history of TBI of any severity on average 30 years after initial evaluation, cognitive decline was observed in 56% of subjects [96]. It is important to note, however, that moderate-to-severe TBI accounted for more than 70% of the injuries, indicating that severity of TBI is important to consider when evaluating later risk of cognitive decline. While follow-up neuropsychological scores were significantly lower in TBI subjects compared to a control group, not surprisingly, generalized cognitive impairment often occurs with moderate-to-severe TBI and thus, differences may have been directly related to these injuries. Nonetheless, neuropsychological profiles among veterans aged 50 years and older with ($n = 88$) and without ($n = 81$) a TBI history, where the majority of injuries were mild in nature (68%), showed significant differences [97]. Individuals with a history of multiple mild TBIs ($n = 41$) or moderate-to-severe TBI ($n = 27$) were found to show lower scores on tasks of processing speed and executive functioning, with mean performances falling nearly 1 standard deviation below the normative sample for both injury groups, whereas those with a single, mild TBI ($n = 19$) did not show this trend. Another study of former retired professional football athletes ($n = 28$), many of whom self-reported a history of multiple concussions, were found to have lower verbal memory performances compared to healthy controls, although mean performances were still within the normal range [30, 98]. Although these findings suggest that a history of multiple mild TBIs or moderate-to-severe TBI may be associated with a vulnerability to diminished cognitive functioning in aging for some individuals, it is important to consider that these studies contained limited sample sizes and require replication in future research investigations before the findings can be appropriately generalized more broadly to other athletes, military personnel, or the public.

Along these lines, two recent multi-center studies provide support for using caution when making assertions about the influence of TBI on cognitive decline later in life. An investigation of 432 participants with normal cognition and 274 with probable AD in the NACC compared performances on a neuropsychological battery over an 8-year time span between those with and without a history of TBI with LOC [99]. A history of TBI was not associated with a greater rate of cognitive decline on any neuropsychological test for those with normal cognition, nor was TBI history associated with more rapid declines for those with AD. Another investigation using the NACC dataset examined the risk for progression to AD and course of decline among 2,719 participants with prodromal AD (i.e., MCI) with and without a TBI history with LOC [100]. Despite a history of TBI with LOC being linked to an earlier onset of symptoms, TBI history was not associated with faster conversion to AD or a greater rate of decline on a measure of global cognition. Also, the annual rate of progression from MCI to AD was similar, at 8% for individuals with a TBI history versus 10% for those without.

CONCLUSION

Moderate-to-severe TBI has garnered several lines of evidence for TBI being a risk factor for the later development of dementia and neurodegenerative disorders. Degeneration of neuronal structures and white matter connections seen on MRI scans and Aβ and *p*Tau found at autopsy have been observed years after injury in those with moderate-to-severe injuries that is greater than would be expected for aging-related effects. Taken together with studies reporting an increased incidence of dementia, there is a significant amount of support to suggest that moderate-to-severe TBI can increase the risk for AD, FTD, and other types of dementia later-in-life in some individuals. Mechanism(s) underlying this risk remains unclear at present, though triggering of a chronic inflammatory process and/or an accumulation of pathological proteins in these conditions may be important. Generally, studies investigating whether TBI is associated with a progressive accumulation of pathological markers seen in AD and related dementias, structural/functional brain-related changes, and cognitive decline many years after injury have been few. For example, the majority of studies have only investigated structural brain changes at two time points, which limits interpretations given that it is difficult to determine whether any changes may have stopped shortly after the initial scan, plateaued after a few years, or progressed for several years thereafter. Although some studies have included additional serial scans to assess for brain changes, the duration of these have been limited to <5 years following a moderate-to-severe injury, and it is unclear at present whether neurodegenerative changes can continue beyond this timeframe. It is thus unknown whether moderate-

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to-severe TBI may increase the risk for developing neurodegenerative conditions by contributing to the accumulation of pathological proteins of an already present neurodegenerative disorder such as AD, lowering one's cognitive/neuronal reserve, and/or may initiate a progressive process ultimately leading to the development of a neurodegenerative dementia in some individuals.

Mild TBI has received some support for increasing the risk for disrupted brain connections/function and neurodegenerative conditions. More complex injuries that include brain hemorrhage, post-traumatic stress disorder, and/or blast exposure as well as presence of cognitive impairment later-in-life (MCI and dementia) may explain the majority of the findings in structural/functional imaging studies. Thus, later-in-life brain changes in individuals having a history of mild TBI without these complications have not been established at present. Furthermore, methodological challenges in studies examining risk for dementia following TBI limit the conclusions that can be drawn, and therefore provide modest or equivocal evidence for mild TBI as an established risk factor at present. Questions about whether a dementia process was already present at the time of injury or related to some condition completely separate from the injury remain to be resolved. Unlike moderate-to-severe TBI, there is also an absence of studies demonstrating whether mild TBI is associated with a greater buildup of pathological markers in AD and related dementias to suggest a pathological link between mild TBI and risk for dementia. Future research investigating presence of AD-related pathological biomarkers and longitudinal structural/functional imaging after mild TBI is needed to better understand the long-term implications in aging.

When one considers that less than 7% of individuals with TBI of any severity (i.e., mild and moderate-to-severe) later develop dementia across the literature, most people have a very high chance for *not* developing dementia as they age following TBI. Thus, the growing public health concern about developing neurodegenerative disorders after a TBI seems disproportionate to the risk estimates currently reported in the scientific literature. Widespread media coverage about CTE pathology in former professional athletes has no doubt contributed to the public's fear about later-in-life effects of TBI. However, there is much that is still unknown about CTE despite it being an established neuropathological syndrome. Questions about the clinical significance of CTE pathology, whether a pathological threshold is needed, and possible confusion of symptoms with alternative neurological, medical, psychiatric, and other neuropathological conditions remain to be answered in future studies. It will be particularly important to clarify whether CTE pathological lesions are related to clinical symptoms before identifying potential risk factors, and a review as well as validation of the initial consensus CTE diagnostic criteria is needed. TBI as a risk factor for long-term brain changes and cognitive decline is a rapidly growing area of research and future studies are needed to answer many of the questions and concerns expressed by military personnel, athletes, parents of young athletes, and the general public.

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Traumatic Brain Injury and Age of Onset of Dementia with Lewy Bodies

Trung P. Nguyen[a,*], Jeff Schaffert[b], Christian LoBue[b], Kyle B. Womack[a,b], John Hart[a,b,c] and C. Munro Cullum[a,b,d]

[a]*Department of Neurology and Neurotherapeutics, The University of Texas Southwestern Medical Center, Dallas, TX, USA*
[b]*Department of Psychiatry, The University of Texas Southwestern Medical Center, Dallas, TX, USA*
[c]*School of Behavioral and Brain Sciences, University of Texas at Dallas, Dallas, TX, USA*
[d]*Department of Neurological Surgery, The University of Texas Southwestern Medical Center, Dallas, TX, USA*

Abstract.
Background: Traumatic brain injury (TBI) with loss of consciousness (LOC) has been associated with earlier onset of mild cognitive impairment, frontotemporal dementia, Parkinson's disease, and Alzheimer's disease (AD), but has not been examined as a risk factor for earlier onset of dementia with Lewy bodies (DLB).
Objective: The purpose of this study was to assess the association between a history of TBI and the age of onset of DLB.
Method: Data from 576 subjects with a clinical diagnosis of DLB were obtained from the National Alzheimer's Coordinating Center (NACC). Analyses of Covariance examined whether self-reported history of remote TBI with LOC (i.e., >1 year prior to the first Alzheimer's Disease Center visit) was associated with earlier DLB symptom onset.
Results: Controlling for sex, those with a history of remote TBI had an approximately 1.5-year earlier clinician-estimated age of onset ($F = 0.87$, $p = 0.35$) and 0.75-years earlier age of diagnosis ($F = 0.14$, $p = 0.71$) of DLB compared to those without a history of TBI, though the differences did not reach statistical significance. Analysis of subjects with autopsy-confirmed diagnoses was underpowered due to the low number of TBI+ subjects.
Conclusions: Remote TBI with LOC was not significantly associated with DLB onset, despite being a significant risk factor for cognitive decline and earlier age of onset in other neurodegenerative conditions. Replication of these results using a larger cohort of DLB subjects with and without a TBI history who have undergone autopsy is indicated, as our TBI+ subjects did show a slightly earlier onset of about 1.5 years. Further investigations into other potential DLB risk factors are also warranted.

Keywords: Age of onset, dementia, dementia with Lewy bodies, traumatic brain injury

INTRODUCTION

A history of traumatic brain injury (TBI) with loss of consciousness (LOC) is increasingly recognized as a risk factor for all-cause dementia [1, 2], Alzheimer's disease (AD) [3–9], mild cognitive impairment (MCI) [10], and frontotemporal dementia (FTD) [11–13]. However, there is limited research investigating the relationship between TBI and dementia with Lewy bodies (DLB). As a syndrome, DLB is characterized by the presence of at least two of the following core clinical features: fluctuating cognition, recurrent visual hallucinations, REM sleep behavior disorder, and spontaneous features of parkinsonism. Pathologically, this neurodegenerative disorder is defined by the presence of cortical Lewy bodies consisting of α-synuclein [14]. Most studies examining TBI as a risk factor for synucleinopathies have focused on the related disorder of Parkinson's disease (PD), with mixed results

*Correspondence to: Trung P. Nguyen, 5323 Harry Hines Blvd, Dallas, TX 75390-9129, USA. Tel.: +1 214 648 3670; E-mail: trungp.nguyen@utsouthwestern.edu.

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[15–19]. A 2013 meta-analysis of 22 studies investigating head trauma and PD found that TBI may increase the risk of PD by a pooled factor of 1.57 [16]. Additionally, a study of sibling pairs with PD found that sustaining a TBI of any severity was associated with an average 3.3-year earlier onset of PD [20]. A meta-analysis published in 2014 focused on mild TBI and PD analyzed five of the studies included in the 2013 meta-analysis [16] and reported a lack of association between mild TBI and PD [18]. The two largest population studies in these meta-analyses, from Denmark [19] and Sweden [21], both reported the risk for PD to be greatest soon after the head injury and dissipates over time, raising concerns for reverse causation, where head injuries preceding the PD diagnosis may be a consequence of prodromal PD rather than causing PD. To address this issue, a more recent investigation [15] assessed the risk of PD associated with TBI and non-TBI physical traumas, as well as different TBI factors, including age of injury, severity, frequency and the interval between injury and PD diagnosis, and reported a 44% increase in risk of being diagnosed with PD after TBI compared to non-TBI trauma, with significantly higher risk proportional to severity and frequency that supports a dose-response relationship. A subsequent study has also reported that an earlier age at first head injury is associated with PD, though the overall association between head injury and PD was not significant [17]. Several studies have reported on the accumulation of α-synuclein following TBI, both in cases of fatal head injury [22] as well as non-human TBI models [23, 24]. Along these lines, a recent study by Crane and colleagues reported that a history of TBI with LOC may be a risk factor for increased Lewy bodies at autopsy as well as the onset and progression of PD [25]. This evidence would suggest that TBI might also increase the risk for developing DLB or hasten its onset due to the accumulation of Lewy body pathology. However, despite this potential link, investigations into the relationship between TBI and DLB are limited.

To our knowledge, only one exploratory analysis, by Boot and colleagues, has evaluated TBI as a risk factor for developing DLB [26]. Those authors compared subjects with a clinical diagnosis of probable DLB ($n = 147$) to sex- and age-matched healthy controls ($n = 294$) in three longitudinal studies of aging and dementia at Mayo Clinic. In that investigation, TBI of any severity was not found increase the likelihood of DLB [26]. Recently, we have published a series of papers investigating the onset of neurodegenerative disorders in those with and without a history of TBI and LOC, showing an earlier onset of MCI, AD, and FTD in those with a history of TBI [10, 27–29]. To add to the sparse literature examining TBI history in DLB, the current study evaluated the relationship between a self-reported remote history of TBI with LOC and symptom onset in DLB.

MATERIALS AND METHODS

Subjects

Data were obtained from the National Alzheimer's Coordinating Center (NACC) Uniform Data Set (UDS) and Neuropathology Data Set (NDS). NACC has collected demographic, genetic, clinical, and autopsy data on healthy controls and subjects diagnosed with various dementias via approximately annual visits from National Institute on Aging-funded U.S. Alzheimer's Disease Centers (ADC) since September 2005 [30]. Subjects with data from UDS versions 1 and 2 who were aged 45 years or older and had a primary clinical diagnosis of DLB were selected for inclusion. A team of multi-disciplinary ADC clinicians or a single clinician at each ADC formulated the diagnosis of DLB using established diagnostic criteria [31]. In total, data were drawn from 31 ADCs in this sample, using data from September 2005 to March 2015. Available autopsy diagnoses using the 2005 3rd consortium criteria [31] were reviewed to determine if the accuracy of clinical diagnoses was similar to recently published frequencies [32].

Measures

The NACC dataset consisted of three questions related to TBI history that were answered by the subject and/or informant during ADC visits. ADC clinicians asked subjects if they had ever sustained a TBI resulting in LOC < 5 minutes or ≥ 5 minutes, or if their TBI resulted in a chronic deficit. Responses were coded as *absent*, *recent/active* (TBI < 1 year ago or required ongoing treatment), *remote/inactive* (TBI ≥ 1 year ago with recovery or no current treatment), or *unknown*. Data comparing a history of single versus multiple TBIs were not available in this version of the NACC dataset. In addition, injury severity cut-offs in the available data were arbitrary, and TBIs resulting in LOC < 5 minutes or ≥ 5 minutes were combined into a single category of TBI with any LOC. To reduce the effect of recent injuries and

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to examine the effect of remote TBI, only those who reported a TBI occurring ≥ 1 year prior to the time of diagnosis were included. Subjects who sustained a TBI occurring < 1 year prior to the initial visit or anytime thereafter were excluded to reduce the likelihood of more acute effects. Finally, to reduce the possibility that residual cognitive deficits influenced the diagnosis of DLB, those who reported chronic deficits from their TBI were excluded.

The age of DLB onset was assessed using the NACC variable of the estimated age of symptom onset, defined as the ADC clinician's estimated age of cognitive decline based on subject/informant report, medical records, and observation. Factors including demographics (e.g., sex, race, education), cardiovascular factors (e.g., smoking and history of stroke), first-degree family member with dementia, apolipoprotein E ε4 status (APOE4; number of alleles), and depression (i.e., recent and lifetime history) were examined as potential covariates.

Statistical analyses

Chi-square and independent *t* tests were used to assess whether subjects with a history of TBI with LOC (TBI+) and those without (TBI–) differed by demographics, number of years smoking, history of remote stroke, number of Apoe4 alleles, family history of dementia, and history of depression. Any significant differences that were identified in these variables were used as covariates in primary analyses. Analyses of Covariance (ANCOVA) were used to assess whether age of DLB onset significantly differed between TBI+ and TBI– groups. Missing data were assessed for each individual analysis and subjects missing relevant data were excluded. All analyses were conducted using IBM SPSS Statistics V24 (IBM Corp, SPSS Statistics V24, Armonk, New York, USA, 2015) with $p < 0.05$ as the level of significance.

RESULTS

Clinical, demographic, and sample characteristics

After excluding six subjects as outliers for time of dementia onset to diagnosis (i.e., ≥3 SDs above the mean), a total of 576 subjects with available TBI history and DLB diagnosis were included. Autopsy data concerning Lewy body disease were available in 191 of these subjects. Of these, 73% ($n = 140$) had an *intermediate* or *high likelihood* that the pathologic findings were associated with DLB clinical syndrome according to the 2005 Consortium Criteria [31]. The frequencies obtained in this sample are similar to recently published information [32].

Among the study sample, 526 reported no history of TBI (TBI–) and 50 reported a history of remote TBI with no chronic deficit (TBI+). Demographic information is provided in Table 1. The sample was largely Caucasian and generally well educated, with an average of 14.61 years of education (Table 1). Missing data were as follows: one subject was missing information regarding race, 13% ($n = 76$) were missing family history of dementia information, 31% ($n = 181$) were missing Apoe4 status, 7% ($n = 38$) were missing number of years smoking cigarettes, 4% ($n = 25$) were missing history of stroke, and 3% ($n = 16$) were missing recent and lifetime depression history. No subjects were missing education, sex, age of diagnosis, or age of estimated onset. There were significantly more males in the TBI+ group (86%) than the TBI- group (70%, $p = 0.014$), so sex was used as a covariate in subsequent analyses. The TBI+ and TBI– groups did not differ in race, years of education, number of years smoking cigarettes, family history of dementia, number of APOE4 alleles, lifetime depression, depression within 2 years of diagnosis, or remote history of stroke (Table 1).

Of subjects with autopsy data with an intermediate or high likelihood that the pathologic findings were associated with DLB clinical syndrome, 131 reported no history of TBI (TBI–) and 9 reported a history of remote TBI with no chronic deficit (TBI+). Due to the limited sample size of TBI+ subjects, statistical analyses comparing groups were not carried out in this neuropathological sample.

TBI history and age of DLB symptom onset and diagnosis

The average estimated age of DLB onset was 1.49 years earlier for the TBI+ group ($M = 68.08$, $SD = 8.96$) compared to the TBI– group ($M = 69.57$, $SD = 8.09$). Similarly, the TBI+ group ($M = 73.75$, $SD = 9.34$) had a 0.75-year earlier diagnosis than the TBI– group ($M = 74.5$, $SD = 7.71$). Controlling for sex, no significant differences between TBI+ and TBI– groups (mean age of estimated DLB symptom onset, $F(1, 573) = 0.87$, $p = 0.35$; mean age of DLB diagnosis, $F(1, 573) = 0.14$, $p = 0.71$) were observed.

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Table 1
Demographics of a clinically-diagnosed DLB sample with and without a history of remote TBI

	TBI+ ($n = 50$)	TBI– ($n = 526$)	p
Males, n (%)	43 (86)	366 (67)	0.014*
Caucasian, n (%)	49 (98)	455 (87)	0.06
Education in years, M (SD)	15.3 (3.7)	14.5 (3.6)	0.17
Years smoking, M (SD)	13.7 (14.1)	11.2 (15.7)	0.25
Family history of dementia, n (%)	24 (50)	256 (57)	0.38
Number of APOE ε4 alleles			0.63
Zero APOE ε4 alleles, n (%)	22 (56)	181 (51)	
One APOE ε4 allele, n (%)	13 (33)	146 (41)	
Two APOE ε4 alleles, n (%)	4 (10)	29 (8)	
Depression			
Lifetime, n (%)	27 (56)	305 (60)	0.65
Within 2 years of diagnosis, n (%)	22 (46)	286 (56)	0.18
Remote history of stroke, n (%)	3 (6)	39 (8)	0.74

*$p<0.05$. *APOE* ε4, apolipoprotein E ε4.

DISCUSSION

A history of TBI has been found to be related to an increased risk and earlier onset of several neurodegenerative conditions. In this study, we compared the average age of DLB onset in those with and without a history of TBI in 576 subjects with the clinical syndrome DLB in the NACC database. While the 50 subjects with DLB and a history of TBI had an approximately 1.5-year earlier onset of symptoms and 0.75-years earlier diagnosis of DLB compared to those without a history of TBI, the differences did not reach statistical significance.

This study marks only the second time in the literature that TBI has been investigated as a potential risk factor for DLB. The previous study was an exploratory investigation into 19 risk factors that may contribute to DLB conducted by Boot and colleagues [26], which showed that, in a cohort of 147 subjects with DLB, 18 of whom reported a history of head injury, a self-reported history of head injury was not associated with a significantly increased risk of developing DLB compared to control subjects (odds ratio of 1.4, 95% confidence interval 0.7 – 2.6, p value 0.33). Despite methodological differences, our findings are in keeping with the prior negative findings for a relationship between TBI and increased risk of DLB and support the conclusion that a history of TBI is not associated with significantly earlier onset or risk of developing DLB.

The current findings with respect to DLB, however, are in contrast with our previous findings between TBI history and onset of several other neurodegenerative conditions. In our study of subjects with a well-characterized clinical history of AD, a history of TBI was associated with a significantly earlier average age of onset by approximately 2.5 years [27, 29]. Those findings are consistent with some [3–9], but not all studies investigating TBI in AD [33–35]. Similarly, TBI has been associated with an approximately 3-year earlier age of FTD symptom onset and diagnosis [10], supporting other studies that have found a significant relationship between TBI and increased risk of FTD [11–13]. In PD, TBI was concluded to be a risk factor (pooled OR = 1.57) for the development of PD in a meta-analysis of 22 studies [16]. The negative findings in this study are curious when compared to one study that found a 3-year earlier age of PD onset [20], which is neuropathologically similar to DLB. Taken together, these findings suggest that TBI may increase the risk of several neurodegenerative diseases to varying degrees, perhaps due to disease-specific neuropathological mechanisms.

Although TBI may be a risk factor for earlier onset of AD, FTD, and PD, why TBI did not appear to be significantly associated with the earlier onset of DLB remains unclear. PD has a similar neuropathological profile to DLB, with the accumulation of α-synuclein-containing Lewy bodies and Lewy neurites, and several studies have shown an increased risk of developing PD after TBI [15–17]. Interestingly, in their recent analysis of data from three prospective cohort studies with a total of 7,130 subjects, Crane and colleagues showed that a history of TBI with LOC was associated with incident PD, progression of parkinsonism, and findings of cortical Lewy bodies at autopsy. TBI history was not, however, related to cognitive impairment or dementia in PD that is typically associated with cortical Lewy bodies [25]. Gardner and colleagues have hypothesized that TBI may decrease motor reserve or accelerate a

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pre-existing neurodegenerative cascade, or even trigger a *de novo* cascade [15]. Jafari and colleagues posited neuroinflammation, mitochondrial dysfunction, or glutamate toxicity as mechanistic possibilities for the association between TBI and PD [16]. If these factors are indeed playing a role, then perhaps the pathological changes instigated by TBI promote either an earlier or more prevalent movement disorder manifestation of synucleinopathy to result in a PD phenotype as opposed to the cognitive manifestations of DLB. Alternatively, the impact of TBI on synucleinopathy may be restricted to brainstem-predominant α-synuclein pathology typical of PD, but not sufficient for more diffuse cortical spread of α-synuclein to the degree necessary to induce cognitive impairment and dementia seen in DLB. Clearly, further investigation is warranted to investigate the differential impact of TBI on neurodegenerative diseases.

There are several limitations and caveats to the present study. First, while the difference in age at onset and diagnosis of DLB in those with a history of TBI was not statistically significant, the TBI+ sample did have an onset 1.49 years earlier than the TBI- group, and showed a symptom onset that was 0.75 years earlier. When compared to our previous studies investigating earlier onset in other neurodegenerative diseases in relation to a history of TBI, substantially smaller group sizes due to the lower frequency of DLB in the NACC dataset could make it difficult to detect a statistically significant difference in the present sample. Although this is the largest study to date investigating TBI and risk of DLB, future studies could aim to investigate this relationship in larger samples, perhaps using combined datasets. In addition, due to the confines of the available data, age of injury was not obtained in the previous versions (i.e., UDS versions 1 and 2) of NACC's data collection forms, making it impossible to explore the interactions between more recent versus remote TBIs. Similarly, other injury-related information, such as the mechanism of injury, subsequent injury-related medical complications, TBI severity, or history of multiple TBIs were not available in the previous versions of NACCs data collection forms and thus were not incorporated into our analysis. These are important factors for characterizing TBI, and should be assessed in future studies to better understand the role of TBI and risk of neurodegenerative diseases including DLB. Also, given the limited autopsy data available at this time, the diagnosis of DLB was based on clinical criteria. Lastly, as with many other NACC-based investigations, subjects in the current study were largely well-educated, male Caucasians who were recruited via clinician referral or self-referred into a voluntary longitudinal observational study, which may not accurately represent the general population.

In conclusion, our findings presented here do not suggest a statistically significant relationship between a self-reported history of remote TBI with LOC and an earlier onset of DLB symptoms or diagnosis. This is consistent with previous findings suggesting that TBI history is not associated with developing DLB [26]. Our present findings, however, contrast with our previous studies indicating that a history of remote TBI with LOC was associated with a significantly earlier onset of MCI, AD, and FTD by approximately 2.5 to 3 years [10, 27–29]. The mechanisms by which TBI may increase the risk of developing different neurodegenerative diseases, or even the phenotypic expression within a neuropathological process, remains poorly understood and warrants further investigation. Such studies should focus on comparing the effects of TBI on AD and FTD as well as the motor and cognitive presentations of synucleinopathies.

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Prevalence of Traumatic Brain Injury in Early Versus Late-Onset Alzheimer's Disease

Mario F. Mendez[a,b,c,*], Pongsatorn Paholpak[a,b,c], Andrew Lin[a,b,c], Jeannie Y. Zhang[b] and Edmond Teng[a,b,c]
[a]*Department of Neurology, University of California Los Angeles (UCLA), Los Angeles, CA, USA*
[b]*David Geffen School of Medicine, University of California Los Angeles (UCLA), Los Angeles, CA, USA*
[c]*Department of Neurology, Neurobehavior Unit, V.A. Greater Los Angeles Healthcare System, Los Angeles, CA, USA*

Abstract.
Background: Traumatic brain injury (TBI) is the most established environmental risk factor for Alzheimer's disease (AD), but it is unclear if TBI is specifically associated with early-onset AD (EOAD).
Objective: To evaluate the relationship between TBI and EOAD (<65 years).
Methods: We identified 1,449 EOAD, 4,337 late-onset AD (LOAD), and corresponding EOAD-matched and LOAD-matched normal controls (NC) in the National Alzheimer's Coordinating Center Uniform (NACC) database and compared the prevalence of any history of TBI as well as measures of cognition, function, behavior, and neuropathology. For validation, we determined TBI prevalence among 115 well-characterized clinic patients with EOAD.
Results: Part A: The prevalence of any TBI in the NACC-database EOAD participants (13.3%) was comparable to that observed in the clinic EOAD patients (13.9%) but significantly higher than in the NACC-database LOAD participants (7.7%; $p < 0.0001$) and trended to higher compared to EOAD-matched NC (11.1%; logistic regression $p = 0.053$). Part B: When we compared EOAD patients with documented non-acute and non-residually impairing TBI to EOAD without a documented history of prior TBI, those with TBI had significantly more disinhibition. Part C: Autopsies did not reveal differences in AD neuropathology based on a history of TBI.
Conclusions: These findings suggest, but do not establish, that TBI is a specific risk factor for EOAD and may lead to disinhibition, a feature that often results from the frontal effects of head injury. This study recommends further research on the effects of TBI in EOAD in larger numbers of participants.

Keywords: Aging, Alzheimer's disease, concussion, dementia, epidemiology, head injury, memory loss, neurodegeneration, risk factors, traumatic brain injury

INTRODUCTION

Alzheimer's disease (AD) is an increasing health problem worldwide, with the prevalence of the disorder projected to rise as people continue to live longer lives [1, 2]. Advancing age is the most important risk factor for AD, as most affected individuals are 65 years of age or older [1, 3]. Consequently, as therapeutic interventions have lagged in development,

*Correspondence to: Mario F. Mendez, MD, PhD; Neurobehavior Unit (691/116AF), V.A. Greater Los Angeles Healthcare Center, 11301 Wilshire Blvd., Los Angeles, CA 90073, USA. Tel.: +1 310 478 3711/Ext. 42696; Fax: +1 310 268 4181; E-mail: mmendez@UCLA.edu.

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there has been increasing emphasis on identifying strategies for prevention in younger life, before the development of the disease. This emphasis on prevention increases the importance of identifying risk factors, other than aging, that can be the focus of intervention or modification.

After normal aging, a history of traumatic brain injury (TBI) is the most established environmental risk factor for dementia [4]. Studies have challenged this association [5–8], but the weight of the evidence appears to favor an increased risk for late-life AD after a single moderate-to-severe TBI involving loss of consciousness (LOC) in early or middle life [9–17]. Although there is limited support for a single mild TBI as a risk factor for dementia [18], repetitive mild TBIs can also result in chronic traumatic encephalopathy (CTE), a progressive neurodegenerative disorder with AD-like neurofibrillary tangle (NFT) formation [19–22]. Together, these studies strengthen the link between TBI and AD and the possibility that TBI promotes the deposition of AD neuropathology.

A lowering of the age of onset of AD and a consequent increased risk for early-onset AD (EOAD) may be evidence for the promotion of AD neuropathology by TBI. A number of studies have reported that a history of TBI lowers the age of onset of dementia [11, 17, 23–26], suggesting that TBI may be a particular risk factor for AD presenting at a relatively young age or for EOAD, defined as AD with onset <65 years of age [9, 27]. However, only a few studies have examined a history of TBI as a risk factor for EOAD, and their results have been contradictory [6, 28–30].

We aimed to investigate whether TBI is a specific risk factor for EOAD relative to the more typical late-onset AD (LOAD). This study could have clinical impact by renewing efforts to prevent head injuries and greater vigilance and cognitive evaluations of individuals <65 years of age who have had one or more TBIs. We investigated the effects of TBI in EOAD in a three part study from the large National Alzheimer's Coordinating Center (NACC) database. Part A determined the prevalence of a history of TBI among NACC-database EOAD participants (age of onset <65), NACC-database LOAD participants (age of onset $\geq$65), and matched normal controls (NC), and, for validation, a cohort of carefully evaluated EOAD patients from our university-based clinic. Part B investigates whether a history of TBI altered the neurobehavioral symptoms of NACC-database EOAD participants, compared to EOAD participants without a history of TBI. Part C investigated whether a history of TBI resulted in qualitative differences in neuropathology among EOAD and LOAD participants in the NACC-database.

METHODS

The NACC-database compiles and maintains the Uniform Data Set (UDS) and Neuropathological database (NPD), which contain standardized clinical and pathologic data collected from participants evaluated at National Institute of Aging (NIA) funded Alzheimer's Disease Centers (ADCs) throughout the United States [31]. The ADCs are funded, in part, for their established expertise in the accurate diagnosis of AD during life. We analyzed UDS and NPD data accumulated from September 2005 through the May 2014 data freeze. Detailed descriptions of the UDS and the NPD have previously been published [31] and are available at http://www.alz.washington.edu. This study was submitted and approved by the institutional review board at our institution. Furthermore, as part of the NACC-database, there was written informed consent from each participant and/or their authorized representative and approval by the Institutional Review Board at each participating ADC.

First, we identified NACC-database participants who were diagnosed with established criteria for "clinically probable AD dementia" at their ADC most recent UDS visit [32]. The ADCs carefully apply these criteria and, hence, these patients are representative of patients with clinically probable AD. In a NACC-database study, 438 of 526 patients with clinically probable AD met neuropathological criteria for AD, for a sensitive of the diagnosis of clinically probable AD dementia of 70.9%, specificity of 70.8%, and positive predictive value of 83.3% [33]. We defined EOAD as clinically probable AD with onset of cognitive decline <65 years of age and LOAD as clinically probable AD with onset of cognitive decline $\geq$65 years of age. Patients with familial or autosomal dominant AD from known genetic mutations, when evident in the record, were excluded. We then identified NC in the NACC-database comprised of participants with normal cognition on their initial visit. The NC were divided into younger (<65) and older ($\geq$65) groups at the initial visit and matched with the corresponding younger (EOAD) or older (LOAD) participants on gender and years of education (within three years). This process yielded 1,449 EOAD participants with 1,449 EOAD-matched NC

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Table 1
Group characteristics of NACC-database participants

	EOAD	EOAD-matched NC	LOAD	LOAD-matched NC
Number	1,449	1,449	4,337	4,337
Gender (% male)	40.8%	40.8%	40.5%	40.5%
Age of Initial Visit (years)*	62.7 ± 6.8	58.8 ± 6.1	79.2 ± 6.3	75.0 ± 6.8
Age of Onset (years)	57.3 ± 5.7	N/A	74.5 ± 6.3	N/A
Education (years)	15.3 ± 3.0	15.4 ± 3.0	14.8 ± 3.3	15.0 ± 3.1
White/Black/Asian/Other (%)	85/10/2/2	82/13/3/2	83/15/1/1	82/15/2/1

*Significant difference in Age of Initial Visit between: EOAD versus EOAD-matched controls: $t = 16.25$, df 2896, $p < 0.0001$. LOAD versus LOAD-matched controls: $t = 29.84$, df 8672, $p < 0.0001$. EOAD, early-onset Alzheimer's disease; LOAD, late-onset Alzheimer's disease; NC, normal controls.

and 4,337 LOAD participants with 4,337 LOAD-matched NC (see Table 1).

Part A

We ascertained the prevalence of a history of Any TBI in the NACC-database EOAD and LOAD groups and their NC. Our initial analyses included Any history of one or more TBI surveyed in the NACC-UDS questions. In all groups, 75% or more had TBIs that were reported as remote with less than 5 min of LOC with most of the others suggested to have LOC less than 30 min without residual disability; however, remote and retrospective determination from patients and informants on exact TBI characteristics and LOC was not clearly reliable as the NACC-database was not designed for precise characterization of TBIs. Because of the problem of possible recall bias in the NACC-database retrospective dataset, we included a validation comparison group of 115 EOAD patients who were carefully evaluated in a university-based subspecialty clinic for early-onset dementias [34]. These patients had a uniform, physician-based assessment that specifically assayed and characterized a history of prior TBI; 88.7% of their TBIs were associated with LOC of <5 min) and 96.5% with LOC of <30 min.

Part B

We further evaluated the impact of prior TBI among the NACC-database EOAD participants. Within the NACC-database EOAD group, we compared cognitive, functional, and behavioral indices between participants with a clear and documented history of TBI and those with no history of TBI. In order to increase the reliability of the reported TBI for possible effects on EOAD, we surveyed the EOAD participants with TBI and included only those ($n = 178$) with documented non-acute ("remote" TBI >1 year per NACC-UDS terminology) TBI that did not leave residual impairments ("chronic dysfunction/disability" per NACC-UDS terminology). The 178 EOAD participants with documented TBI were comparable to the 1,234 without Any TBI in age of presentation (62.13 ± 6.77 versus 62.76 ± 6.84) and years of education (15.5 ± 3.03 versus 15.21 ± 3.03) but had a greater percentage of men (60% versus 37.8%, $\chi^2 = 36.34$; $p < 0.001$). Cognitive tests included Mini-Mental State Examination, logical memory (IA and IIA) of the revised Wechsler Memory Scale (WMS-R), digit spans (forward and backward) from the WMS-R, category fluency (animals and vegetables), the Boston Naming Test, the digit symbol subtest of the Wechsler Adult Intelligence Scale, and Trail-Making tests (A and B) [31, 35]. Functional scales included the Functional Activities Questionnaire and the Clinical Dementia Rating Sum of Boxes [36, 37]. Behavioral symptoms were evaluated with the Neuropsychiatric Inventory (NPI-Q), which was completed by caregivers or other informants [38].

Part C

Neuropathological data from the NACC-NPD was available on 241 (13.5%) EOAD and 626 (14.9%) LOAD participants. We evaluated for the presence of Consortium to Establish a Registry in AD (CERAD) criteria for definite or other AD [39], as well as for the presence of other neuropathological diagnoses.

Data analysis

Statistical analyses were performed using SPSS 22 for Windows (IBM, Armonk NY). Demographic variables were compared between the EOAD and LOAD groups using χ^2 tests for nominal variables (gender, education, race), and unpaired *t*-tests for continuous variables (age, years of education). Dis-

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tribution of data were tested with Shapiro-Wilk and Kolmogorov-Smirnov Tests. We subsequently used logistic regression to adjust for possible confounding effects due to differences in gender, age at initial visit, years of education, and race (percent White). The results of the cognitive, functional, and NPI assessments were non-normally distributed; hence they were analyzed with nonparametric tests, including their median and interquartile ratio (IQR) results, using Mann-Whitney U analyses. We used the false discovery rate (FDR) to correct for multiple comparisons.

RESULTS

Part A

There were no significant differences in group characteristics except in older age of initial visit for the EOAD and LOAD participants compared to their respective NC (see Table 1). The prevalence of a history of Any TBI in the NACC-database EOAD group was 13.3% (see Table 2). This was significantly higher than the prevalence of a history of Any TBI in the NACC-database LOAD group (7.7%; $\chi^2 = 45.43$, $p < 0.0001$), but comparable to the prevalence of a history of Any TBI in the EOAD patients evaluated in the early-onset dementia subspecialty clinic (13.9%; n.s.). The prevalence of a history of Any TBI trended toward significantly higher in the NACC-database EOAD group compared to EOAD-matched NC (11.1%; $\chi^2 = 3.64$, $p = 0.056$). The prevalence of a history of Any TBI was not significantly different in the NACC-database LOAD group compared to LOAD-matched NC (8.7%; $\chi^2 = 2.73$, $p = 0.100$).

Logistic regression adjusted for possible confounding effects due to differences in gender, age, years of education, and race (% White): 1) When comparing NACC-database EOAD and LOAD groups, a history of Any TBI was significantly more common in the EOAD group (β 0.56; $p < 0.001$; OR 1.75, 95% CI 1.47–2.07) and in men (β 0.82; $p < 0.001$; OR 2.28, 95% CI 1.92–2.70); 2) When comparing NACC-database EOAD and the EOAD-matched NC, a history of Any TBI still trended to significance (β 0.22; $p = 0.053$; OR 1.28, 95% CI 1.00–1.57) despite the group differences in age (see Table 1); 3) When comparing NACC-database LOAD and the LOAD-matched NC, a history of Any TBI was not significant (β –0.12; $p = 0.132$; OR 0.90, 95% CI 0.76–1.04). Other variables (gender, age, years of

Table 2
Prevalence of TBI in NACC-database groups

	EOAD	EOAD-matched NC	LOAD	LOAD-matched NC
No previous TBI	1234 (85.2%)	1279 (88.3%)	3954 (91.2%)	3931 (90.6%)
Any TBI*	193 (13.3%)	161 (11.1%)	335 (7.7%)	379 (8.7%)
Insufficient information on TBI	22 (1.5%)	9 (0.6%)	48 (1.1%)	27 (0.6%)

*Trend toward significance for EOAD versus EOAD-matched controls: $\chi^2 = 3.64$, $p = 0.056$. TBI, traumatic brain injury; EOAD, early-onset Alzheimer's disease; LOAD, late-onset Alzheimer's disease; NC, normal controls.

Table 3
Median and interquartile range of cognitive test scores for NACC-database EOAD participants

	EOAD with History of TBI	EOAD without History of TBI	Mann-Whitney U	*p*-value
Mini-Mental State Examination	21 (9)	21 (9)	95734	n.s.
Logical Memory IA	4 (6)	3 (5)	67839	$p < 0.001$*
Logical Memory IIA	1 (4)	0 (3)	70200	$p < 0.05$
Digit Span - Forward	6 (1.75)	6 (2)	86793	n.s.
Digit Span - Backward	3 (2)	3 (2)	85314	n.s.
"Animal" Category Fluency	11 (8)	10 (8)	82655	n.s.
"Vegetable" Category Fluency	6 (5)	6 (7)	85608	n.s.
Boston Naming Test	24 (8)	23 (10)	77974	n.s.
WAIS-R Digit Symbol Subtest	28 (25)	23 (25.75)	49680	n.s.
Trail Making A Test‡	54 (69)	58 (78.25)	62826	n.s.
Trail Making B Test‡	214.5 (205.25)	240.5 (184)	32366	n.s.

Non-normally distributed. Medians and Interquartile Range (IQR). *Still significant after False Discovery Rate correction for multiple comparisons but no longer significant after further accounting for the age differences between the two groups. ‡Excludes those who did not complete Trail Making A Test in ≤150 s and Trail Making B Test in ≤300 s. TBI, traumatic brain injury; EOAD, early-onset Alzheimer's disease; WAIS-R, Wechsler Adult Intelligence Scale; n.s. = non-significant.

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Table 4
Median and interquartile range of functional measures for NACC-database EOAD participants

	EOAD with History of TBI	EOAD without History of TBI	Mann-Whitney U	*p*-value
Total FAQ Score	19 (14)	19 (15)	44748.5	n.s.
CDR Sum of Boxes Score	5 (3)	5 (5)	109508	n.s.

Non-normally distributed. Medians and Interquartile Range (IQR). TBI, traumatic brain injury; EOAD, early-onset Alzheimer's disease; FAQ, functional activities questionnaire; CDR, Clinical Dementia Rating; n.s., non-significant.

Table 5
Available data for neuropathology in NACC-database EOAD and LOAD participants

Clinical Diagnosis	No.	CERAD Neuropathological Criteria		Other Non-AD Neuropathology
		Definite AD	All AD Criteria*	
EOAD with Any TBI	29	23 (79.3%)	24 (82.8%)	5 (FTLD = 1, HS = 2, Other = 2)
EOAD without TBI	212	195 (92%)	201 (94.8%)	11 (FTLD = 6, HS = 1, DLB = 2, PSP = 1; Other = 1)
LOAD with Any TBI	79	54 (68.4%)	70 (88.6%)	9 (FTLD = 3, HS = 1, DLB = 1, VASC = 2; Other = 2)
LOAD without TBI	736	536 (72.8%)	679 (92.3%)	57 (FTLD = 10, DLB = 10, HS = 9, VASC = 5; PSP = 4, NORM = 3, Other = 16)

*Definite + Probable + Possible AD; TBI, traumatic brain injury, EOAD, early-onset Alzheimer's disease; LOAD, late-onset Alzheimer's disease; CERAD, The Consortium to Establish a Registry for Alzheimer's Disease; FTLD, frontotemporal lobar degeneration; HS, hippocampal sclerosis; DLB, dementia with Lewy bodies; PSP, progressive supranuclear palsy; VASC, vascular pathology; NORM, normal brain; Other, otherwise undefined.

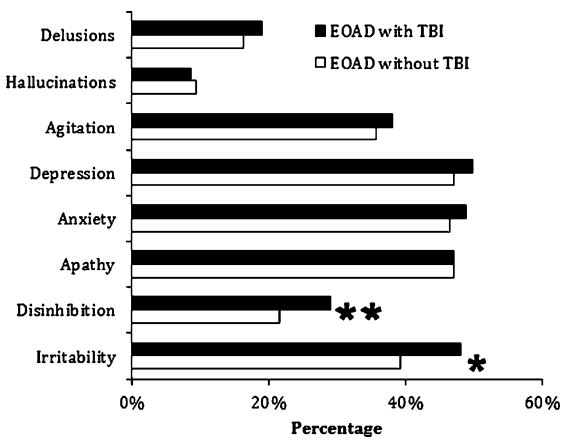

Fig. 1. Prevalence of neuropsychiatric symptoms among NACC-database early-onset Alzheimer's disease (EOAD) participants: Eight main behavioral measures of the Neuropsychiatric Inventory. *Significance to $p = 0.01$ level for disinhibition (29.0% versus 21.6%; $\chi^2 = 6.19$) and for irritability (48.1% versus 39.3%; $\chi^2 = 6.40$). **Only disinhibition remained significant after application of FDR cut-off for multiple comparisons. TBI, traumatic brain injury.

education, race) were not significant other than as noted.

Part B

Among EOAD participants, we evaluated the impact of TBI on cognitive, functional, and behavioral performance by comparing EOAD participants with documented TBI to those without a history of Any TBI. After FDR adjustment for multiple corrections, there were no significant differences between groups on cognitive or functional scores, except for paradoxically better logical memory IA scores among those with TBI compared to those without a history of Any TBI (see Tables 3 and 4). However, the significant difference in logical memory IA scores did not survive logistic regression. Age differences accounted for the significant difference in logical memory IA scores between groups (β –0.12; $p = 0.011$; OR 0.89, 95% CI 0.81–0.97).

On the NPI-Q behavioral subscales, EOAD participants with documented TBI, when compared to those without a history of Any TBI (see Fig. 1), had a significantly higher prevalence of disinhibition even after FDR correction for multiple comparisons (29.0% versus 21.6% respectively; $\chi^2 = 6.19$, $p = 0.011$). These findings remained significant after logistic regression (β 0.36; $p = 0.027$; OR 1.50, 95% CI 1.04–1.96). Disinhibition was also significantly more likely among males (β –0.27; $p = 0.022$; OR 0.76, 95% CI 0.60–0.96), but was unaffected by age, years of education, or race.

Part C

Neuropathological data were available for 241 EOAD participants (29 with TBI and 212 without TBI) and 815 LOAD participants (79 with TBI and 736 without TBI). Overall, the vast majority (92.4%) of the EOAD and LOAD participants had some form

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of AD on neuropathology (94.2% for EOAD versus 91.9% for LOAD; n.s.). The remaining exhibited a range of other neuropathologies (see Table 5). The EOAD participants were significantly more likely to have a diagnosis of definite AD on neuropathology (77% versus 71%) than the LOAD participants ($\chi^2 = 37.96$, $p < 0.0001$). There were no significant differences in the prevalence of definite or any AD between participants with and without a history of Any TBI, either within or across the EOAD and LOAD groups.

DISCUSSION

In the NACC database, a history of TBI was significantly more common in EOAD than in LOAD and trended to significance ($p = 0.056$) when compared to EOAD-matched normal controls. Furthermore, among the EOAD participants, those with a history of documented TBI, compared to those without a history of TBI, had a higher prevalence of disinhibition. Disinhibition is a characteristic clinical sign of frontal lobe dysfunction that may be influenced by the neuropathological process of TBI. Autopsies of EOAD and LOAD participants did not show qualitative differences related to a history of TBI but did not exclude quantitative effects on the neuropathology of AD. Together, these findings suggest an effect of TBI in promoting the development of EOAD.

Although a number of reports have failed to document an association between TBI and increased rates of AD [5–8], the weight of the evidence suggests that moderate-to-severe TBI with LOC is a risk factor for dementia and probably for AD [40]. An early meta-analysis of 11 case control studies confirms the association of TBI with AD, but primarily in men [11, 41], and other studies show a greater risk for AD or dementia with increasing numbers and/or severity of moderate-to-severe TBIs [12, 14]. Among U.S. Veterans, a cohort study indicates that prior moderate-to-severe TBI have an increased risk of AD [14] and, among Veterans $\geq$55 years, there may be a 60% increase in the incidence of dementia after 9 years [26]. In another retrospective cohort study, those with moderate-to-severe TBI at $\geq$55 years or mild TBI at $\geq$65 years have an increased risk of developing dementia after 5–7 years [42]. One study with a mean follow-up of one year reports an increased risk of dementia after a single mild TBI [18], but other evidence for mild TBI precipitating subsequent AD or dementia is less clear. A recent meta-analysis of studies on mild TBI (defined as loss of consciousness of <30 min and post-traumatic amnesia of <24 h) finds no association with dementia [8]. Other cohort studies have also failed to find an association between lifetime history of any TBI and an increased risk of developing dementia or AD [6, 7, 25]. In sum, the relationship between TBI and AD remains somewhat controversial; though there is greater support for a relationship between moderate-to-severe TBI and subsequent dementia.

Assuming that TBI is a risk factor for AD, one mechanism may be to hasten the age of AD onset [9, 27], and a number of studies indicate an association between a history of TBI and lower age of dementia onset [11, 17, 23–26]. The early meta-analysis of TBI in AD reported an earlier age of AD onset in individuals below 70 years of age [11]. Two early studies with small sample sizes reported a younger mean age of AD onset if they had a history of TBI [23, 24], and a community based prospective study found a younger age of AD onset among individuals with prior TBI [17]. In a well-designed, population-based investigation of all documented episodes of TBI from 1935–1984 in Olmsted County, Minnesota, the observed time from TBI to AD was less than the expected time to onset of AD (median 10 versus 18 years, $p = 0.015$), and, when stratified by age of AD onset, the number of TBI cases with EOAD was more than twice that expected [25]. Furthermore, in the aforementioned study of U.S. veterans with 9 years of follow-up, a history of TBI lowered the age of dementia onset by 2.1 years [26].

Our findings suggest that TBI may be a more specific risk factor for EOAD, as compared to LOAD. While the vast majority of cases of AD are 65 years of age or older, a smaller cohort of 4-5% are younger than 65 [1], and these EOAD cases, which are generally sporadic and not related to presenilin mutations or apolipoprotein ε4 allele [40, 43], can be phenotypically distinct from LOAD [44]. Few other studies have specifically examined a history of TBI as a risk factor for EOAD, and the results have been somewhat contradictory [28, 29]. In a recent investigation of over 800,000 Swedish men conscripted for military service and followed for 33 years, "young onset dementia," but not specifically AD, is linked to a history of TBI, with greater risk seen with more frequent or severe TBI [28]. The current investigation has the advantage of using the NACC-database, which is one of the largest and most comprehensive databases of its kind, with a specific focus on individuals diagnosed with AD [31].

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A previous investigation by Sayed and colleagues using the NACC database failed to find a relationship between TBI and an earlier age of dementia onset [30]; however, they compared dementia participants with and without a history of TBI, rather than comparing prevalence of TBI among those with EOAD compared to LOAD. They also found an increased risk for dementia among participants with dementia associated with TBI with chronic deficit/dysfunction and concluded that these participants may differ from AD because of higher rates of neuropsychiatric symptoms. Similarly, we also found that EOAD participants with determined TBI had more disinhibition than those without TBI.

There are several possible mechanistic explanations for the association between TBI and EOAD, including a reduction of cognitive reserve, diffuse axonal injury facilitation of tau deposition, frontotemporal contusions, and, most specifically, the promotion of AD-related neurodegeneration [29]. Severe TBI increases the likelihood of AD-related neuropathology changes [43]. Widespread NFTs and amyloid-β plaques are present in up to a third of patients surviving a year or more after a single TBI, even in those who are relatively young, thus supporting the role of TBI in accelerating the age of onset of AD [9]. Amyloid-β plaques can emerge hours after a single TBI, but they are usually diffuse and not fibrillar as in AD [45], and recede several months after the injury. CTE, an AD-like neurodegenerative process with extensive tau-immunoreactive NFTs, has been linked to repetitive mild TBI among boxers, other athletes, veterans, and others [46–48]. In CTE, NFTs initially deposit perivascularly and in superficial cortical layers, especially in the frontal lobes [46, 48], and CTE may manifest with early clinical "frontal" symptoms such as disinhibition, irritability, and mood disturbances, and even suicidal behavior [48, 49]. These neurobehavioral changes are similar to the disinhibition and other behavioral changes present in our EOAD participants and other demented individuals who have a history of TBI [50, 51].

A number of important issues may limit the interpretation of our results. First, we cannot completely exclude the possibility that younger age in general accounts for a higher prevalence of prior TBI among the NACC-database EOAD participants; we report only a trend to significance ($p = 0.05$) when they are compared to normal controls. Moreover, male gender, as in prior studies [11], significantly accounts for much of the difference in TBI between EOAD and LOAD participants. Second, the NACC-database does not define the nature and characteristics of the TBI, including time course, the presence of multiple TBIs, and clearly reliable LOC and severity information. Although the NACC-database does not sufficiently and reliably characterize the TBIs, it is reasonable to presume, based on similar clinic patients and the inclusion criteria for Part B, that most TBIs are mild. A third concern regarding the NACC-database is the use of retrospective ascertainment for prior TBI, a methodology fraught with recall bias, with the possibility that informants for AD participants more readily recall a previous TBI than for the normal controls. Our inclusion of a "validation" group of well-studied EOAD clinic patients, who reported a similar prevalence of prior TBI as the NACC-database EOAD participants, mitigated, but did not eliminate, the potential effects of recall bias. Finally, the inclusion of clinically probable AD does not guarantee the presence of AD on neuropathology [33].

Despite the reservations, our data suggest, but do not prove, that TBI is associated with EOAD and recommends further research on how TBI may interact with other risk factors to accelerate the onset of AD [25]. Our findings also suggest that a prior history of TBI may promote the neuropsychiatric presentation of EOAD, such as disinhibition. The results emphasize the importance of assessing TBI history and evaluating neuropsychiatric symptoms in patients who present with AD at an early age. From a public health standpoint, our findings underscore the value of public health efforts in reducing the incidence of TBI, a potentially preventable risk factor for AD, and also recommend following-up this study with further research employing larger numbers of participants in clarifying the role of TBI in accelerating the age of onset for AD.

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Autonomic Nervous System Dysfunctions as a Basis for a Predictive Model of Risk of Neurological Disorders in Subjects with Prior History of Traumatic Brain Injury: Implications in Alzheimer's Disease

Lap Ho[a,b], Marc Legere[c], Tongbin Li[d], Samara Levine[a], Ke Hao[e], Breanna Valcarcel[a] and Giulio M. Pasinetti[a,b,*]

[a]*Department of Neurology, Icahn School of Medicine at Mount Sinai, New York, NY, USA*
[b]*Geriatric Research, Education & Clinical Center, James J. Peters Veterans Affairs Medical Center, Bronx, NY, USA*
[c]*PATCH Chiropractic, Philadelphia, PA, USA*
[d]*AccuraScience, Johnston, IA, USA*
[e]*Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, USA*

Abstract. Autonomic dysfunction is very common in patients with dementia, and its presence might also help in differential diagnosis among dementia subtypes. Various central nervous system structures affected in Alzheimer's disease (AD) are also implicated in the central autonomic nervous system (ANS) regulation. For example, deficits in central cholinergic function in AD could likely lead to autonomic dysfunction. We recently developed a simple, readily applicable evaluation for monitoring ANS disturbances in response to traumatic brain injury (TBI). This ability to monitor TBI allows for the possible detection and targeted prevention of long-term, detrimental brain responses caused by TBI that lead to neurodegenerative diseases such as AD. We randomly selected and extracted de-identified medical record information from subjects who have been assessed using the ANS evaluation protocol. Using machine learning strategies in the analysis of information from individual as well as a combination of ANS evaluation protocol components, we identified a novel prediction model that is effective in correctly segregating between cases with or without a documented history of TBI exposure. Results from our study support the hypothesis that trauma-induced ANS dysfunctions may contribute to clinical TBI features. Because autonomic dysfunction is very common in AD patients it is possible that TBI may also contribute to AD and/or other forms of dementia through these novel mechanisms. This study provides a novel prediction model to physiologically assess the likelihood of subjects with prior history of TBI to develop clinical TBI complications, such as AD.

Keywords: Alzheimer's disease, autonomic nervous system, biomarker, neurodegenerative disorders, risk factor, traumatic brain injury

*Correspondence to: Giulio Maria Pasinetti, MD, PhD, 1468 Madison Avenue, Annenberg Building, Room 20-02, New York, NY 10029, USA. Tel.: +1 212 241 7938; Fax: +1 212 876 9042; E-mail: giulio.pasinetti@mssm.edu.

INTRODUCTION

Various central nervous system structures affected in Alzheimer's disease (AD) are also implicated in autonomic nervous system regulation, and it has

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been hypothesized that the deficit in central cholinergic function observed in AD could likely lead to autonomic dysfunction [1–3]. Several feasible tests can be used in clinical practice for the assessment of parasympathetic and sympathetic functions, particularly in terms of cardiovascular autonomic modulation. Dysfunctions of the sympathetic nervous system (SNS) as well as an increased risk of developing neurodegenerative diseases, such as AD, post-trauma have both been implicated as risk factors in response to traumatic brain injury (TBI) [4–8].

Because evidence suggests a definite presence of autonomic dysfunction in dementia at various degrees, a method to assess TBI by monitoring of the autonomic nervous system (ANS) would allow for the detection and possible development of interventions, should TBI be identified to be part of the clinical history, of any subject at risk for developing AD. The SNS, together with the parasympathetic nervous system (PNS), form the ANS which is charged with unconsciously regulating internal organs and bodily functions. The terms paroxysmal sympathetic storm and sympathetic storming have been used to describe the presence of overly active sympathetic signaling after a severe TBI [5]. This sympathetic hyperactivity has been associated with pupil dilation, tachycardia, and hypertension [5]. On top of these symptoms, several other physical symptoms are known to present in an individual who has experienced a TBI [9]. Physical symptoms include, but are not limited to, those listed in Table 1.

In addition to these symptoms, an increase in sympathetic firing also leads to an increase in muscular tonicity, and therefore disproportionate muscular development and body positioning. This phenomenon is linked to the excitatory: inhibitory ratio (EIR) model, where the brain stem EIR is overwhelmed by excessive brain activity causing the spinal EIR to become overwhelmed as well and lose its protective effect, making it unable to balance and/or inhibit the increased catecholamine circulated to peripheral organs. This manifests in end-organ dysautonomia of various end organs, including smooth muscle (rigidity) or cardiac muscle (tachycardia) [10]. These phenomena affect how the body looks during a physical examination.

In cases where a TBI has occurred that only affects one side of the head and brain, a hemispheric presentation can occur where a change in physical input and output develop ipsilaterally [11]. This ipsilateral hemisphericity can present as a change in muscle tone and limb angulation on only one side of the body. In addition to head trauma through contact, TBI can occur through non-impact trauma such as a blast or explosion. The blast waves that impact the brain can cause physical changes as well. The presentations of this type of TBI include symptoms that have been addressed above, such as vertigo and motion intolerance [12].

There are many theories regarding the underlying molecular mechanisms behind sympathetic storming. Nonetheless, the physical manifestation appears to result from an exaggeration of the SNS with a diminished PNS response or a disruption in communication within the two ANS components [13]. It is likely that such TBI-mediated ANS dysfunction may contribute to some of the TBI clinical phenotypes over shorter or longer terms. These long-term effects of trauma-induced brain damage are of increasing concern to a large portion of the population, from athletes, military personnel, to younger members of society who are physically active and at risk for head injury. Therefore, novel approaches that can identify TBI patients would also allow for an increased opportunity for future preventative therapeutic interventions for AD and other forms of dementia, which those with a history of TBI may be at risk of developing. In this study we therefore present a novel predictive model to assess patients' risk of developing clinical complications in the future, based off of prior evidence that TBI may lead to changes in the ANS.

The protocol presented here provides a first step in the investigation of potential noninvasive TBI monitoring methods that can identify subjects who would be in need of more in-depth examinations in the long-term to monitor their risk of developing future

Table 1
TBI physical symptoms

Incontinence	Ataxia	Dementia
Headaches	Vomiting	Confusion
Drowsiness	Ipsilateral Pupil Dilation	Ipsilateral Hemiparesis
Contralateral Hemiparesis	Pain	Decreased Range of Motion
Peripheral nerve compression	Lymphedema	Hypertension
Vein Thrombosis	Anosmia	Vertigo
Nystagmus	Blurred vision	Spasticity

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neurological complications such as AD and other forms of dementia.

MATERIALS AND METHODS

We randomly selected and pulled de-identified medical records from 50 individuals seeking chiropractic therapy at PATCH Chiropractic offices, a large private chiropractic clinic that is located in Philadelphia, PA, who had been evaluated using the simple ANS evaluation protocol. From each of the cases, we extracted only information that was gathered by using the ANS evaluation protocol, as well as information on self-reported history of TBI exposure. Among the 50 randomly selected cases, self-reported TBI history was documented for 18 of the cases and was not documented for the remaining 32 cases. As we describe in more detail below, our ANS evaluation protocol is comprised of multiple components (Table 2). Using machine learning strategies, we assess whether information gathered from individual components (or a combination of components) of the ANS evaluation protocol may correctly segregate cases with or without a documented self-reported history of TBI exposure. Studies were approved by the Ethical Committee and Institutional Review Board at the Icahn School of Medicine at Mount Sinai.

ANS evaluation protocol

As indicated above, our ANS evaluation protocol is comprised of a simple set of self-report questionnaires and a simple set of physical examinations that are designed to address 12 general categories: posture, face-eye features, pupil reaction, vital measurements, breakdown of pursuits, optokinetic measures, vestibuloocular reflexes, pain sensation, sympathetic muscle tone, abdominal characteristics, balance while standing, and deep tendon reflexes (Table 2). For each of the general categories, multiple subcategories were evaluated (Table 2). For example, under the general category of "posture", subcategory assessments included characteristics pertaining to gait/sway, head tilt, heal perception, angulation of limbs, arm swing, and head rotations. In addition to these questionnaires, individual participants were subjected to simple physical examinations, including manual examination of joints/posture/range of motion/temperature, manual muscle examination for joint/motion/grade, objective observations of orthopedic characteristics, and deep tendon reflexes. Each of the physical examinations included multiple subcategories. As part of the examination for deep tendon reflexes in particular, the following were assessed: reflexes for biceps, triceps, thenar eminence, patellar, achilles, and blink reflex (BR). For example, the thenar test is when the thenar eminence is stroked briskly with a thin stick using moderate pressure, from proximal to distal locations. A positive response is considered if there is a single visible twitch of the ipsilateral mentalis muscle. Collectively, our ANS evaluation protocol encompasses 102 subcategories (Table 2), which we used as independent variables for segregating cases based on presence or absence of a documented history of TBI. For example, the discrete independent variable of convergence is tested when you have the patient follow an object, like the tip of a pen, with their eyes (Table 3). The pen is held about 1-2 feet away from the patient, placing the pen between the eyes, and then moved closer to the patient as they are instructed to maintain eye contact on the object. As the person follows the pen tip, it would make them move their eyes in a way that is similar to them crossing their eyes. The pen is moved all of the way in until both eyes stop moving inward and it is noted whether one eye keeps moving inward and the other one either stops or starts to act differently. The feature E/N (Table 3) is used to state if there are any abnormalities with how one eye moves compared to the other in this test, such as shaking or twitching. Upon review, E/N is used to indicate abnormal findings when testing independent variables, such as convergence, while on the other hand, blank or negative features are denoted to show when no abnormalities were found (Table 3).

Table 2
Summary of major categories and subcategories that were addressed by the simple autonomic nervous system evaluation protocol. Shown are the 12 major categories assessed by the protocol. The table also shows the number of minor categories that were monitored for each of the major categories. Collectively, the autonomic nervous system evaluation protocol assesses a total of 102 subcategories

Major Categories	# Subcategories (Independent variables)
Posture	7
Face-eyes	5
Pupil reaction	13
Vitals	11
Breakdown of pursuits	8
Optokinetic testing	7
Vestibuloocular reflex testing	4
Pain sensation	13
Muscle tone-sympathetics	4
Abdominal	4
Standing	12
Deep tendon reflex	14

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Table 3
Features that were defined based on discrete independent variables. Shown are 62 features that were defined as independent variables in machine learning strategies to segregate 49 cases based on the presence/absence of documented traumatic brain injury (TBI) history. ACC, accelerometer; L, left; R, right; BR, blink reflex; E/N, abnormality observed; neg. or blank, no abnormal observation; V:A R (or L) = 1 (or 2, or 3), width of the vein to width of the artery ratio in the right (or left) eye is 1:1 (or 1:2, or 1:3)

Gait-Sway = L	V:A L = 2 or 3	Crude Hearing = E/N	BR-L = absent
Gait-Sway = R	Abdominal = intact	Crude Hearing = blank	Thenar-R = 2 (in a 0–4 scale where 2 = normal)
Head Tilt = L	Abdominal = blank	Gillets+ = neg.	Thenar-R = blank
Head Tilt = R	Absent Bowel Sounds = present	Gillets+ = LorR	Thenar-L = 2 (in a 0–4 scale where 2 = normal)
Head Perception = ACC measurement	Absent Bowel Sounds = blank	Biceps-R = 2	Thenar-L = blank
Head Perception = blank	Tympany = none	Biceps-R = blank	Patellar-R = 2 (in a 0–4 scale where 2 = normal)
Angulation of Limbs = E/N	Tympany = blank	Biceps-L = 2 (in a 0–4 scale where 2 = normal)	Patellar-R = 1 (in a 0–4 scale where 2 = normal)
Angulation of Limbs = not E/N	Sway Eyes Closed = none	Biceps-L = blank	Patellar-L = 2 (in a 0–4 scale where 2 = normal)
Arm Swing = L	Sway Eyes Closed = L or R	Triceps-R = 2 (in a 0–4 scale where 2 = normal)	Patellar-L = 1 (in a 0–4 scale where 2 = normal)
Arm Swing = R	Neck = E/N	Triceps-R = blank	Achilles-R==2 (in a 0–4 scale where 2 = normal)
Head Rotation = E/N	Neck = absent of carotid bruit	Triceps-L = 2 (in a 0–4 scale where 2 = normal)	Achilles-R = 1 (in a 0–4 scale where 2 = normal)
Head Rotation = L or R	Temples = E/N	Triceps-L = blank	Achilles-L = 2 (in a 0–4 scale where 2 = normal)
V:A R = 1	Temples = blank	BR-R = normal	Achilles-L = 1 (in a 0–4 scale where 2 = normal)
V:A R = 2	Convergence = E/N	BR-R = blank	
V:A L = 1	Convergence = blank	BR-L = normal	

Data pre-processing

Information for some of the ANS evaluation subcategories was missing among the individual de-identified medical records we randomly retrieved. We therefore identified and removed subcategories for which information was missing among $\geq$80% ($\geq$40) of the 50 cases. Following these pre-processing steps, the dataset contained only 31 remaining ANS evaluation subcategories which we decided to use as independent variables to segregate cases based on the presence or absence of documented history of TBI. Moreover, we removed one case (a case with a self-reported TBI history) from the study entirely because information was missing for 84 of the 102 subcategories. Thus, we ended up using 31 ANS evaluation subcategories as independent variables in machine learning classification of 49 cases (18 cases with documented history of TBI and 31 cases without).

Case segregation using machine learning strategies

We tested three classification methods using de-identified medical record information generated by the ANS evaluation protocol to classify the 50 cases into two groups: the presence or absence of a documented history of TBI exposure. These three classification methods are (1) support vector machine (SVM), (2) random forest (RF), and (3) naive Bayes (NB), which we will discuss in more detail below.

Support vector machine

SVM [14] is a distinguished family of classification methods that focus on defining an optimal hyperplane to separate the samples of different classes. It adopts the "kernel trick" in hyperplane definition, and introduces the "margin concept" as an effective strategy to counter the overfitting problems suffered from by many earlier classification methods. In this study, we used the SVM methods implemented in the *e1071 Vector Machine Support Package* [15] in R, and selected the radial basis function (RBF) kernel, which has been shown to produce outstanding grouping performances in previous publications [16]. The RBF kernel function could be defined as:

$$K(x,\ y) = e^{|-y||x-y|^Z}$$

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Here, x and y are samples with gene expression and γ is the kernel parameter. In this study, the kernel parameter γ and SVM penalty parameter C were optimized by nested cross-validation over the γ value ($2^{-15}, 2^{-13}, \ldots, 2^{1}$) and C value ($2^{-5}, 2^{-3}, \ldots, 2^{15}$).

Random forest

RF [17] is a classification algorithm that uses an ensemble of unpruned decision trees, each of which is built on a bootstrap sample of the training data using a randomly selected subset of variables. RF uses both bagging (bootstrap aggregation) for combining unstable learners and random variable selection for tree building. RF has been successfully applied in classification problems of biological data, and has demonstrated excellent predicting performance even when feature variables were highly noisy [18]. We employed the RF implementation in the R package *randomForest* [19] in this study. There are three important parameters whose values need to be determined: mtry (the number of input variables tried at each split), ntree (the number of trees to grow for each forest), and node size (the minimum size of the terminal nodes). We considered different parameter configurations for their values of mtry = (0.5, 1, 2), ntree = (500, 1000, 2000), and node size = 1, as recommended in a previous study [20]. The best-performing parameters were selected by a 2-layer cross-validation scheme, which will be described in a later section.

Naive Bayes

NB [21] is a rule generator based on Bayes' rule of conditional probability. It uses all attributes and allows them to contribute to decision-making as if they were all equally important and independent of one another. We used the following conditional probability formula in which P(H/E) denotes the probability of event H (conditional on event E), and En denotes the nth attribute of the instance:

$$P(H|E) = \frac{P(H1|H).P(H2|H)\ldots P(En|H)}{P(E)}$$

The NB implementation provided in the e1071 package in R was used in this analysis, in which the parameter "Laplace" (positive double controlling Laplace smoothing) was set by default (disables Laplace smoothing).

Selection of most efficacious ANS evaluation features for classifying cases using machine learning strategies

Feature selection is a domain of active research in machine learning. Feature selection methods are developed with the observation that not all features are contributing to the predicting performance of the classification method. A properly designed feature selection method intelligently identifies a small subset of the features, which, in conjunction with a proper classification method, are more effective in distinguishing the samples with differing class labels. Generally, established feature selection methods can be broadly categorized into three types: (1) filter methods, (2) wrapper methods, and (3) embedded methods [22]. Filter methods usually rank the features based on univariate measures and select the most significant feature genes. Wrapper methods search for a subset that is found to be optimal with respect to a subset evaluator such as a classifier. Embedded methods incorporate the search algorithm into the classifier. In this study, we attempted three feature selection methods: the Chi-squared method, the correlation-based feature selection method, and the SVM-based recursive feature elimination method.

Chi-squared method

The Chi-squared method is a filter method. It evaluates features individually by measuring their chi-squared statistic with respect to the classes, and ranks all features. The gene expression values are first discretized into several intervals using an entropy-based discretization method, and the chi-square statistic of each feature is defined as follows:

$$X^2 = \sum_{i=1}^{m}\sum_{j=1}^{K} \frac{(A_i \cdot \frac{R_i \cdot c_j}{N})^2}{\frac{R_i \cdot c_j}{N}}$$

where m denotes the number of intervals; k is the number of classes; N is the total number of patterns; R_i represents the number of patterns in the ith interval; C_j is the number of patterns in the jth class; and A_i denotes the number of patterns in the ith class.

Correlation-based feature selection method (CFS)

The CFS method [23] is also a filter method. This method adopts a heuristic to measure the correlation between attributes and rewards that features subsets

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in which each feature is highly correlated with the class and uncorrelated with other subset features. Given a feature subset S consisting of k features, the merit of the subset is defined as:

$$\text{Merit}_{S_k} = \frac{k\,\overline{r_{cf}}}{\sqrt{K + k\,(k-1)\,\bar{r}_{ff}}}$$

where $\overline{r_{cf}}$ is the average value of all feature-classification correlations, and $\overline{r_{ff}}$ is the average value of all feature-feature correlations. The CFS criterion is defined as follows:

$$\text{CSF} = \frac{r_{cf1} + r_{cf2} + \ldots + r_{cfk}}{\sqrt{K + 2(r_{f1/f2} + \ldots + r_{fi\ fj} + r_{fk\ fl})}}$$

where the r_{cfi} and r_{fifj} are referred to as correlations.

SVM-based recursive feature elimination method (SVM-RFE)

SVM-RFE [18] (abbreviated as RFE in the remainder of this manuscript) is a wrapper method. It is an iterative algorithm that fits SVM classification models by discarding genes with small impacts on classification and selecting the smallest subset of genes that participate in the best performing classification model. The iterative procedure of this method is:

1. Train the SVM classifier.
2. Compute the ranking criterion for all features.
3. Remove the feature with the smallest ranking criterion.

Features were ranked by evaluating how well an individual feature contributes to the separation.

Ranking selected features using a two-layer cross-validation strategy

A five-fold cross-validation was used to estimate the performance of the classification algorithms (outer-loop). In order to find the best parameters, another 'nested' leave-one-out cross-validation was performed on the five original training sets (inner-loop). For each combination of the classifier parameters, we first obtained cross-validation performances and selected the best performing parameters inside this inner loop of cross-validation. Then we built a classification model with the best parameters on the original training set and applied this model to the original testing set.

This two-layer, nested cross-validation scheme was first proposed by Liu et al. [16] and it is an effective procedure that curbs the overfitting problem while objectively evaluating the performance of the classification models for unobserved data. The two-layer cross-validation implemented in this study was carried out with a locally developed R script.

Classification performance evaluation metrics

We used four classification performance metrics: accuracy, sensitivity, specificity, and Mathew's correlation coefficient (MCC). The formulas used to derive these values are:

$$\text{Accurancy} = \frac{(TP + TN)}{(TP + FN + FP + TN)}$$

$$\text{Sensitivity} = \frac{TP}{(TP + FN)}$$

$$\text{Specificity} = \frac{TN}{(FP + TN)}$$

$$\text{MCC} = \frac{TP \times TN - TP \times FN}{\sqrt{(TP + FP)(TP + FN)(TN + FP)(TN + FN)}}$$

where TP stands for true positive, TN for true negative, FP for false positive, and FN for false negative. Of these metrics, MCC is not used frequently outside the machine learning field. Comparing with accuracy, MCC is a more objective measure of the predicting performance when the sample sizes between classes are very unbalanced.

RESULTS

Our ANS evaluation protocol encompasses 102 subcategories (Table 2). However, information for some of the ANS evaluation subcategories was missing among individual's de-identified medical records we randomly retrieved. As we described in the Materials and Methods, we identified and removed subcategories for which information was missing among $\geq$80% of the cases. This resulted in 31 ANS evaluation subcategories as independent variables in machine learning classification of 49 cases (18 cases with documented history of TBI and 31 cases without).

Among the selected 31 ANS evaluation subcategories, 2 were continuous variables, and 29 were discrete variables. For the discrete variables, a commonly applied "sparse coding" scheme [16] was employed to define 2 features from each variable. For instance, for the discrete variable "Gait-Sway", which could take one of two possible values, "L"

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and "R", we defined two features, namely "Gait-Sway = L" and "Gait-Sway = R". The values for these two features were equal to 1 and 0, respectively, for participants whose "Gait-Sway" value was "L", and were 0 and 1, respectively, for participants whose "Gait-Sway" value was "R". A total of 58 features were defined based on these 29 discrete independent variables (Table 3). For the two continuous independent variables, "BP L" and "BP R" (which represent two sets of blood pressure readings, both high and low, taken from the left and right arms), we treated the four readings—high/L, low/L, high/R, and low/R—separately. For each reading, we scaled the values across all participants into the scale of [0,1]. An important consequence of this scaling is that the values of features, those generated from discrete variables and those generated from continuous variables, were precisely in the same range. Blank values were imputed using the mean value for all other participants. This procedure resulted in the definition of 4 additional features. In total, 62 features defined for the 49 remaining cases (18 cases with and 31 cases without a documented history of TBI exposure) were used for machine learning classification.

Performance prediction of three classification methods

The task of constructing a prediction model for a group classification problem and objectively evaluating its predicting performance for future untested samples, while still controlling for an overfitting problem, can conceptually be considered to comprise of the following components: (1) selecting a proper classification method, (2) selecting a proper feature selection method, (3) determining the proper number of features to include (for those feature selection methods that do not choose the "optimal" feature numbers automatically), (4) selecting proper model parameter values, and (5) evaluating prediction model performance. In practice, we carried out this task in a streamlined manner, where we examined a total of 9 classification methods: feature selection method combinations (3 classification methods and 3 feature selection methods)—integrating components (1) and (2) in a single step. In addition, we integrated components (4) and (5) together using a two-layer cross-validation scheme established by Liu et al. [16] and accomplished this (3) by monitoring changes of the model performance as the feature number decreased.

One of the three feature selection methods we included in this study, CFS, selects the "optimal" feature numbers automatically. The testing of model performance with decreased feature numbers was conducted for the other two feature selection methods, RFE and Chi-squared. We expected that as the number of features decreased, the model performance would show a biphasic characteristic. It would first increase, as the feature selection method eliminates those features that do not "help" the model performance. They are the features that add noise rather than contribute to the predicting accuracy of the model, thus elimination of these features would lead to improvement of the model performance. After all the "bad" features have been eliminated, the model performance then enters the second phase, where further removing features would result in degradation of the model performance. This is because at this time, all remaining features are contributing to the model performance, and removal of some of them would "hurt" the model. As shown in Fig. 1, despite considerable noise levels (which could be expected due to the relatively small number of samples included in this study), the biphasic characteristic was clearly visible for some of the classification methods, such as feature selection method combinations, including SVM-RFE (Fig. 1A), SVM:Chi-squared (Fig. 1B), and RF:Chi-squared (Fig. 1C). Some other classification methods, involving feature selection method combinations, in particular (NB:Chi-squared, Fig. 1D) showed a small downfall phase at the end of the curve, but lacked the uprising phase. Other classification methods of feature selection method combinations (NB:RFE, Fig. 1E, and RF:RFE, Fig. 1F) showed a complete absence of the biphasic characteristic, suggesting that the feature selection method did not work effectively in these cases.

Best predicting performance achieved

Table 4 summarizes the best predicting performance achieved for each of the 3 classification methods without feature selection, as well as in conjunction with each of the 3 feature selection methods. Among the combination of classification models and diverse feature selection approaches that were tested, SVM-RFE achieved the most impressive predicting performance: at 27 remaining features, it achieved an accuracy of 0.98, and MCC of 0.96, i.e., of the 49 participants, only 1 was misclassified in the cross-validation based testing (Table 4). A detailed list of

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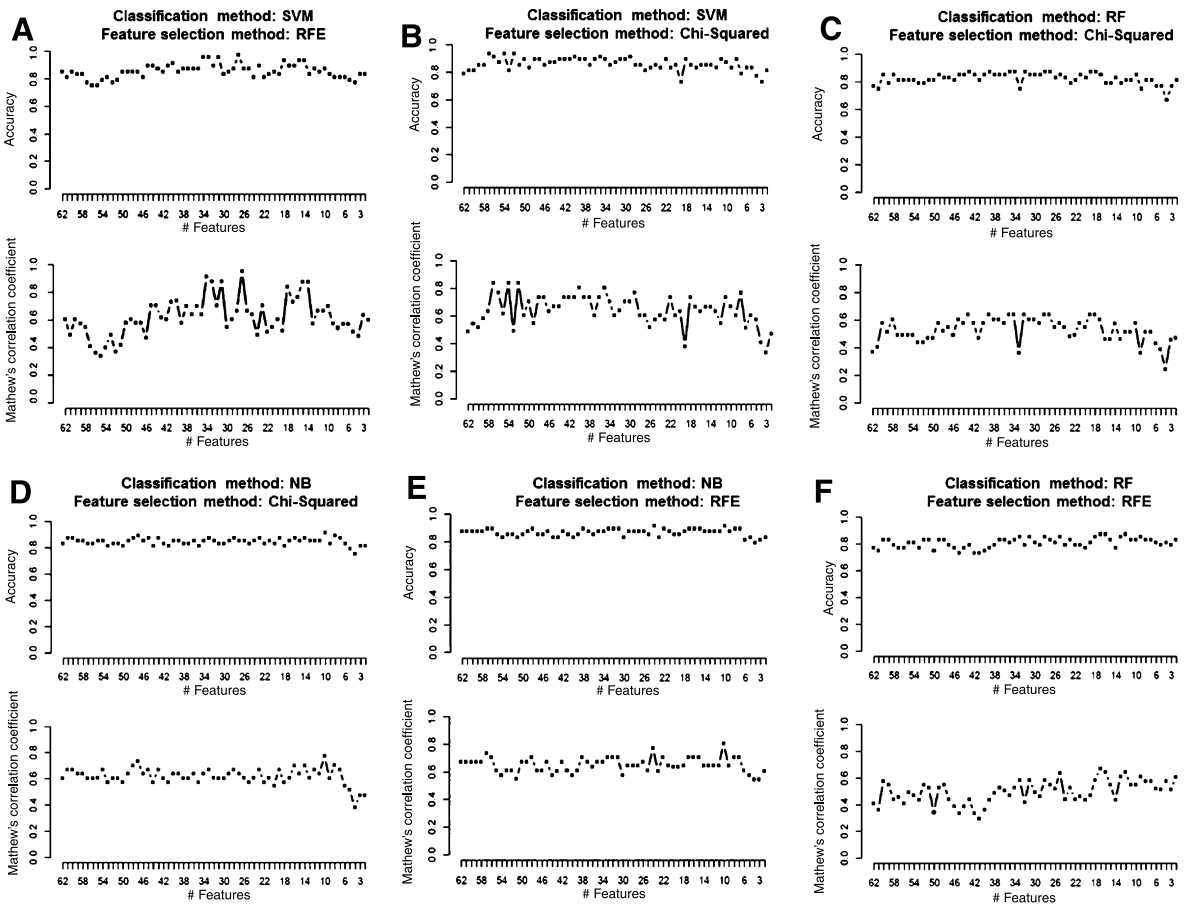

Fig. 1. Model performance changes with number of features remaining when using a combination of classification methods and feature selection methods. A-C) A biphasic characteristic is observed for some of the classification methods: feature selection method combinations including SVM-RFE (A), SVM:Chi-Squared (B), and RF:Chi-Squared (C). D) A classification:feature selection method combination using NB:Chi-Squared showed a small downfall phase at the end of the curve, but lacked the uprising phase. E,F) A classification:feature selection method combination using NB:RFE (E) and RF:RFE (F) showed a complete absence of the biphasic characteristic. For each of the classification:feature selection method combinations shown, the top panel depicts model accuracy and the bottom panel depicts Mathew's correlation coefficient. SVM, support vector machine; RFE, SVM-based recursive feature elimination method; RF, random forest; NB, naive Bayes.

the 27 features that were used in SVM-RFE is shown in Table 5.

Model validation

The data used in this study are slightly unbalanced, i.e., among the 49 participants, 18 were documented for a history of TBI exposure and 31 were not. For unbalanced datasets like this, MCC, instead of accuracy, would be a more proper performance metric to examine. However, for ease of explanation, we choose to focus on accuracy in the following discussion because of its simple and intuitive physical interpretation; the accuracy is essentially the proportion of cases whose group labels are correctly predicted. For instance, the best SVM-RFE model achieved an accuracy of 0.98, which means that of the 49 participants, $0.98 \times 49 = 48$ were correctly classified. One way to understand how good a constructed predicting model is, is by putting it into the context of comparison with a "random guessing" model; the latter can be regarded as equivalent to a "negative control" for researchers more familiar with experimental research. For an unbalanced dataset that consists of a 31:18 splitting of participants between groups, the best "random guessing" model would produce an accuracy of $31/(31+18) = 0.63$. Thus, a model of 0.98 accuracy represents a substantial improvement in predicting performance as compared to the "random guessing" model.

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Table 4
Best model performance achieved by each classification method with each corresponding feature selection method. CFS, correlation-based feature selection method; MCC, Mathew's correlation coefficient; NB, naive Bayes; RF, random forest; RFE, SVM-based recursive feature elimination method; SVM, support vector machine

Classifier	Feature selection	Sensitivity	Specificity	Accuracy	MCC	# Features
SVM	All Features	0.78	0.90	0.86	0.61	62
	RFE	1.00	0.97	0.98	0.96	27
	Chi-Squared	0.94	0.94	0.94	0.84	57
	CFS	0.78	0.77	0.78	0.49	4
RF	All Features	0.50	0.94	0.78	0.37	62
	RFE	0.83	0.90	0.88	0.67	17
	Chi-Squared	0.78	0.94	0.88	0.64	43
	CFS	0.56	0.90	0.78	0.39	4
NB	All Features	0.83	0.84	0.84	0.61	62
	RFE	0.94	0.90	0.92	0.81	10
	Chi-Squared	0.89	0.94	0.92	0.77	10
	CFS	0.72	0.90	0.84	0.55	4

Table 5
A listing of 27 features that supported the best predicting performance. Among all models, support vector machine-based recursive feature elimination method achieved the best prediction performance using these 27 features. ACC, accelerometer; L, left; R, right; BR, blink reflex; E/N, abnormality observed; blank, no abnormal observation; V:A R (or L) = 1 (or 2, or 3), width of the vein to width of the artery ratio in the right (or left) eye is 1:1 (or 1:2, or 1:3); triceps (or biceps, or patellar)-L = 2 (normal in a scale of 0–4); BP R/high, blood pressure from the right arm/high readings

Gait-Sway = L	Tympany = none	Head Perception = blank
Head Tilt = R	Tympany = blank	Triceps-L = 2
V:A L = 2 or 3	Sway Eyes Closed = none	Abdominal = intact
Head Rotation = E/N	Absent Bowel Sounds = present	Biceps-L = blank
Head rotation = L or R	Absent Bowel Sounds = blank	Patellar-L = 2
Gillets = L or R	BR = L = 2	BP R/high
Convergence = not E/N	BR-L = blank	Head Perception = ACC measurement
Head Tilt = L	Triceps-L = blank	
V:A R = 2	Biceps-L = 2	
Sway Eyes Closed = L or R	Abdominal = blank	

DISCUSSION

There is large evidence associating ANS perturbations to AD as well as ANS perturbations to TBI [1, 8, 24–29]. However, despite the large body of literature suggesting that TBI might pose a risk for accelerating the onset and possibly the progression of AD dementia [6, 7, 30, 31], little is known about ANS dysfunctions as a potential link associating the two conditions. Our present study was designed to investigate a readily-available ANS evaluation protocol as a way to identify and eventually monitor subjects at risk for TBI complications.

Initially, when a subject presents with short-term physical complications post-TBI, there are different presentations which can be bilateral or ipsilateral in nature. If a subject presents with greater sympathetic muscular tone on one side of their body, or with bad balance on the contralateral side of their body due to nerves crossing at the cerebellum, further investigation would be performed on the side of the muscular imbalances. If there are random presentations on both sides of the body, it is customary to then search for a more "centralized" area of TBI. For any subject presenting with an abnormality in sympathetic tone, a battery of tests would be conducted in order to rule in/out the likelihood of TBI on either side of the brain. The more positive the results that are returned from an examination, the more "weight" would be put into a TBI diagnosis, subsequently signaling the need for further testing at the very least. The protocol presented here therefore provides a simple first step in the investigation of potential TBI identification methods and, as such, will be an invaluable tool in ascertaining subjects who would be in need of more in-depth examinations in the long-term, to monitor their risk of developing future cognitive complications.

Evidence from our study supports the feasibility that our simple ANS evaluation protocol may reflect a history of TBI exposure, thereby identifying those at risk of developing long-term, detrimental brain responses later in life caused by head injury, such as

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AD. In particular, we identified a prediction model, established on the application of the SVM-based recursive feature elimination (SVM-RFE) method in the analysis of a subset of 27 select features from the ANS evaluation, which is effective in correctly segregating cases with or without a documented history of TBI exposure with an accuracy of 0.98, and an MCC of 0.96. With the implications of TBI in the etiopathology of neurodegenerative disorders such as AD [6, 32, 33], the long-term effects of trauma-induced brain damage is of increasing concern to a large portion of the population. Therefore, the ability of this novel prediction model to identify subjects with prior TBI history will allow for an increased opportunity for future preventative therapeutic interventions in neurodegenerative disorders and age-related cognitive decline.

We note that the model performance evaluations made in this study were conducted in a 2-layer cross-validation setting, thus they are objective evaluations of the model performance when they are tested on unknown future samples, as long as those future unknown samples are assumed to be taken from the same population of samples as were the ones used in our model construction work—the weakest assumption that can be made in this type of study.

The performance measurements obtained in this study should be understood in lieu of the high noise levels in the plots presented in Fig. 1 (which was expected because of the small sample number included in this study). Although at 27 remaining features, an accuracy of 0.98 was achieved. It is an overstatement that when tested using future samples, the model is expected to consistently achieve 0.98 accuracy. Following the smoothing of the curves, it appears that accuracy around the level of 0.90 would be more realistically achievable for future samples possessing the same population characteristics as the samples used in model construction.

Collectively, results from our present study demonstrating the efficacy of our prediction model, based on SVM-RFE analysis of select features from the ANS evaluation in correctly differentiating between cases with or without a documented TBI history, are consistent with the hypothesis that trauma-induced ANS dysfunctions may contribute to clinical TBI features. Our ANS evaluation protocol is designed as a simple assessment approach for potential disturbances in the ANS to identify and monitor those with a prior record of TBI. The study will therefore provide a novel prediction model as a readily applicable physiological assessment of the likelihood of experiencing potentially negative impacts of prior TBI injury in subjects who may also be at risk of developing AD and other forms of dementia.

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Self-Reported Traumatic Brain Injury and Mild Cognitive Impairment: Increased Risk and Earlier Age of Diagnosis

Christian LoBue[a,*], David Denney[a], Linda S. Hynan[a,b], Heidi C. Rossetti[a], Laura H. Lacritz[a,c], John Hart Jr.[a,c,d], Kyle B. Womack[a,c,d], Fu L. Woon[a] and C. Munro Cullum[a,c]
[a]*Department of Psychiatry, University of Texas Southwestern Medical Center, Dallas, TX, USA*
[b]*Department of Clinical Sciences, University of Texas Southwestern Medical Center, Dallas, TX, USA*
[c]*Department of Neurology and Neurotherapeutics, University of Texas Southwestern Medical Center, Dallas, TX, USA*
[d]*Center for BrainHealth, School of Behavioral and Brain Sciences, University of Texas at Dallas, Dallas, TX, USA*

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Abstract. This study examined whether history of traumatic brain injury (TBI) is associated with increased risk and earlier onset of mild cognitive impairment (MCI). Subjects with MCI ($n = 3{,}187$) and normal cognition ($n = 3{,}244$) were obtained from the National Alzheimer's Coordinating Center database. TBI was categorized based on lifetime reported TBI with loss of consciousness (LOC) without chronic deficit. Logistic regression was used to examine TBI history as a predictor of MCI, adjusted for demographics, apolipoprotein E-ε4 (ApoE4), a composite vascular risk score, and history of psychiatric factors. ANCOVA was used to examine whether age at MCI diagnosis and estimated age of onset differed between those with (TBI+) and without (TBI–) a history of TBI. TBI history was a significant predictor ($p < 0.01$) and associated with increased odds of MCI diagnosis in unadjusted (OR = 1.25; 95% CI = 1.05–1.49) and adjusted models, accounting for age, education, ApoE4, and a composite vascular score (OR = 1.32; 95% CI = 1.10–1.58). This association, however, was largely attenuated (OR = 1.14; 95% CI = 0.94–1.37; $p = 0.18$) after adjustment for reported history of depression. MCI was diagnosed a mean of 2.3 years earlier ($p < 0.001$) in the TBI+ group, and although TBI+ subjects had an estimated mean of decline 1.7 years earlier, clinician-estimated age of onset failed to differ ($p = 0.13$) when gender and psychiatric factors were controlled. This is the first report of a possible role for TBI as a risk factor in MCI, but its association may be related to other factors such as gender and depression and requires further investigation.

Keywords: Age of onset, mild cognitive impairment, risk factors, traumatic brain injury

INTRODUCTION

Each year, approximately 1.7 million persons sustain a traumatic brain injury (TBI) [1]. While many survive the initial insult, cognitive, psychiatric, and physiological dysfunction often follows and may be transient or persistent, depending in part on injury severity [2]. Whether full recovery is achieved or not, sustaining a TBI has been implicated as a risk factor for later development of neurodegenerative disorders [3], including Alzheimer's disease (AD) [3, 4], with evidence demonstrating that risk increases with greater length of loss of consciousness [5, 6]

*Correspondence to: Christian LoBue, MS, 5323 Harry Hines Blvd., Dallas, TX 75208-8564, USA. Tel.: +1 214 422 3760; Fax: +1 214 648 5297; E-mail: christian.lobue@utsouthwestern.edu.

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and severity [7]. In addition, repetitive mild TBIs have been controversially linked to chronic traumatic encephalopathy [8, 9], and while this link remains in question, recent evidence has shown that mild TBI may invoke a higher risk for dementia [10].

The possible mechanism(s) associated with TBI in early or midlife and later development of AD remains unclear, but it is hypothesized that TBI activates a neurodegenerative process [11] which may interact with age and other factors over time. Nearly all severities of closed head injuries consist of damage to white matter tracts, i.e., diffuse axonal injury, which acutely disrupt biochemical and cytoskeletal functions that may contribute to long-term damage to neurons and/or neuronal transmission [12]. As a consequence of diffuse axonal injury, amyloid-β protein precursors are suspected of accumulating in the axon and forming amyloid-β (Aβ) plaques [12], while tau proteins aggregate into neurofibrillary tangles (NFT) [13], both believed to be involved in the pathogenesis of AD [14]. Previous studies have found that within hours of acquiring a single, severe TBI, Aβ plaques are present in up to a third of severely injured patients, even children [15]. It has also been noted that at autopsy, several years after the initial injury a significant proportion of TBI patients had Aβ plaques present that were fibrillary or a mixed diffuse/fibrillary pattern, unlike the predominantly diffuse plaques present in normal controls [16]. With respect to tau pathology, NFT appearance is seemingly delayed, as prior studies report that NFTs were not present within 4 weeks following a single, severe TBI [17] but were present when patients were examined after at least 1 year post-injury [16]. Additionally, persons who had sustained even a single TBI in that sample had more extensive NFTs and at a much earlier age relative to age-matched controls [16]. Such findings support the notion of TBI as a potential risk factor in the neurodegenerative process; however, a direct link to AD continues to be lacking as the long-term development of AD-like Aβ plaques remains to be studied in humans and the interactive effects with normal aging are poorly understood.

Mild cognitive impairment (MCI) is often a prodromal stage of AD [18]. While previous research has emphasized the relationship between a history of TBI and AD [6, 19, 20], little is known about the potential impact of TBI on the development of MCI. For example, Guskiewicz et al. reported a relationship between MCI symptoms and a history of three or more concussions in a small sample of retired athletes [21]. In addition, a recent finding showed that in a small sample of individuals diagnosed with MCI, those with a history of TBI and loss of consciousness showed greater amyloid deposition than those without, suggesting TBI may be associated with AD-related neuropathology [22]. Similarly, in a meta-analysis of 15 studies examining history of TBI as a risk factor for the later development of AD, Fleminger et al. found an odds ratio of 1.58, suggesting an increased risk, although the study is rather dated and does not include findings published over the last 12 years [6]. Furthermore, an earlier age of onset has been observed in some studies of patients with dementia [23], but these results have varied widely, ranging from 6 months to 8 years [3, 20, 24]. It is not known whether an increased risk for cognitive decline or an earlier age of onset may also be associated with MCI.

The current study sought to examine whether a history of TBI was a significant predictor of MCI diagnosis and was associated with increased risk for MCI. In addition, we assessed whether a reported history of TBI was linked with an earlier age of MCI diagnosis and clinician-estimated age of onset. Determining whether TBI is a risk factor for MCI and is associated with an earlier age of onset would support an emerging literature that remote TBI may be associated with later cognitive decline in some individuals.

MATERIALS AND METHODS

The National Alzheimer's Coordinating Center (NACC) has maintained a centralized database of demographic and clinical information pooled from National Institute of Aging (NIA) - funded Alzheimer's Disease Centers (ADC) across the U.S. since September 2005. For this study, the NACC Uniform Data Set (UDS) [25] was queried for subjects aged 50 years or older with initial and follow-up visits completed between September 2005 and December 2013. Subjects diagnosed with amnestic or non-amnestic MCI ($n = 3{,}187$) at the initial visit were selected for examination. Subjects with normal cognition were selected as a comparison group; however, in order to account for the possibility that some subjects may have progressed to MCI/dementia beyond the initial visit, only subjects with three or more visits completed ($n = 3{,}244$) were included. Cognitive status and clinical diagnosis was determined by ADC clinicians using NINCDS/ADRDA guidelines.

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A clinical diagnosis of amnestic or nonamnestic MCI was made if there was impairment in memory or another cognitive domain (i.e., language, attention, executive function, visuospatial) and criteria for dementia were not met. Normal cognition was defined as the lack of neurocognitive impairment and failure to meet published clinical criteria of MCI, dementia, or other neurological conditions. For the present study, only data obtained at the initial ADC visit were examined, as follows: age, education, gender, race, number of apolipoprotein E-ε4 (ApoE4) alleles, and cigarette smoking history (number of years smoked and average number of packs/day). Age at initial visit corresponded with age of diagnosis for MCI subjects. In addition, clinician-estimated age of onset was examined, and was determined by ADC clinicians via subject/informant report for when cognitive abilities began declining.

The NACC database contains three questions related to TBI based on subject/informant interview. Subjects were asked if they ever sustained a TBI resulting in a) <5 min loss of consciousness (mLOC), b) ≥5 mLOC, and c) if they were left with a chronic deficit/dysfunction as a result of the injury. Each question was answered as *absent*, *recent/active* (defined as occurring within 1 year of visit or currently requiring treatment), *remote/inactive* (defined as occurring >1 year before visit and either having recovered from the injury or no current treatment is underway), or *unknown*. As the present study sought to examine the effect of a remote history of TBI on MCI diagnosis, only those individuals who reported either an absence of TBI history or a remote (defined as >1 year of visit) TBI with LOC of any duration and no chronic deficits were included. We attempted to limit potential confounding for cognitive decline that may be directly attributable to a TBI by excluding subjects who reported any history of TBI resulting in chronic deficit/dysfunction ($n = 16$) or a *recent/active* TBI ($n = 65$).

In order to examine the potential influence of vascular risk factors, a history of the following conditions was obtained via self/informant report and coded as absent or present: heart attack, atrial fibrillation, angioplasty, cardiac bypass, pacemaker, congestive heart failure, stroke, transient ischemic attack, hypertension, hypercholesterolemia, and diabetes. Possessing multiple cardiovascular or cerebrovascular conditions has been associated with increased risk for AD [26], and the use of composite vascular risk scores has proven useful in assessing the additive implications of multiple vascular factors on cognitive impairment [27]. As such, a composite vascular risk score was created from the above conditions to control for the potential influence in MCI. The number of above conditions was totaled to create a composite vascular risk score ranging from 0 to 11, with 11 indicating the highest risk.

NACC also contains some limited information about histories of depression and substance abuse as obtained via subject/informant report. Subjects are asked if they ever a) had a history of depression occurring >2 years prior to the initial visit and b) had clinically significant impairment in occupational, social, or legal activities due to alcohol (i.e., alcohol abuse) or drug use (i.e., drug abuse). Episodes of depression were coded as *absent* or *present* and defined as *consulting a clinician about depressed mood, taking antidepressant medication, or receiving a mood disorder diagnosis (i.e., major depression, dysthymia, bipolar*). Like the TBI information, the alcohol and drug abuse questions were coded as *absent*, *recent/active*, *remote/inactive*, or unknown; only *remote/inactive* histories of alcohol and drug abuse were examined.

All analyses were conducted using IBM© SPSS Statistics V22 (IBM Corp, SPSS Statistics V22, Armonk, NY, 2013) with $p < 0.05$ as the cutoff for significance. Four logistic regression models were used, serially controlling for different factors linked to cognitive decline, to examine the utility of history of TBI as a predictor of a diagnosis of MCI (versus normal cognition). In the first model (unadjusted), TBI was the only predictor. A second, adjusted model included history of TBI and six other covariates: age, years of education, number of ApoE4 alleles, cigarette smoking history (number of years smoked and average number of packs/day), and composite vascular risk score. Covariates remained in the model if $p < 0.15$. This was to include variables related to a MCI diagnosis, but to eliminate measures that potentially reduced the fit of the models. A third, adjusted model additionally included gender and race as covariates. A fourth model included all of the significant variables in previous models plus psychiatric conditions (i.e., histories of depression, alcohol abuse, and drug abuse).

The Hosmer-Lemeshow statistic was used to examine the fit of the models to the data. The criterion of an acceptable fit was defined as $p > 0.10$ and a good fit as $p > 0.40$ [28]. Receiver operating characteristic (ROC) analyses compared the area under the curve between pairwise models using

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the predicted scores from each logistic regression model. Using the ROC results, the best cut-score for group predictions was determined using a combination of criteria: sensitivity, specificity, accuracy, and maximum perpendicular distance above the 45° line of equality [29]. The added predictive value of the adjusted models was evaluated using Net Reclassification Improvement (NRI) analyses based on the ROC cut-scores [30]. This method was used to determine whether models with additional predictors significantly improved classification accuracy for MCI diagnosis versus normal cognition.

Two ANCOVAs were performed to examine if age at diagnosis of MCI and estimated age of cognitive decline onset were different between those with a self-reported history of TBI (TBI+) and those without (TBI–). Demographic characteristics, number of ApoE4 alleles, cigarette smoking history (number of years smoked and average number of packs/day), vascular risk factors, and histories of psychiatric conditions were compared between the TBI+ and TBI– groups using chi-square or independent samples *t*-tests. History of drug abuse was not compared due to the limited number of TBI cases reporting a positive history ($n = 3$). Because history of TBI is associated with later development of psychiatric conditions, especially depression [31], variables that differed between the groups were entered as covariates in a stepwise process, first controlling for relevant demographics and secondly, demographics plus medical/psychiatric factors. A Bonferroni correction of $p < 0.016$ was used to account for multiple comparisons.

Assumptions for all tests were reviewed, and unequal variances were observed between the TBI+ and TBI– groups when comparing age of MCI diagnosis. A Welch ANOVA was used in order to determine if unequal variances resulted in a Type I error.

RESULTS

The MCI cohort included 3,187 subjects that were 51% female, well educated ($M_{Educ} = 15.0$, $SD = 3.3$), had a mean age of 74.4 years ($SD = 8.9$), were 84% Caucasian, and possessed approximately two vascular-related conditions on average (Median = 2.0, $SD = 1.5$, range = 0–10). History of TBI with LOC was reported in 10% of those with MCI. Forty-four percent of MCI subjects had no ApoE4 allele, 36% had only one ApoE4 allele, and 8% had two ApoE4 alleles. There were 3,244 normal cognition subjects who, like the MCI cohort, were 86% Caucasian, well educated ($M_{Educ} = 15.7$, $SD = 2.8$), and possessed approximately two vascular-related conditions on average (Median = 1.0, $SD = 1.3$, range = 0–9). The normal cognition subjects had a higher proportion of women (68%) and were slightly younger (mean age = 72.3 years, $SD = 8.9$) at the initial visit. History of TBI with LOC was reported in 8% of normal cognition subjects, 72% had no ApoE4 allele, 26% had only one ApoE4 allele, and 2% had two ApoE4 alleles. Demographic and psychiatric characteristics for the study samples can be found in Table 1.

Odds ratios, 95% confidence intervals for odds ratios, and characteristics for each logistic regression model can be found in Table 2.

Logistic regression for the unadjusted model showed that a history of TBI alone was a significant predictor for MCI diagnosis (OR = 1.25; 95% CI = 1.07–1.51; $p = 0.01$) However, ROC analysis revealed that TBI history alone did not discriminate between the two groups, as the area under the ROC curve (AUC) was 0.51 and non-significant ($p = 0.22$). In the second logistic regression model, age, education, number of ApoE4 alleles, composite vascular score, and history of TBI (OR = 1.32; 95% CI = 1.10–1.58) were all significant predictors (p's < 0.01) of diagnosis. Cigarette smoking history (number of years smoked and average number of packs/day) was removed from the model due to the degree of non-significant prediction (p's = 0.75 and 0.81, respectively). When gender and race were included in the third model, there was only a trend for history of TBI (OR = 1.19; 95% CI = 0.99–1.43; $p = 0.07$;), while gender, race, and all of the previous covariates were significant predictors (p's < 0.001) of diagnosis. Further, history of TBI was a non-significant predictor of diagnosis in the fully adjusted model (OR = 1.14; 95% CI = 0.94–1.37; $p = 0.18$), while history of depression was a significant predictor along with gender, race, age, education, ApoE4 alleles, and a composite vascular score (p's < 0.001). Because of the degree of non-significant predictions for histories of alcohol and drug abuse (p's = 0.34 and 0.86, respectively), these variables were removed from the model. The Hosmer-Lemeshow statistic indicated that all of the adjusted models were an acceptable fit to the data (p's ≥ 0.11; See Table 2). In addition, ROC results showed that for each of the adjusted models, AUCs were ≥0.66 and significantly (p's < 0.001) discriminated MCI from normal

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Table 1
Demographics and medical history of MCI and normal cognition groups

	MCI ($n = 3,187$)	Normal Cognition ($n = 3,244$)
Age, *M (SD)*	74.4 (8.9)	72.3 (8.9)
Education, *M (SD)*	15.0 (3.3)	15.7 (2.8)
% Female	51	68
% Caucasian	83	86
% APOE ε4 alleles		
0 ε4 alleles	56	72
1 ε4 allele	36	25
2 ε4 alleles	8	2
Composite Vascular Risk Score, *M (SD)*	1.7 (1.4)	1.3 (1.2)
% History of Vascular Conditions		
Heart Attack	7	4
Atrial Fibrillation	8	6
Angioplasty	8	5
Cardiac Bypass	6	3
Pacemaker	3	2
Congestive Heart Failure	3	2
Stroke	5	2
Transient Ischemic Attack	6	3
Hypertension	55	47
Hypercholesterolemia	55	46
Diabetes	14	9
Number of Years Smoking Cigarettes, *M (SD)*	10.3 (15.3)	10.1 (15.1)
Average Number of Cigarette Packs/Day		
% No Smoking History	56	55
% <Half Pack	14	16
% Half to 1 Pack	14	14
% 1 to 1.5 Packs	8	8
% 1.5 to 2 Packs	4	4
% ≥2 Packs	4	3
% History of Depression	18	15
% History of Alcohol Abuse	4	2
% History of Other Substance Abuse	1	1
% History of TBI	10	8

MCI, mild cognitive impairment; TBI, traumatic brain injury with loss of consciousness; APOE ε4, apolipoprotein E-ε4.

cognition subjects. Using the cut scores for the adjusted models (Model 2 = 0.486; Model 3 = 0.513; Model 4 = 0.495), the contribution of all the predictors included in each model provided sensitivities ≥58%, specificities ≥63%, and accuracies of ≥62%. NRI analyses revealed that classification accuracies significantly improved when model predictions accounted for additional predictors [Model 1 versus 2, NRI = 0.23; Model 2 versus 3, NRI = 0.03; Model 3 versus Model 4, NRI = 0.01; p's ≤ 0.04], indicating that the fully adjusted model had the greatest classification accuracy.

TBI+ and TBI– groups did not differ by race ($p = 0.29$), education ($p = 0.86$), number of years smoking cigarettes ($p = 0.26$), average number of cigarette packs/day ($p = 0.06$), average composite vascular risk score ($p = 0.70$), or number of ApoE4 alleles ($p = 0.22$; see Table 3).

Because the TBI– group had a larger proportion of females (52% versus 38%) and fewer subjects without a history of depression (17% versus 29%) and alcohol abuse (3% versus 9%) than the TBI+ group (p's < 0.001), gender and psychiatric conditions were used as covariates. Controlling for gender only, the TBI+ group ($M_{Age} = 72.2$, SD = 10.0) was diagnosed with MCI on average 2.3 years earlier ($p < 0.001$) than the TBI– group ($M_{Age} = 74.5$, SD = 9.1). When controlling for gender plus psychiatric conditions (depression and alcohol abuse), the difference remained statistically significant ($p = 0.001$). Based on Levene's test (p's ≤ 0.01), the two groups had significantly unequal variances and a Welch's ANOVA was conducted to account for this discrepancy. Results from Welch's ANOVA showed that despite the unequal variances among the two groups, the TBI+ group was diagnosed with MCI

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significantly earlier ($p < 0.001$) than the TBI– group. Clinician-estimated age of cognitive decline, controlling for gender, was on average 1.7 years earlier for the TBI+ group ($M_{Age} = 68.0$, $SD = 12.4$) compared to the TBI– group ($M_{Age} = 69.7$, $SD = 13.7$), although this was only a trend ($p = 0.05$). When gender and psychiatric conditions were controlled, estimated age of cognitive decline failed to differ between the TBI+ and TBI– groups ($p = 0.13$).

DISCUSSION

A history of TBI with LOC was initially associated with increased odds of a diagnosis of MCI after adjusting for several variables that may have a causal link with cognitive decline; however, this risk was attenuated by history of depression and, to a lesser extent, basic sociodemographic factors (i.e., gender and race). Overall risk for MCI in the present study was greatest when individuals were older, less educated, from a racial background other than Caucasian/African American, ApoE4 carriers, had several lifetime vascular conditions, and had a history of depression. While previous studies have shown depression symptoms in early- and late- life to be associated with increased risk of cognitive decline and dementia [32], the nature of this relationship is unclear. Approximately 24% of TBI patients in general are found to later develop depression [31], and although a larger percentage of our TBI+ sample (29%) reported a depression history relative to the TBI- group (17%), we do not know if the depression was directly attributable, unrelated, or preceded the injury, since this information is based on a single question (i.e., presence/absence of depression episodes prior to 2 years of visit). In addition, depression symptoms are common in patients diagnosed with MCI and dementia (20–30%) [33, 34]. Thus, whether or not depression is caused by other risk factors, a direct result of cognitive decline, the earliest symptoms of neurodegeneration, or is a true risk factor for cognitive decline is unclear and remains an area of investigation [32].

Age of diagnosis was found to be 2.3 years earlier for those with a TBI history among our MCI sample, and while clinician-estimated age of onset was similarly observed to be 1.7 years earlier for the TBI+ group, psychiatric comorbidities attenuated this trend. Late-life depression has been found to be associated with earlier onset of dementia [35], but the reasons for this association remain unclear, as discussed above. Therefore, the overall findings indicate that the risk for MCI that was associated with a history of TBI with LOC may be related to other factors such as gender and depression, though further investigation is needed, as the implications of depression in this equation are unclear and cannot be explored further in the NACC dataset.

Given that MCI is considered a prodromal phase of AD and dementia in general, one would anticipate that TBI history has a similar risk for and association with both MCI and dementia. We did find that the level of risk for MCI associated with a history of TBI was comparable to dementia findings (95% CI odds ratios) [6, 7] prior to adjustment for depression and basic sociodemographic factors (i.e., gender and race). Interestingly, many previous dementia studies have not taken psychiatric comorbidities into account when examining TBI as a risk factor [5–7], suggesting that methodological differences across studies may account for mixed findings. In addition, among previous studies reporting on the association between TBI and earlier dementia onset, limited demographic factors were taken into account, and some reported no association [7, 36], while others found that those with a history of TBI developed dementia approximately 6 months [20, 37] or 8 years [24] earlier than those without. However, a recent investigation included a reasonably large autopsy-confirmed AD sample with a history of TBI ($n = 197$) and found that those with TBI developed dementia 2-3 years earlier than those without [38], similar to our findings in MCI, even after accounting for demographic and psychiatric factors such as depression. Furthermore, in an MCI cohort with and without a history of TBI with LOC, TBI+ individuals did not show faster progression to AD, or a greater rate of cognitive decline compared to those without a TBI history [39], similar to reports in AD [40]. It follows then that methodological differences in the literature likely account for a great deal of the mixed findings, including limited cases, reliance on medical records, different criteria for TBI classification, variation in ages at injury, variation in confounding factors, and dissimilar follow-up intervals. Nonetheless, a TBI history appears to confer similar risk for age of onset and course in MCI and dementia overall.

Several variables related to head injury appear to be important in the associated risk for MCI and dementia. In one small investigation, more severe TBI (i.e., extended LOC or posttraumatic amnesia >24 h) was associated with a 4.5 time higher risk for AD, while moderate TBI (LOC or posttraumatic

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Table 2
Odds ratios, confidence intervals, and fit statistics for logistic regression models plus results from receiver operating characteristics using model predictions

	OR	95% CI for OR	*p*-value	HL p	AUC	SS%/SP%	Accuracy %
Unadjusted Model				–	*0.51*		
TBI history*	1.25	1.05–1.49	0.01*				
Adjusted, Model 2				0.13	0.66	62/63	62
Age*	1.03	1.02–1.03	<0.001*				
Education*	0.94	0.92–0.96	<0.001*				
Composite vascular risk score*	1.20	1.15–1.24	<0.001*				
APOE ε4 alleles							
1 ε4 allele*	1.93	1.72–2.16	<0.001*				
2 ε4 alleles*	5.26	4.00–6.91	<0.001*				
TBI history*	1.32	1.10–1.58	0.003*				
Adjusted, Model 3				0.16	0.69	58/70	64
Age*	1.03	1.02–1.03	<0.001*				
Education*	0.92	0.91–0.94	<0.001*				
Composite vascular risk score*	1.16	1.11–1.20	<0.001*				
APOE ε4 alleles							
1 ε4 allele*	1.94	1.73–2.17	<0.001*				
2 ε4 alleles*	5.28	4.00–6.97	<0.001*				
Gender, Female*	0.47	0.42–0.52	<0.001*				
Race							
African American	1.11	0.95–1.31	0.19				
Other, Non-white*	2.10	1.50–2.94	<0.001*				
TBI history**	1.19	0.99–1.43	0.07**				
Adjusted, Model 4				0.11	0.69	62/67	65
Age*	1.03	1.02–1.04	<0.001*				
Education*	0.92	0.91–0.94	<0.001*				
Composite vascular risk score*	1.15	1.11–1.19	<0.001*				
APOE ε4 alleles							
1 ε4 allele*	1.95	1.74–2.18	<0.001*				
2 ε4 alleles*	5.39	4.08–7.11	<0.001*				
Gender, Female*	0.45	0.41–0.51	<0.001*				
Race							
African American	1.16	0.99–1.37	0.07**				
Other, Non-white*	2.19	1.56–3.08	<0.001*				
History of Depression*	1.49	1.29–1.72	<0.001*				
TBI history	1.14	0.94–1.37	0.18				

HL p, Hosmer-Lemeshow statistic *p*-value; AUC, Area Under the Curve for model predictions; SS/SP, Sensitivity/Specificity for model predictions; TBI, traumatic brain injury with loss of consciousness; APOE ε4, apolipoprotein E-ε4. *$p < 0.05$. **Trend for significance.

amnesia 30 min to 24 h) was associated with a 2.3 fold increase in risk in one preliminary investigation [7]. In contrast, mild TBI (defined as LOC or posttraumatic amnesia <30 min) was not related to an increased risk for AD in that study of 17 subjects with AD. However, a recent report utilizing a large ($N = 51{,}799$) TBI sample reported that a mild or moderate-to-severe TBI was associated with a higher risk for dementia, even after adjusting for several demographic, medical, and psychiatric factors [10]. In addition, TBI occurring later in life (i.e., within 10 years of AD onset), has been found to be related to higher risk estimates and more rapid decline after dementia onset [19]. Yet another related issue is that of repetitive TBI in relation to risk for cognitive decline later in life. Some studies have shown that MCI was more prevalent (24%) compared to the general population in retired football players who sustained an average of three or more mild TBIs during their career [21, 41]. Therefore, TBI severity, age at the time of injury, and repetitive injuries may be important components in the associated risk of MCI.

It should be noted that the NACC data are not population-based, and the present study was limited to subjects with complete data. Since many subjects in the NACC database were missing information for ApoE, generalizability may be limited. In addition, clinician-estimated age of onset of MCI is subjective, non-specific, and may suffer from biased recall.

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Table 3
Demographics, age of diagnosis, and age of cognitive decline in MCI subjects with and without TBI

	TBI+ ($n = 304$)	TBI– ($n = 2883$)	p
MCI Age of Diagnosis, *M (SD)*	72.3 (9.8)	74.6 (8.8)	<0.001*
Estimated Age of Cognitive Decline, *M (SD)*	68.1 (12.2)	69.8 (13.6)	0.05
Education, *M (SD)*	15.0 (3.6)	15.0 (3.3)	0.86
% Female	40	52	<0.001*
% Caucasian	86	82	0.29
% APOE ε4 alleles			0.22
0 ε4 alleles	61	56	
1 ε4 allele	34	36	
2 ε4 alleles	6	8	
Composite Vascular Risk Score, *M (SD)*	1.7 (1.4)	1.7 (1.4)	0.70
% History of Vascular Conditions			
Heart Attack	6	7	
Atrial Fibrillation	11	7	
Angioplasty	8	8	
Cardiac Bypass	4	7	
Pacemaker	3	3	
Congestive Heart Failure	2	3	
Stroke	4	6	
Transient Ischemic Attack	9	5	
Hypertension	53	55	
Hypercholesterolemia	56	55	
Diabetes	14	14	
Number of Years Smoking Cigarettes, *M (SD)*	11.2 (16.1)	10.2 (15.2)	0.24
Average Number of Cigarette Packs/Day			0.07
% No Smoking History	50	56	
% <Half Pack	13	15	
% Half to 1 Pack	18	14	
% 1 to 1.5 Packs	8	8	
% 1.5 to 2 Packs	6	4	
% ≥2 Packs	6	4	
% History of Depression	29	17	<0.001*
% History of Alcohol Abuse	9	3	<0.001*
% History of Other Substance Abuse	3	1	–

TBI+ are subjects with history of traumatic brain injury (TBI) with loss of consciousness; TBI– are subjects without TBI. Age of Diagnosis reflects subjects' age in the initial visit when a diagnosis of MCI was received. *$p < 0.05$.

Moreover, due to the limited data available regarding TBI details in the NACC database (i.e., based upon three questions), we were not able to examine potentially important variables such as TBI severity, age at the time of injury, or repetitive injuries. Even though this study attempted to examine the effect of remote TBI on MCI, it is possible that based on the definition for remote TBI, injuries could have occurred as recently as nearly one year prior to the initial visit. Since falls are one of the leading causes of TBI, particularly in an aging population [42], a number of TBIs in this sample could have been relatively more "recent" as opposed to occurring many years earlier, although we selected those with a history of TBI occurring >1 year earlier. Furthermore, while the majority (66%) of those with MCI self-reported a history of mild TBI (i.e., LOC <5 min), TBI severity could not be assessed for many, as the cutoff of "LOC ≥5 minutes" (34%) has no upper boundary and may include a wide range of severity. Therefore, it is unknown how risk and age of onset may be affected by different levels of injury severity.

In summary, a history of TBI was associated with increased risk for an MCI diagnosis, although this association was attenuated by a reported history of depression. A TBI history was associated with approximately a 2.3 year earlier age of MCI diagnosis but psychiatric comorbidities also attenuated the trend for an earlier age of clinician-estimated onset of MCI. These associations deserve further investigation as much is still unknown, including the implications of depression. Future research should incorporate tools that more thoroughly assess TBI features (e.g. severity, age of injury, presence and duration of LOC, posttraumatic symptoms, etc.) and depression in order to identify which factors may be most associated with increased risk for or earlier onset of MCI.

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Traumatic Brain Injury and Suicidal Behavior: A Review

Abhishek Wadhawan[a,b], John W. Stiller[a,c,d], Eileen Potocki[e], Olaoluwa Okusaga[a,f,g], Aline Dagdag[a,h], Christopher A. Lowry[i,j,k], Michael E. Benros[l] and Teodor T. Postolache[a,k,m,*]
[a]*Department of Psychiatry, Mood and Anxiety Program, University of Maryland School of Medicine, Baltimore, MD, USA*
[b]*Saint Elizabeths Hospital, Psychiatry Residency Training Program, Washington, DC, USA*
[c]*Saint Elizabeths Hospital, Neurology Consultation Service, Washington, DC, USA*
[d]*Maryland State Athletic Commission, Baltimore, MD, USA*
[e]*VA Maryland Healthcare System, Baltimore VA Medical Center, Baltimore, MD, USA*
[f]*Menninger Department of Psychiatry and Behavioral Sciences, Baylor College of Medicine, Houston, TX, USA*
[g]*Michael E DeBakey VA Medical Center, Houston, TX, USA*
[h]*Department of Psychiatry, University of Maryland Medical Center, Baltimore, MD, USA*
[i]*Department of Integrative Physiology and Center for Neuroscience, University of Colorado Boulder, Boulder, CO, USA*
[j]*Department of Physical Medicine and Rehabilitation and Center for Neuroscience, University of Colorado Anschutz Medical Campus, Aurora, CO, USA*
[k]*Rocky Mountain Mental Illness Research Education and Clinical Center (MIRECC), Veterans Integrated Service Network (VISN) 19, Military and Veteran Microbiome: Consortium for Research and Education (MVM-CoRE), Aurora, CO, USA*
[l]*Mental Health Centre Copenhagen, Copenhagen University Hospital, Copenhagen, Denmark*
[m]*Mental Illness Research, Education and Clinical Center (MIRECC), Veterans Integrated Service Network (VISN) 5, VA Capitol Health Care Network, Baltimore, MD, USA*

Abstract. Given the increasing rate of death by suicide in the United States, it is imperative to examine specific risk factors and to identify possible etiologies of suicidal behavior in at-risk clinical subpopulations. There is accumulating evidence to support an elevated risk of death by suicide in individuals with a history of traumatic brain injury (TBI). In this review article, after defining terms used in suicidology, we discuss the associations of TBI with death by suicide, suicide attempt, and suicidal ideation. A model for repetitive TBIs, leading to chronic traumatic encephalopathy, is also discussed as a neuroinflammatory process, with discussion about its possible link with suicide. The review concludes with an overview of interventions to prevent suicidal behavior.

Keywords: Chronic traumatic encephalopathy, death by suicide, inflammation, neuroinflammation, suicidal behavior, traumatic brain injury

*Correspondence to: Teodor T. Postolache, MD, 685 W. Baltimore Street, MSTF Building, Suite # 930, Mood and Anxiety Program, Department of Psychiatry, University of Maryland School of Medicine, Baltimore, MD 21201, USA. Tel.: +1 (410) 706-2323; E-mail: tpostola@som.umaryland.edu.

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This review of the relationship between traumatic brain injury (TBI) and suicidal behavior provides a comprehensive analysis of possible mediating mechanisms and evaluates various pharmacological and non-pharmacological approaches to reducing the risk of suicidal behavior in individuals with TBI and post-TBI. A review of analogous neurological disorders, formally described in both Alzheimer's disease (AD) and chronic traumatic encephalopathy (CTE), may present an example of the neuroinflammatory prototypes that lead to suicidal behavior. To provide uniformity and clarity, we adopted the Centers for Disease Control and Prevention's (CDC) definition of TBI: "a disruption in the normal function of the brain that can be caused by a bump, blow, or jolt to the head, or a penetrating head injury" [1].

The CDC defines suicidal ideation as "thinking about, considering, or planning suicide" (see Box 1) [2]. Suicidal intent is defined as "past or present evidence (implicit or explicit) that an individual wishes to die, means to kill him/herself, and understands the probable consequences of his/her actions or potential actions [3]." Suicidal intent can be determined retrospectively and in the absence of suicidal behavior [3]. Suicide ideation-related behavior (SIRB) is defined as "thoughts related to wishing to die where the evidence of suicidal intent is undetermined [4]." On Patient Health Questionnare-9 (PHQ-9) for screening depression, endorsement of item 9 ("Thoughts that you would be better off dead or of hurting yourself in some way") parallels the presence of SIRB in the individual [3, 5]. Self-directed violence is defined by CDC as "behavior that is self-directed and deliberately results in injury or the potential for injury to oneself" [6]. It is further divided into suicidal, non-suicidal, and undetermined types, and each of these types can be of fatal (leads to death) or non-fatal type (does not lead to death). The CDC defines suicidal self-directed violence (SSDV) as "behavior that is self-directed and deliberately results in injury or the potential for injury to oneself, and there is evidence, whether implicit or explicit, of suicidal intent" [6]. When "behavior that is self-directed and deliberately results in injury or the potential for injury to oneself, and there is no evidence, whether implicit or explicit, of suicidal intent", then it is regarded as non-suicidal self-directed violence [6]. Undetermined self-directed violence is defined as "behavior that is self-directed and deliberately results in injury or the potential for injury to oneself, and when the suicidal intent is unclear, based on the available evidence" [6]. Hence, a range of violent behaviors, including non-suicidal intentional self-injury (i.e., behaviors where the intention is not to kill oneself, as in self-mutilation), and acts of non-fatal and fatal suicidal behavior, are included in self-directed violence [6].

Box 1: CDC uniform definitions for suicide terminology [6], as modified by us

Suicidal ideation: Thinking about, considering, or planning suicide.

Self-directed violence: Behavior that is self-directed and deliberately results in injury or the potential for injury to oneself. It includes:

- *Non-suicidal self-directed violence*: Behavior that is self-directed and deliberately results in injury or the potential for injury to oneself, and there is no evidence, whether implicit or explicit, of suicidal intent.
- *Undetermined self-directed violence*: Behavior that is self-directed and deliberately results in injury or the potential for injury to oneself, and when the suicidal intent is unclear, based on the available evidence.
- *Suicidal self-directed violence*: Behavior that is self-directed and deliberately results in injury or the potential for injury to oneself, and there is evidence, whether implicit or explicit, of suicidal intent.

Suicide attempt/Non-fatal Suicidal self-directed violence: A non-fatal, self-directed, potentially injurious behavior with an intent to die as a result of the behavior; it may or may not result in injury.

Death by suicide/Fatal Suicidal self-directed violence: Death caused by self-directed injurious behavior with an intent to die as a result of the behavior.

Suicidal behavior: Suicidal behavior can include both suicide attempt/non-fatal suicidal self-directed violence as well as death by suicide/fatal suicidal self-directed violence.

Suicide attempt is defined by CDC as "a non-fatal, self-directed, potentially injurious behavior with an intent to die as a result of the behavior; it may or may not result in injury" (see Box 1) [2]. The CDC defines death by suicide as "death caused by self-directed injurious behavior with an intent to die as a result of the behavior" [6]. Suicide, death by suicide, and fatal SSDV are synonymous. Suicidality is limited to suicidal ideation only and not to include suicidal behavior [6]. However, according to the CDC, suicidality is a term that is "often used to refer simultaneously to suicidal thoughts and suicidal behavior" and is considered an unacceptable term, due to the presence of major differences in the associated factors, occurrence, interventions, and consequences related to both of these phenomena (see Box 2) [6].

TRAUMATIC BRAIN INJURY

About 30% of the injury-related deaths in the United States (US) can be attributed to TBIs [7]. In 2013, approximately 2.8 million people, or 1.9% of the US population, had TBI-related emergency

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Box 2: CDC's list of unacceptable terms for suicide terminology [6]

Completed suicide: This terminology implies achieving a desired outcome. Alternate term: suicide.
Failed attempt: This terminology gives a negative impression of the person's action, implying an unsuccessful effort aimed at achieving death. Alternate terms: suicide attempt or suicidal self-directed violence.
Nonfatal suicide: This terminology portrays a contradiction. "Suicide" indicates a death while "nonfatal" indicates that no death occurred. Alternate term: suicide attempt.
Parasuicide: Formally used to refer to a person's self-directed violence, whether or not the individual had an intent to die. However, the World Health Organization is now favoring the term suicide attempt. Alternate terms: non-suicidal self-directed violence or suicidal self-directed violence.
Successful suicide: This term also implies achieving a desired outcome. Alternate term: suicide.
Suicidality: This terminology is often used to refer simultaneously to suicidal thoughts and suicidal behavior. These phenomena are vastly different in occurrence, associated factors, consequences and interventions so should be addressed separately. Alternate terms: suicidal thoughts and suicidal behavior.
Suicide gesture, Manipulative act, and Suicide threat: Each of these terms gives a value judgment with a pejorative or negative impression of the person's intent. They are usually used to describe an episode of nonfatal, self-directed violence. A more objective description of the event is preferable such as non-suicidal self-directed violence or suicidal self-directed violence.

department visits, hospitalizations, and deaths (TBI-EDHDs) [7]. Military personnel, in particular, appear to be at a much higher risk of sustaining TBI. According to the Department of Defense data presented in the 2014 CDC Report to Congress, between 2000 and 2011, about 4.2% of the personnel who had served in various branches of the military had been diagnosed with a TBI [8].

Variability across gender and age in TBI-EDHD incidence rates in the US population in 2013, compared to 2007, suggests that certain risk factors increase vulnerability to acquiring TBI in general. Individuals aged $\geq$75 years (2,232.2/100,000), 0–4 years (1,591.5/100,000), and 15–24 years (1,080.7/100,000) had some of the highest rates of TBI-EDHDs [7]. Females (810.8/100,000) had overall lower age-adjusted rates of TBI-EDHDs than those of males (959.0/100,000) [7]. However, among active duty US military service members (Army, Navy, Air Force, and Marine Corps), it was reported that the average incidence rate of having a TBI diagnosis increases by almost 8.4 times during the first 4 weeks after the end of deployment (1055.8/100,000), as compared to the average rate prior to deployment (119.8/100,000) [9]. Interestingly, the incidence rate of having a TBI diagnosis then declines sharply during the second 4-week period after the end of deployment, yet it continues to remain higher than the average rate prior to deployment [9].

Some of the most common causes of TBI in the general population are: unintentional falls; unintentional motor vehicle accidents; intentional self-harm; assaults/homicides involving firearms, explosives, and other weapons; sports; and work-related injuries [7]. In terms of the most likely causes of injury for all age groups, the age-adjusted rates were highest for falls (413.2/100,000), being struck by or against an object (142.1/100,000), and motor-vehicle accidents (121.7/100,000) [7]. Among Operation Enduring Freedom/Operation Iraqi Freedom (OEF/OIF) service members, blast exposure was the most common mechanism for TBI [10].

Severity of injury in TBI is classified into three types: mild, moderate, and severe, and can be determined by findings on structural imaging, duration of alteration of consciousness (AOC), severity of Glasgow Coma Scale (GCS) scores or an equivalent scale that predicts neurological outcomes [11] at the initial evaluation, duration of posttraumatic amnesia (PTA), and the duration of loss of consciousness (LOC), all of which highlight the extent of disruption in brain function secondary to TBI [12]. According to the Veterans Administration-Department of Defense practice guidelines, TBI is considered to be mild in severity if the structural imaging is normal, AOC continues for no more than 24 hours, GCS score in the first 24 hours is 13–15, PTA persists for 0–1 day, and LOC occurs for 0–30 minutes [12]. However, even though the PTA mostly takes into account the anterograde amnesia after TBI, there is some evidence that retrograde amnesia may also be of some use in predicting outcomes [13]. On the other hand, normal or abnormal structural imaging, as well as AOC continuing for greater than 24 hours, characterize both moderate and severe TBI. Moderate and severe TBIs are differentiated based on the GCS score in the first 24 hours (9–12 for moderate and <9 for severe), duration of PTA (>1 and <7 days for moderate and >7 days for severe), and the duration of LOC (>30 minutes to <24 hours for moderate and >24 hours for severe) [12]. Often, moderate and severe types are counted together [14] to emphasize that the differences among them are generally smaller than the differences between moderate and mild TBI, as well as between severe and mild TBI [14–17].

According to the CDC's report to Congress, 75% of TBIs sustained by the US population in 2003

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were mild in severity [18]. In contrast, an estimated 11–23% of military personnel serving in recent conflicts had either been diagnosed with mild TBI, confirmed by a clinician, or screened positive for TBI [10, 19–21]. A history of mild TBI was reported in approximately 8–22.8% of military personnel deployed to Afghanistan or Iraq [10, 19, 21]. From the year 2000 to 2015, most TBIs suffered by the OEF/OIF service members were mild in severity (82.4%) [22]. Among OEF/OIF service members, only 2.5% of the sustained TBIs were severe, with 8.5% being moderate in severity and 6.6% being not-classifiable [22]. Brenner et al. [23] reported that, based on a TBI screening tool, the prevalence of probable TBI was 45% in a sample of veterans from all eras who had sought Veterans Health Administration (VHA) mental health services. A subset of veteran participants interviewed about the severity of their worst lifetime TBI reported that 27% of them had severe TBI, 16% had moderate TBI, and 57% had mild TBI [23].

The majority of individuals report that they return to baseline functioning within a year of sustaining a mild TBI [24]. However, 7–33% of these individuals report symptoms persisting for more than one year [10]. Among individuals with moderate to severe TBI, only 40% of subjects report being able to recover their capacities for leisure, recreation, and cognition to those of pre-injury levels [17]. Another study demonstrated a significant positive correlation between the severity of TBI and cognitive impairment on measures of executive functioning, processing speed, and memory. These acquired deficits persisted 10 years post-injury [25]. Such deficits in executive functioning have been associated with functional, cognitive, and behavioral outcomes, all of which play an important role in community integration after rehabilitation [26].

A history of TBI, independent of severity, has been associated with higher rates of developing posttraumatic stress disorder (PTSD) [27] and new anxiety and mood-related psychiatric disorders [28, 29]. TBI is also recognized as one of the major sources of disability, morbidity, and mortality among US veterans [19, 30]. Combat-associated mild TBI in US veterans frequently results in PTSD and subsequent difficulties in returning to a civilian lifestyle [27, 31]. Negative mental and physical outcomes, which include suicidal behaviors (i.e., death by suicide and suicide attempt), as well as suicidal ideation, have been associated with a history of TBI [32–34]. TBI consistently has been associated with increased risk of death by suicide, even after adjusting for psychiatric illness, as reported in large European cohorts [33] and US veterans [32, 35]. After the initial event, even though a majority of patients survive, a very high rate of longer-term mortality via death by suicide exists in these individuals [36]. Intentional self-harm became the leading cause of TBI-related deaths in 2013, which is parallel to the ever increasing rates of death by suicide in the US over the last two decades [37].

REPETITIVE MILD TBIS AND DEATH BY SUICIDE

Death by suicide also appears to be a frequent cause of death in CTE [38–44], which is characterized by a widespread deposition of neurofibrillary tangles containing hyperphosphorylated tau in the depths of sulci that occurs secondary to a history of repetitive sub-concussive or concussive TBIs [41]. Surveys have concluded that former National Football League (NFL) players have relatively higher rates of chronic pain and opioid use than the general population [45], and that those with chronic pain and depression tend to have more financial difficulties and higher life-stress [46–49]. A considerable number, although a minority of all ex-players, have psychiatric issues such as major depression [49–51] which, together with chronic pain and higher financial/life stress, have been reported to be risk factors for death by suicide [52–58].

However, some studies have identified a substantially lower rate of death by suicide in professional football players compared to that of the general US population [59–61], which could be related to the lack of financial burden in football players. Focusing on cardiovascular mortality, Baron et al. (2012) examined a cohort of 3,439 professional football players and reported on their mortality resulting from intentional self-harm [59]. The authors reported that death by suicide in this cohort was substantially decreased compared to that of the general population of the US, with a standardized mortality ratio (SMR; the ratio of observed deaths to expected deaths) of 0.41 (95% Confidence Intervals [CI], 0.19–0.78); 21.8 deaths expected, 9 observed [59]. Lehman et al. (2016) reexamined this cohort after adjusting for age, sex, and race, and determined an SMR of 0.47 (95% CI, 0.24–0.82), re-affirming a statistically significant lesser rate of death by suicide in professional football players compared to that of the general population

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of the US [61]. According to a recent retrospective cohort study by Webner et al. (2016), which examined 26,702 NFL athletes over the past 95 years who were either dead, retired, or were still playing NFL, findings from multiple internet queries, obituaries, news reports, and from reviews of athletes' life indicated that a total of 26 male athletes had died by suicide [60]. The authors reported that even though CTE had been widely presumed to result in death by suicide and depression in NFL athletes, their finding of a lower rate of death by suicide in NFL players compared to that of males in the general population did not support this hypothesis [60]. A substantial percentage of deaths by suicide (42.3%) had occurred between 2009 and 2015, and this timing raised questions regarding the possible role played by recurring reports in media on these deaths in reinforcing or eliciting thoughts about suicide in at-risk athletes [60]. The heterogeneity of outcomes in the studies could be attributed to selection bias inherent in football player-only samples, as well as to several other underlying confounding variables, including, but not limited to, inflammation, concomitant PTSD, and infections. Additionally, a recent meta-analysis across 20 studies by Ferguson [62] failed to support evidence for suicide contagion being embedded in fictional media. The authors hypothesized that the absence of such a finding could be attributable to several methodological limitations leading to inter-study heterogeneity. Thus, a complex picture of the associations between CTE and death by suicide is emerging, potentially implying that individual vulnerability and resilience, as well as contextual factors, could contribute towards diverging results.

DEATH BY SUICIDE

Globally, death by suicide leads to approximately 800,000 deaths per year, making it the seventeenth leading cause of worldwide mortality in 2015 [63]. In 2015, the global age-adjusted standardized rate for death by suicide was 10.7/100,000 annually [64]. In fact, preventing suicidal behaviors was recognized as a priority for global health by the World Health Organization (WHO) in 2014 [65]. Data from the CDC have revealed that, with close to 43,000 deaths in a year, death by suicide was the tenth leading cause of mortality in the US in 2014 [66]. Death by suicide claimed about 45,000 lives in 2016 in the US, with about 13.5 individuals per 100,000 of the population (age-adjusted rate) dying from suicide [37]. Comparatively, in 2016, the overall age-adjusted death rate from all causes in the US was 728.8 per 100,000 [67], which is almost 54 times higher than the age-adjusted death rate attributable to death by suicide. Reflecting the ever-increasing rates of death by suicide in the US, the rates of death by suicide increased across 44 of the 50 states (88%) in the US from 1999-2016 [37]. Rates of death by suicide have shown a >30% increase in 25 states across the US from 1999-2016, with the absence of a known mental health condition in about 54% of the individuals who died by suicide across 27 states in 2015 [37]. Additionally, greater life stressors, such as criminal/legal matters, relationship problems, recent or impending crises, and eviction/loss of home, were present in individuals without a known mental health condition [37]. Data from military and veteran samples indicate that, in the year 2015, an estimated 20.6 non-activated Guardsmen or Reservists, active-duty Service members, and "other veterans" died by suicide each day, with 16.8 of these deaths occurring in "other veterans" [68]. Among veterans and non-veteran adults from the US, the age- and gender-adjusted death by suicide rates in 2016 were reported to be 26.1/100,000 and 17.4/100,000, respectively, thereby indicating a 1.5 times higher rate of death by suicide in veterans as compared to non-veterans [69]. In comparison to their adult civilian peers, after adjustment for age and sex, veterans were 22 percent more likely to die by suicide in 2014 [70]. Among veterans using VHA services, the age-adjusted death by suicide rates (per 100,000 Person-Years) were reported to be higher than those of the general US population for both males and females, with female veterans having a greater magnitude of increase (age-adjusted suicide rate ratios being 2.28 for females versus 1.66 for males) compared to that of the general US population [68]. With minor fluctuations, an overall upward trend in death by suicide rates has been reported among the veterans of OEF, OIF, Operation New Dawn (OND), and OEF/OIF/OND who also used VHA services, with the death by suicide rates (per 100,000 Person-Years) rising from 26.8 in 2003 to 47.8 in 2014 [70]. Younger male (between the ages of 18–29 years) OEF/OIF/OND veterans using VHA services were reported to have the highest rates of death by suicide (110.3 per 100,000 Person-Years in ages 18–24 years and 56.3 per 100,000 Person-Years in ages 25–29 years) [70]. Following similar trends, since 2007, among veterans using VHA services, the death by suicide rates in 2014 were reported to be higher in OEF/OIF/OND active duty veterans (54.9 per 100,000 Person-Years) compared to those of

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veterans of the National Guard or Reserve components (35.6 per 100,000 Person-Years) [70].

SUICIDAL IDEATION AND SUICIDAL BEHAVIOR

The rate of suicide attempt is estimated to be ten-times higher than death by suicide, thus making non-fatal suicidal behavior a substantial public health concern [71]. According to the data from the National Survey on Drug Use and Health (NSDUH), which involved in-person interviews of participants in a nationwide survey, in 2016, about 4.0% (9.8 million individuals) of adults aged >18 years had serious thoughts of suicide in the past year. Meanwhile 1.1% (2.8 million individuals) made any suicide plans in the past year, and 0.5% (1.3 million individuals) attempted suicide in the past year [72]. Among all age groups, adults aged 18–25 years had the greatest percentages for having serious thoughts of death by suicide in the past year (8.8%; 3.0 million individuals), making any suicide plans in the past year (2.9%; 1 million individuals), as well as attempting suicide in the past year (1.8%; 0.6 million individuals) [72]. Among all racial backgrounds, individuals reporting two or more races had the highest prevalence for having serious thoughts of suicide (7.5%; 298,000 individuals), for making any suicidal plans (3.3%; 130,000 individuals), and for attempting suicide (0.8%; 31,000) [72]. In comparison, data from VA's Suicide Prevention Applications Network indicated that between 2012–2014, the monthly non-fatal suicide attempt among VHA patients increased from slightly above 600 in May 2012 to about 900 reported attempts in August 2014 [68].

RISK FACTORS FOR SUICIDAL BEHAVIOR

Death by suicide has been previously linked to psychiatric disorders, such as depression and substance use disorders [56, 73]. A number of deaths by suicide occur due to impulsivity in response to crises mediated by negative life events, including, but not limited to, chronic pain [46], relationship loss in the recent past [74], and acute stress at the time of death [75]. Death by suicide has been recognized as the second leading cause of mortality among individuals between 15–29 years of age [76]. Living alone, being white, male gender, and having a chronic medical illness leading to perception of poor health are demographic factors associated with that increased risk of death by suicide [74]. Notably, the rates of death by suicide in young adult males are almost quadruple those of females [37, 66].

The most important and greatest risk factor for future suicide attempt is previous suicide attempt(s) [77, 78]. Suicidal behavior has also been associated with social isolation [79], severe natural disasters [80], partial insomnia (difficulty falling asleep, sleep continuity disturbance and early morning awakening), severe anxiety and/or panic attacks, global insomnia, anhedonia, feelings of hopelessness, helplessness, or worthlessness, chronic worsening of medical illness, failure to keep a job or being a student, and being diagnosed with a life-threatening disorder, such as acquired immunodeficiency syndrome or cancer [74]. In fact, trait aggression/impulsivity and neurocognitive dysfunction have been recognized as the endophenotypes of suicidal behavior [81].

Increased social stress, economic, and political factors [82], an elevated frequency of drug and alcohol use [83–85], and high divorce rates [86] have been implicated as possible risk factors for the marked rise in the rates of death by suicide. A diagnosable psychiatric disorder is present in more than 90% of suicide attempters. Mood disorders are the most common diagnoses in individuals who complete suicide and account for about 60% of all suicides [77, 87, 88]. Other psychiatric disorders, such as personality disorders [77, 89, 90], alcoholism [91], schizophrenia [92], and substance use [93], are also related to suicide. Frequency of suicide is lower (about 2%) in the outpatient psychiatric populations [94, 95] compared to the hospital populations that have been discharged from care, ranging from 5–10% in individuals with antisocial or borderline personality disorders and about 20% in individuals with bipolar disorder or manic depression [90, 96–98].

Suicidal behavior carries a number of non-modifiable risk factors, including sex, age, unexpected harmful life events, and a personal or family history of suicide [71, 99]. It becomes essential to recognize unique risk factors for suicidal behavior, which could be identified and managed in a clinician's office to reduce overall risk of occurrence. Some of these modifiable risk factors include mood, psychotic and anxiety symptoms, substance use disorders, personality disorders, chronic worsening of medical illness, chronic pain, and sleep disturbances.

Interestingly, a number of the above-mentioned risk factors for death by suicide and suicidal behavior also overlap with certain risk factors for TBI, such as

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male gender, being an adolescent or a young adult, having chronic health conditions, a history of mental health diagnoses at baseline, and problematic alcohol and drug use [100–103]. Moreover, a history of previous TBI also elevates the risk for future TBI [102, 104], just as the risk for death by suicide is elevated by a history of previous suicide attempt(s).

MODELS OF DEATH BY SUICIDE

The contributing factors for suicidality and death by suicide can be categorized into state- and trait-dependent factors, or "proximal and distal stressors," reiterating the high complexity and multifactorial nature of suicidality and death by suicide [105]. Various models have been proposed and investigated; these have been supported by empirical findings, and help to explain the psychopathology of suicidality and death by suicide [106]. Among these models, the interpersonal theory [107] and the stress-diathesis model [71, 108] are discussed below:

Interpersonal theory of suicide

Joiner [109] proposed that the simultaneous presence of desire for suicide and the acquired capability for death by suicide is required for occurrence of lethal or near-lethal suicidal behaviors in individuals [107, 109]. When present simultaneously, two interpersonal constructs, i.e., thwarted belongingness ("*I am alone*") and perceived burdensomeness ("*I am a burden*"), can lead to the desire for death by suicide if an individual develops hopelessness regarding both these states and starts to perceive them as unchanging and stable [107, 109]. Loneliness, absence of reciprocal care, subjective experience of not being part of one's peer group or family, and social alienation, are all-synonymous with thwarted belongingness [107, 109]. On the other hand, the notion that one's death is worth more than one's life to others (liability), self-hate, and one's perceived conviction of being a burden to family and peer system constitutes perceived burdensomeness [107, 109]. According to this theory, an individual may develop passive suicidal ideation in the presence of either thwarted belongingness or perceived burdensomeness alone [107, 109]. However, transformation of suicidal desire into suicidal intent occurs only when an individual's fear for death is lowered, together with the presence of desire for death by suicide in that individual [107, 109]. The fear of death, injury, and pain may be attenuated in individuals who have experienced prior pain, violence, or trauma repeatedly, such as by having attempted suicide [110, 111], childhood maltreatment [107], self-injury as part of eating disorders [112, 113], combat exposure [114, 115], experiencing proxy-violence and death (physicians) [116, 117], or conducting animal euthanasia [118]. This process has been referred to as an acquired capability for death by suicide by Joiner [107, 109]. Joiner [109] stated that a higher threshold for tolerating pain and decreased fear of death are the two major characteristics of having a high capability for death by suicide. An individual's capability for suicide might also be enhanced by personality traits, such as impulsivity [119].

In summary, Joiner's theory states that a combination of hopelessness, regarding both thwarted belongingness and perceived burdensomeness, may precipitate suicidal desire (ideation), which may further interact with elevated tolerance for physical pain, as well as reduced fear of death by suicide, to increase the likelihood of occurrence of serious suicidal behavior (i.e., lethal or near lethal suicide attempt) [107, 109]. We further interpret Joiner's model of suicidal behavior as being a consequence of an occurrence of readiness to die and readiness to kill (acquired capability to kill self in Joiner's model) as explained further in Fig. 1. As further developed later in the article, TBI leads to both readiness to kill and readiness to die which, in turn, elevates the risk of suicide.

Stress-diathesis model

Mann et al. (1999) proposed a stress-diathesis model, which states that predisposing vulnerability factors (diathesis or "distal risk factors") interact with triggering stress factors ("proximal risk factors") to ultimately escalate death by suicide-risk [108]. Both proximal and distal risk factors are required to reach the threshold for suicidal behavior. Some of the proximal risk factors are psychiatric disorders, including substance abuse, exacerbation of psychiatric illness, adverse psychosocial events, marital or financial issues, and emotional pain [71, 108, 120]. Distal risk factors are comprised of impulsive-aggressive personality traits, TBI, cognitive inflexibility, sensitivity to social stress, pessimism/hopelessness, childhood abuse, and family history of suicide [71, 108, 120].

Despite the heterogeneity of suicidal behavior, the neurobiological, clinical, and demographic characteristics shared by both death by suicide and non-fatal suicide attempt with high lethality suggest the

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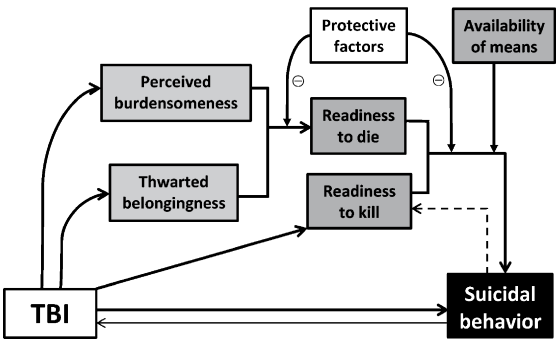

Fig. 1. Modified model of Joiner's interpersonal theory of suicide linking cognitive distortions induced by traumatic brain injury (TBI) to suicidal behavior. TBI may lead to physical incapacitation and cognitive distortions of perceived burdensomeness ("*I am a burden*") and/or thwarted belongingness ("*I am alone*"). Both of these cognitive distortions, when present together, may lead to "readiness to die" (modification of the "desire for suicide" in Joiner's model). "Readiness to kill" (modification of the "acquired capability for suicide" in Joiner's model) may develop with repeated exposure to pain, violence or trauma, including TBI, leading to a higher threshold for tolerating pain and decreased fear of death. Impulsivity and aggression, endophenotypes of suicidal behavior, may also elevate readiness to kill. In the presence of both "readiness to kill" and "readiness to die", an individual's likelihood of engaging in suicidal behavior increases substantially. The model of "readiness to die" and "readiness to kill", which we are first proposing in this publication, is often characterized by a diminution of the internal conflict between intent to die and intent to live, and of the painful ambivalence that is so common in individuals with suicidal ideation, intent and even plan. Moreover, with each subsequent act of non-fatal suicidal behavior, one's "readiness to kill" increases due to a concomitant habituation of the fear of death and dying, as well as to an increase in tolerance for pain (dotted line). Although, certain protective factors/deterrents, such as social supports, having children to raise etc., may reduce the risk for suicidal behavior, the availability of means to kill oneself elevate the risk for suicidal behavior, with the availability of lethal means elevating the risk of death by suicide. Also, suicidal behavior may contribute to TBI, due to head trauma resulting from certain suicide attempts, as well as long-term accident proneness, thereby creating a vicious circle.

existence of a possible shared diathesis between them [120]. In addition to a psychiatric disorder, diathesis is an important risk factor for death by suicide, as most individuals with a major psychiatric illness do not engage in suicidal behavior [120]. Nearly half of the death by suicide-risk attributable to diathesis is heritable, with men possibly having a lesser heritability than women [121, 122]. Risk of suicidal behavior can be enhanced by the epigenetic effects of childhood adversity, including sexual, physical, or emotional abuse, and subsequent mood-related psychiatric illnesses on an individual's diathesis [123].

Interestingly, individuals with histories of substance and alcohol abuse, as well as those who are more impulsive and aggressive, are likely to have more frequent head injuries. A possible bidirectional relationship likely exists between these variables. Head injuries, aggressive behaviors, and alcohol and substance use [124–126] may potentiate the diathesis-induced increased risk for death by suicide [71].

In summary, this model attempts to integrate the cognitive and clinical perspectives with the neurobiological phenotypes of suicidal behavior. Diathesis in Mann's model probably overlaps, at least partly, with the acquired capability of death by suicide in Joiner's model [127].

ASSOCIATION BETWEEN TBI AND DEATH BY SUICIDE, SUICIDAL BEHAVIOR, AND SUICIDAL IDEATION

The earliest accounts of the association of TBI and death by suicide was in Finnish veterans from the Second World War, who were studied by Hillbom [128] and Achte et al. [129]. Subsequently, in a civilian population, Fahy et al. [130] reported suicidal impulses/ideation in four of the 32 survivors of severe head-injuries mainly related to road-traffic accidents. Since then, more evidence has been gathered in support of TBI being a risk factor for death by suicide [4, 32, 33, 131–136], suicidal behavior [133, 137–141], and suicidal ideation [34, 138, 139, 141, 142].

Relationship of TBI to death by suicide

Robust data exist supporting a positive association between TBI and death by suicide. Results of a retrospective cohort study based on Danish population registers identified that individuals with TBI had 3–4 times greater risk of death by suicide than those who had not suffered a TBI [33]. A three-times greater risk of death by suicide was identified in adults with a diagnosis of concussion in a recent longitudinal cohort study spanning across a 20-year period [136]. A Swedish study comparing the death records of 200,000 individuals having TBI with those of 2 million control subjects reported that death by suicide was three-times more likely in individuals having TBI compared to the general population control subjects [131]. Even though individuals with TBI have a higher comorbidity of substance use and psychiatric disorders, both of which are independent risk factors that can increase suicide-risk, the elevated rate of death by suicide in individuals with TBI still prevailed, even after adjustment

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for substance use and psychiatric disorders [131]. Madsen et al. (2018) recently performed a registry-based retrospective cohort study in Denmark and reported that a statistically significant increased risk of death by suicide was present in individuals having medical contact for TBI when compared with the general population without TBI [fully adjusted incidence rate ratio (IRR) = 1.90; 95% CI, 1.83–1.97] [134]. It was speculated that the IRR in this study was lesser than the difference between suicide-rates in individuals with vs. without TBI, measured as hazard rates, standardized mortality rates, or odds ratios, in previous studies [33, 36, 131, 136], probably due to a more extensive adjustment model [134]. The authors reported that, in a fully adjusted model, the absolute rates, as well as the IRRs for death by suicide increased with greater severity of TBI [134]. Also, upon adjustment for multiple confounders, a trend for an increasing risk of death by suicide was reported with increasing number of medical contacts for TBI [134]. Interestingly, even after adjustment for several confounders, a temporal relationship was identified between TBI and increased risk of death by suicide ($p < 0.001$), with the highest reported risk for death by suicide within the first 6 months (IRR: 3.67; 95% CI, 3.33–4.04) after the last medical contact for TBI. It continued to remain high even 7 years (IRR: 1.76; 95% CI, 1.67–1.86) after the last medical contact related to TBI [134]. Furthermore, the association between TBI and suicide was not merely due to injury proneness or systemic trauma rather than brain trauma, as the suicide-risk was substantially higher after TBI in comparison to that after non–central nervous system-related fractures [134]. Bahraini et al. (2013) completed a systematic review of suicidal ideation/behaviors/death by suicide after TBI and reported that, after adjustment for psychosocial, psychopathological, demographic, and socio-economic factors, which could potentially confound the relationship between these variables, a significantly higher rate of death by suicide after TBI was present [135]. In another systematic review, Simpson and Tate [133] reported that, in comparison to individuals with concussion, individuals with severe TBI had a higher risk of death by suicide (hazard ratio = 1.4; 95% CI, 1.15–1.75). However, the increased risk of suicide in those with concussion is far from negligible. Specifically, based on a meta-analysis of 17 studies examining the risk for death by suicide following a mild TBI/concussion, Fralick et al. [143] reported that, as compared to individuals who had not been diagnosed with TBI/concussion, individuals diagnosed with mild TBI/concussion had about a 2-fold higher relative risk of suicide (2.03 [95% CI, 1.47–2.80]; $p < 0.001$).

While the risk of suicide is elevated with TBI in both civilian and military/veteran populations, it appears that, against expectations, the magnitude of the association is not as high in the military/veteran studies as in their civilian counterparts. Bryan and Clemans [144] investigated the association of cumulative TBIs with death by suicide-risk in a clinical sample of 161 deployed military personnel referred for a TBI evaluation. They reported that greater numbers of TBIs were associated with greater risk for death by suicide (β [SE] = 0.214 [0.098]; $p = 0.03$) after controlling for the effects of TBI, depression, and PTSD symptom severity [144]. Brenner et al. [32] reported a greater hazard ratio [1.55 (95% CI, 1.24–1.92)] of death by suicide in veterans with histories of TBI, which resisted adjustment for psychiatric diagnoses, including PTSD. Moreover, the meta-analysis by Fralick et al. (2018) indicated a significant risk for suicide in individuals with history of mild TBI/concussion in civilians (2.36 [95% CI, 1.64–3.40], but not in military/veteran samples (OR 1.46 [95% CI, 0.80–2.58]) [143]. It is possible that non-TBI suicide risk factors in military/veteran populations (such as PTSD) have a greater representation than in the civilian population, and as such, reduce the TBI-control separation in odds ratio for suicide.

Relationship of TBI to suicide attempt

According to a study by Simpson and Tate (2002), over a mean period of 5 years, about 17.4% of the outpatients with TBI had attempted suicide [137]. In another study utilizing the data from the Traumatic Brain Injury Model Systems (TBIMS) funded by National Database National Institute on Disability and Rehabilitation Research (NIDRR), Fisher et al. [138] looked at the rate of suicide attempt in the previous year and reported that 0.8 to 1.7% of the study participants, from a large cohort of patients who had sustained moderate-to-severe TBI, had attempted suicide throughout the 20 years of follow-up [138]. It is intriguing to note that these rates fall barely outside the broad-range of prevalence rates for suicide attempt (2–60%), as reported by Simpson and Tate (2007) in their systematic review of studies spanning various cohorts [133]. However, this wide variation in the prevalence rate of suicide attempt in various studies included in the review by Simpson and Tate (2007) was attributed to significant challenges associated

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with identifying suicide attempt, particularly in subjects having a history of TBI [133, 137].

Among those who have served in the military, less work has been done to study the relationship between TBI and suicidal ideation/suicide attempt. Gutierrez et al. (2008) looked at a sample of 22 veterans with post-TBI psychiatric hospitalization and reported that 27.3% of the subjects had attempted suicide [139]. In a study by Breshears et al. [140], an interdisciplinary TBI team evaluated a sample of 154 military veterans. In the 2 years following TBI (mild, moderate, or severe), suicide attempt was documented in the VA medical records of 7.1% of the study subjects [140]. However, Gutierrez et al. [139] had reported a much higher rate of post-TBI suicide attempt of 27.3% in a much smaller sample of 22 veterans who had inpatient psychiatric admissions after TBI, or who had a history of TBI (primarily moderate or severe) [139].

Using VA administrative data, Finley et al. [141] identified veterans who had served in OEF/OIF and who had received VA care in the fiscal years 2009–2011. They found a significant increase in the risk for suicidal attempt in veterans who had depression, substance use disorder, or a combination of PTSD and TBI [141]. On the other hand, an increased risk of suicide attempt was not associated with TBI alone [141]. This discrepancy was hypothesized to be due to the means of data collection, i.e., data on both TBI and suicide attempt was based on diagnostic codes documentation in clinical care and not on primary data collection in clinical research [141].

Recently, for the first time, Dreer et al. (2017) examined the participants eligible for and enrolled in one-year, post-injury follow-up in the VA Polytrauma Rehabilitation Centers TBI Model Systems, in order to assess rates of pre-/post-TBI mental health utilization, SIRB, and suicide attempt in these individuals [4]. One year post-injury, about 3% of the surveyed study subjects had attempted suicide [4]. Additionally, higher risk for suicidal behavior was reported independent of the presence or absence of a history of premorbid mental health care utilization or a pre-injury suicide attempt [4].

Relationship of TBI to suicidal ideation

In a systematic review, Simpson and Tate [133] reported that the prevalence rate of suicidal ideation was between 3–33% in individuals who had sustained TBI, with most studies reporting a rate of suicide ideation being more than 10%. A recent study by Mackelprang et al. [142] examined the predictors and rates of SIRB in a cohort of adult patients with a complicated mild to severe TBI, within the first 12 months after they sustained TBI. During the year following TBI, one-quarter of the study subjects reported SIRB during $\geq$1 assessment period [142]. In an analysis of retrospective data, Tsaousides et al. [34] had also reported similar findings in a community sample. They identified that the prevalence rate for suicidal ideation in individuals with different severities of TBI was 28.3% [34]. In another study utilizing the data from the TBIMS that was funded by NIDRR, Fisher et al. [138] evaluated rates of SIRB and reported that these rates varied from 7.0% to 10.0% over the 20-year follow-up period. Gutierrez et al. (2008) looked at a sample of 22 veterans with post-TBI psychiatric hospitalization and reported that suicidal ideation was present in 72.7% of the subjects [139]. As stated above, Finley et al. [141] had also reported a significant increase in the risk for suicidal ideation in veterans who had depression or substance use disorder, or a combination of PTSD and TBI [141]. Similar to the results for suicide attempt, increased risk of suicidal ideation was not associated with TBI alone [141], a finding attributed to similar limitations of the study, as described above. Furthermore, Dreer et al. (2017) stated that about 13% of their subjects ($N = 284$) had reported having SIRB one-year post-TBI [4].

Hence, in terms of predicting rates of suicidal ideation and suicide attempt in individuals with a history of TBI, much less definitive research has been done [135]. This could be related, in part, to limitations, such as reliance on retrospective data (e.g., existing medical records), challenges associated with measuring suicide ideation/suicide attempt, variable follow-up periods, and wide-ranging strategies for measuring outcomes [4].

NEUROPSYCHOLOGICAL DEFICITS AS MEDIATORS FOR INCREASE IN RISK OF SUICIDAL BEHAVIOR AFTER MODERATE TO SEVERE TBI

TBI often results in focal injuries to the temporal and frontal lobes [145]. Hence, behavioral, cognitive, and affective deficits are commonly present in individuals with moderate to severe TBI [145–147]. Also, impulse control problems, personality changes, disinhibition, attention modulation deficits, and lack of social awareness may be present in those with a history of TBI [148]. Keilp et al. [149] published

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a landmark study on unmedicated patients with major depression (divided into three groups: previous suicide attempts of low lethality, previous suicide attempts of high lethality, and no history of suicide attempts) and healthy controls. They reported that the subjects with high-lethality suicide attempt in the past had poorer results on all measures of executive functioning, when compared to all other 3 groups (2 groups of patients and healthy controls) [149]. Also, on tests of memory, general intellectual functioning, and attention, the group of patients with high-lethality previous suicide attempt was the only one that had performed significantly worse than healthy controls [149]. These results suggested that impairment of executive functioning, beyond that typically found in major depression, was present in the depressed patients with high-lethality prior suicide attempt [149], indicating that widespread cognitive deficits may well be a risk factor for severe suicide attempt in the context of depression [149]. Moreover, attention deficits in patients with depression have also been linked to suicidal behavior [150].

Jollant et al. [151] hypothesized that during periods of increased stress, suicidal crises could be facilitated by dysfunction in three neuropsychological domains: facilitation of acts in emotional contexts, altered modulation of value attribution, and reduced regulation of cognitive and emotional responses. These neuropsychological domains translate clinically into: a) facilitation of acts in emotional contexts—challenges or impulsivity associated with disinhibiting behavior; b) value attribution—increased sensitivity to others, the environment; and c) reduced regulation of emotional and cognitive responses—poor problem-solving skills and lack of cognitive flexibility [151, 152]. Jollant et al. (2011) supported this hypothesis with neuroanatomical and neuropsychological findings from a review of various studies examining relationships of neuroanatomical alterations with neuropsychological domains and SSDV [151].

Facilitation of acts

Disinhibition and impulsivity are characteristic of TBI. Some of the features of the neuropsychological syndrome associated with TBI are impulsivity, environmental dependency, a lack of empathy, aggressive or abusive behavior, selfishness, and lability [148, 153–156]. This syndrome could be understood best as an inability to integrate feedback into one's behavior in a meaningful way and as a problem with impulse control [157]. Damage to the orbitofrontal region that mediates social comportment and intelligence mediates this behavioral syndrome [155, 158, 159]. Furthermore, facilitation of acts in an emotional context are facilitated by impulsiveness and disinhibition, which could lead to violent behavior directed toward others or self [151]. Research has identified that, in comparison to individuals with other injuries, subjects with a history of TBI have increased aggression toward others [160]. Moreover, individuals with TBI to frontal lobes were found to have greater rates of aggression, compared to the individuals who had TBI located elsewhere in the brain [160]. Additionally, diminished capacity for empathy has also been reported in individuals with TBI [154]. In addition to various premorbid conditions, many other factors might influence violent behavior post-TBI. However, an individual's ability to inhibit such behaviors might be lowered by an injury to the orbitofrontal cortex [161].

A similar pattern of a decreased ability to inhibit behavior might also follow in SSDV, including death by suicide, via increased aggression [162], increased disinhibition, and altered impulse control [71]. Emphasized by a possible interplay with the deficits in emotional regulation and value attribution (as described below), the orbitofrontal cortex is also activated by social exclusion, as well as other situations involving social rejection and self-blame [163]. Thus, in an individual who may already have a tendency towards increased perseveration about the external environment or other triggers, the diminished ability to inhibit SSDV may enable a suicidal act. Impulsivity and a higher rate of aggression both have been recognized as the endophenotypes for suicidal behavior [71] and can result from TBI-mediated orbitofrontal cortical damage.

Altered modulation of value attribution

Rumination has been found to have a robust association with suicidal behavior and suicidal ideation, as well as with impaired problem-solving ability, depression, and hopelessness [164]. In a study by Homaifar et al. (2012), veterans with a history of TBI and no suicide attempt had significantly fewer perseverative errors on the Wisconsin Card Sorting Test compared to the veterans with a history of TBI and at least one suicide attempt [165]. In fact, the suicide attempters with reduced problem-solving ability have been reported to have 'cognitive rigidity' [166]. In turn, the ability to precisely assign value

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to internal and external events might be affected by increased rumination [151]. Interestingly, the anterior and posterior medial prefrontal cortex (PFC) areas that are known to be frequently damaged in TBI [145] have also been correlated with rumination and self-referential processes that characterize rumination [167, 168]. In individuals having suicidal ideation, there may be difficulties in regulation of rumination, secondary to disruption of these areas in the brain via TBI. Thus, TBI-related perseverative errors might hinder attending to goal-directed behavior, as the individual would be unable to stop ruminating over suicidal thoughts, which may potentially influence the higher risk of suicide in TBI patients. However, more research is needed in this area, as most of the studies highlighted above had small sample sizes and did not effectively take into consideration various preexisting comorbidities, including those that could be a consequence of TBI, including depression.

Impaired regulation of emotional and cognitive responses

One of the identifying features of TBI is impaired regulation of cognitive and emotional responses, which includes depression [169] and emotional instability or lability [170]. Post-TBI, individuals may have increased vulnerability for suicidal behaviors and suicidal ideation, due to their impaired ability to regulate their cognitive and emotional responses. Executive functions, such as problem-solving ability and cognitive flexibility, which also have been implicated in risk for suicidality [152], are further disrupted by impaired emotional regulation induced by TBI. The anterior cingulate cortex, an area that, due to its location, is often damaged in TBI, is primarily believed to be involved in emotional regulation [171]. TBI may also lead to disruption in working memory [172, 173] and prolonged negative emotional states, secondary to increased demands to regulate emotional and cognitive processes. Disruption in working memory in individuals with TBI would require differential and compensatory recruitment of more areas in the brain to complete tasks assigned to them. In fact, in comparison with normal subjects, individuals with moderate to severe TBI have blood flow abnormalities in their frontal lobes on functional magnetic resonance imaging, indicating that, in order to complete the same working memory task, they need to recruit more areas in their brain [173]. Mild TBI also elicits this need for increased activation of frontal lobes during subjects' task completion [174]. Also, the medial PFC has been reported to have a higher activity related to negative moral feelings, including shame, guilt, and embarrassment [175].

In addition to involvement of areas of the brain located more medially in the frontal lobe, laterally located areas, such as dorsolateral and ventrolateral PFC, may also be damaged in TBI and potentially, the lesions in these areas may lead to dysexecutive syndrome [176]. In this syndrome, impairments in monitoring, organization, set-shifting, planning, and reasoning often co-occur [177]. Researchers have also identified that individuals with TBI have decision-making problems on the Iowa Gambling Test [178]. Compounding the deficits in working memory and emotional regulation due to medial PFC damage, injury to lateral PFC leads to difficulty in 'set-shifting' and contributes to increased rumination [167, 168, 176, 177]. Additionally, greater problems with rumination also may be partially exacerbated by impaired working memory. Poor working memory, which likely exacerbates the already impaired regulation of cognitive and emotional responses, and increased rumination in individuals with TBI, likely increases their risk for suicidal behaviors and ideation.

In summary, the person's inability to regulate cognitive and emotional responses, combined with facilitation of acts and altered modulation of value attribution due to the frontotemporal injuries commonly seen in TBI, might together explain the increased risk for suicidal thoughts and behaviors in individuals with a history of TBI. Hence, in individuals with a history of moderate and severe TBI, all these factors could interplay together and contribute to an increased risk for SSDV [151].

RELATIONSHIP BETWEEN ALZHEIMER'S DISEASE, TBI, AND SUICIDAL BEHAVIOR

Given the aims and scope of the *Journal of Alzheimer's Disease*, we also reviewed the relationship between Alzheimer's disease (AD), TBI, and suicidal behavior. AD, a chronic neurodegenerative disorder, is the leading cause of dementia in humans [179]. Intracellular aggregates of aberrantly phosphorylated tau protein, called neurofibrillary tangles, together with extracellular deposits of amyloid-β, called senile plaques, constitute the two major neuropathological features of AD [180, 181].

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Association between TBI and dementia

A robust association between a single moderate to severe TBI and increased risk of developing progressive cognitive deficits, which may ultimately lead to a later-onset of dementia, has been demonstrated in a number of epidemiological studies [182–193]. However, such an association was not found in several other epidemiological reports, with results possibly explained by recall bias inherent in the retrospective design of these studies [194–202]. Nevertheless, prospective studies support the notion of TBI being a risk factor for dementia [190, 192]. Also, the idea of a 'dose-response' relationship between TBI and dementia has emerged, described as increased risk of dementia in individuals with a history of loss of consciousness versus without loss of consciousness [188], as well as increased risk of dementia in survivors of severe versus moderate TBI [190]. Evidence also indicates that onset of dementia may be accelerated by a history of TBI [189, 203–205]. Frequently, dementia associated with TBI has been reported as being of the Alzheimer-type [206].

Histopathological features of TBI and AD

The common pathological features of TBI and AD also support the fact that TBI may precipitate a dementia-like process in the brain. Survival from a single TBI is frequently characterized by generalized brain atrophy, which can be observed 6 months after the injury and may continue to progress for a number of years [207–211]. Histological features of acute TBI, however, are not similar to those of the tau pathology in CTE resulting from chronic repetitive mild TBI [212]. In a study by Johnson et al. (2012), age-matched controls were compared to 39 survivors from a single moderate to severe TBI after $\geq$1 year of original injury [213]. The authors reported that about 30% of the TBI survivors had a wider distribution and greater density of neurofibrillary tangles, which were observed in a hierarchical distribution similar to that of AD [213]. In addition, acute and chronic neuronal loss and white matter degeneration are also characteristics of all severities of TBI [214].

Increased risk of death by suicide in dementia

As discussed in earlier sections, similar to the increased risk of death by suicide in individuals with TBI, subjects with dementia have also been reported to have a higher risk of death by suicide [215]. In a Danish register-based study, Erlangsen et al. [215] evaluated the records of about 2.5 million participants aged >50 years over a period of 11 years and found that an elevated risk for death by suicide was present in older adults, who were diagnosed with dementia during their hospitalization. The authors reported that death by suicide occurred in 136 persons who had been previously diagnosed with dementia [215]. Also, relative risk of death by suicide was higher in women (10.8 [95% CI, 7.4–15.7]) compared to men (8.5 [95% CI, 6.3–11.3]) aged 50–69 years, both having hospital presentations with dementia [215]. A three-times higher risk of death by suicide was identified in subjects $\geq$70 years old with dementia, when compared to subjects without dementia of similar age [215]. The risk for death by suicide among subjects with dementia remained significant after controlling for mood disorders. Heightened risk for death by suicide was associated with the time shortly after the diagnosis of dementia, with about 14% of women and 26% of men dying by suicide within the first 3 months after the diagnosis [215]. Later, more than 3 years after the diagnosis, about 41% of women and 38% of men had died by suicide [215]. It has been proposed that alterations in serotonergic function in the brain of patients with dementia may lead, at least in part, to suicidal behavior via impulsive aggression [108, 216, 217]. Also, increased insight into the disorder, may itself lead to increased hopelessness [218] and hence, elevate the risk for suicidal behavior [219]. Interestingly, a bidirectional positive association between insight and depression has also been reported in individuals with TBI and may partly explain the increased risk of suicidal behavior associated with TBI [220]. Likewise, other types of dementia, such as Huntington's disease [221] and frontotemporal dementia [222], have also been associated with suicidal behavior. As highlighted above, both TBI and AD (along with other types of dementia) are characterized by cognitive deficits, including executive dysfunction [25, 149, 223] and deficits in decision-making capacity [148, 178, 224–227], which in turn have been related to suicidal behavior.

OTHER MODERATING FACTORS IN THE RELATIONSHIP BETWEEN TBI AND SUICIDAL BEHAVIOR

TBI is frequently accompanied by disruption in sleep [228, 229], which may worsen or lead to a number of co-morbid conditions like cognitive deficits, depression, irritability, anxiety, fatigue, functional

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impairments and pain [230, 231]. Additionally, sleep disruption may decrease an individual's quality of life by impeding an individual's return to baseline functioning and by compromising his/her recovery [229–232]. Co-morbid conditions associated with TBI (like depression) [233], and trauma-induced biochemical and physiological changes [230] could lead to sleep disruption, which could also be further influenced by sleep issues present prior to TBI [234]. In a meta-analysis of 21 studies, Mathias and Alvaro [235] reported that after TBI, about 50% of individuals reported some form of sleep disturbance. Also, prevalence rates of a diagnosed sleep disorder, including obstructive sleep apnea, insomnia, and hypersomnia, were about 25–29% and were far greater in the individuals with TBI compared to the rates observed in the population in general [235]. Also, early awakenings, nightmares, problems with sleep efficiency and maintenance, sleep walking, and excessive sleepiness were 2–4 times more likely to be present in individuals with TBI [235].

Similarly, a number of commentaries [236, 237] and review articles [238–240] have emphasized the possible association between disrupted sleep and suicidal behavior/ideation, with nightmares and insomnia being the most frequently implicated sleep disturbances that had an association with suicidal behavior/ideation [241]. In fact, a number of medical and psychiatric conditions that are associated with suicide (e.g., major depressive disorder) [242] are also associated with sleep disturbances [243]. In a meta-analysis of 39 studies, Pigeon et al. [241] reported that, in unadjusted studies, the relative risk for death by suicide, suicide attempt and suicidal ideation together, was significantly higher (ranging from 1.95 [95% CI, 1.41–2.69] to 2.95 [95% CI, 2.48–3.50]) in individuals having a sleep disturbance. Similarly, in adjusted studies, these associations remained highly significant; however, they were smaller in magnitude [241]. Also, the association between suicide variables and sleep disturbances was not moderated by depression [241]. The authors speculated that the cognitive deficits related to sleep problems might lead to increased negative rumination, impulsivity and behavioral disinhibition, and potentially may lead to suicidal behaviors and thoughts [241]. Since TBI has also been associated with cognitive deficits [151], it could be possible that this association is moderated by sleep disturbances, at least partly, which could potentially lead to death by suicide, suicidal behaviors and suicidal thoughts.

TBI also has been related to higher scores on measures of cognitive and personality traits, such as aggression and impulsivity [148, 224, 244–246], impaired decision-making [148, 224–226], and increased sense of hopelessness [137], all of which have been linked to death by suicide.

PATHOPHYSIOLOGY OF TBI AND THE ROLE OF INFLAMMATION IN ITS RELATIONSHIP TO SUICIDAL BEHAVIOR

Mechanisms underlying the injuries induced by impact(s) on the head are believed to result from the brain being pushed against the sides of the cranium, and thus, leading to two types of injuries: 1) direct injury at the location of impact; and 2) indirect injury at the opposite side of the cranium, relative to the location of the impact [145]. Since the human skull is inflexible and hard in nature, susceptibility to direct injury is particularly higher along the temporal and frontal lobes of the brain, as here the anterior and middle fossae are divided by the sphenoid bone, which could lead to frequently observed TBI-related deficits in frontal and temporal lobe functions [247]. Rotational forces incurred at the time of injury could also lead to shear injury [248]. Diffuse axonal injury can result from these rotational forces, which might include injury at multiple locations in the brain [249]. A surge in the discharge of excitotoxic chemicals, vascular compromise and edema [145] might further influence the emotional, cognitive and physical compromise seen after TBI [250].

Outcome after a TBI depends upon: 1) severity of the early "primary injury" and the magnitude of other associated factors, such as intracranial fluid dynamics, hypotension, and hypoxemia [251]; and 2) "secondary injury" manifested by activation of damaging molecular cascades [252]. Distinct pathways comprise the "secondary injury," which include: 1) glutamate-induced excitotoxicity [253]; 2) systemic and local inflammation [254]; and 3) free radical production leading to damage of neurites and neuronal membranes [255].

Inflammation and TBI

TBI consistently has been linked with neuroinflammation, in spite of its pathophysiologic heterogeneity [251, 256–258]. Moreover, TBI is used in animal models to induce neuroinflammation [259, 260].

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Inflammation itself might play a role, at least partly, in mediating the greater risk of SSDV in TBI, as even after adjusting for depression and its severity, inflammation has been linked predictively to SSDV [256–258]. Even though activation of an immune response is required for healing post-TBI, a poor prognosis is associated with prolonged and intense inflammation after TBI [261–263]. Tardive changes, such as neuronal cell death, delayed neuroinflammation, systemic inflammation, and impaired neurological function are also induced by TBI [263, 264]. Head trauma results in highly complex molecular and cellular responses, with the neuroinflammatory response being responsible for much of this complexity [251, 265]. Injury to the cerebral vascular system [266], a high degree of disruption of the blood-brain barrier [267] (allowing peripheral inflammation to spread easily in the central nervous system), and damage to the white matter tracts [268] are some of the other more specific factors that differentiate inflammation following TBI from other causes of neuroinflammation.

A primed profile of microglia is sometimes established during the post-acute neuroinflammation phase, which is comprised of long-term changes in microglial reactivity, mobility, morphological and biochemical properties [269], and an increased MHC II mRNA and protein expression [270, 271]. TBI, via production of a proinflammatory response, can cause a decrease in the priming threshold for microglial cells [272, 273], or might trigger previously primed microglial cells. A secondary immune challenge, such as allergy, autoimmune disease, or infection, which may be encountered by the already primed microglial cells, could lead to a higher risk for SSDV, as a prolonged and ample state of immune activation might result from these secondary insults that could affect neurons, neurotransmitters and neuronal circuits. Studies in rodents have demonstrated that cognitive deficits [274] and affective dysregulation develop if an immune challenge follows a vulnerable period after TBI [270].

Interestingly, pathophysiology of AD may also involve a significant role of neuroinflammation, especially early in the course of this illness [275–280]. Specifically, clusters of microglia around amyloid plaques, as well as a wide-variety of inflammatory proteins, such as proinflammatory cytokines, acute-phase proteins and complement factors, have been shown to be present in both human subjects with AD and its transgenic models [279]. Also, the pathophysiology of TBI and AD overlap in terms of oxidative stress [281–287], and the double-hit concept of neuronal damage [288–290]. Risk of AD is increased by certain single nucleotide polymorphisms in the genes that encode microglia-specific proteins, which are involved in protein degradation pathways, as well as phagocytosis [291, 292]. Neuroinflammatory response is further aggravated by accumulation of reactive astrocytes around the fibrillary amyloid plaques [293], which operate similarly to microglia, in terms of releasing potentially cytotoxic molecules and cytokines after being exposed to amyloid-β [280]. Increasing age has also been strongly associated with the prevalence of AD, and changes in microglia related to aging have also been postulated to be involved in its pathology [294]. Recent reports have also highlighted a reduction in coverage of brain tissue by microglial cell processes, as well as shortening of microglial cell processes in AD and normal aging [295]. Both experimental and clinical studies have reported a shift towards a pro-inflammatory state in the aged brain [272, 296]. Higher levels of circulating cytokines, like interleukin (IL)-1β, interferon γ and tumor necrosis factor (TNF), in association with an increased microglial and astrocyte reactivity, have been characteristically seen in age-associated neuroinflammation [273, 297].

Inflammation, depression, and suicidal behavior

An increasing amount of evidence has linked inflammation and depressive symptomatology [257, 298–300]. Multiple animal models have also confirmed a causal relationship between depressive-like symptoms and inflammation [301–306]. Recent meta-analyses have concluded that there is also evidence for immune activation in the blood and cerebrospinal fluid of suicide attempters and in post-mortem brain samples of individuals who died by suicide [307–309]. The senior author's (TP) team was the first to identify mRNA transcripts of cytokines in the orbitofrontal cortex of suicide victims [310]. The cytokine profile in this study was consistent with the results from the senior author's reports on outcomes in rodents sensitized and exposed to aeroallergens, including 1) increased prefrontal cortex cytokine mRNA expression [311], 2) impairment in social interactions [311], and 3) aggressive-behavior after stress [312], mimicking risk factors and endophenotypes of SSDV [71]. Endophenotypes associated with suicidal behavior, such as aggression [81], have

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been exacerbated (at least partially) by the effects of individual cytokines [301, 303, 313]. A second postmortem study in the same year found pronounced microgliosis in the brains of patients who died by suicide [314]. More recently, increased levels of IL-1β, IL-6, and TNF, cytokines that are excessively produced by primed microglia, were observed at both the mRNA and protein levels in the anterior prefrontal cortex of teenagers who died by suicide [315]. Also, significantly elevated levels of IL-6 in the cerebrospinal fluid of suicide attempters further supports the role of neuroinflammation in contributing to suicidal behavior [316]. Additionally, the protein and mRNA expression of certain Toll-like receptors, which are involved in the production of cytokines, has also been reported to be increased in depressed individuals who died by suicide, as compared to the normal control subjects [317]. Moreover, an increased gene expression of these cytokines in rodents after a peripheral immune challenge has also been reported by a team led by the senior author [312]. In fact, the exaggerated glucocorticoid responses to stress observed after this immune challenge [312] are one of the central and highly replicated pathophysiological features of suicide attempters in comparison to those of non-attempter psychiatric controls [71].

How inflammation triggered by TBI can lead to suicidal behavior?

Transport of tryptophan through the blood-brain barrier occurs via a transporter for large neutral amino acids. Tryptophan is metabolized in the brain through two pathways, which involve synthesis of either kynurenines or serotonin. The rate-limiting enzymes involved in metabolism of tryptophan in the kynurenine pathway are indoleamine-2, 3-dioxygenase (IDO) and tryptophan-2, 3-dioxygenase. Inflammation within the brain induces the expression of IDO. This further increases the production of metabolites downstream along this pathway, including that of kynurenic acid and quinolinic acid [318]. Kynurenic acid serves as a free radical scavenger and antagonizes glutamate to confer neuroprotection. However, quinolinic acid is an agonist at N-methyl-d-aspartate (NMDA) receptors and produces free radicals, which ultimately lead to toxic effects on neurons. The macrophages that infiltrate the brain along with the microglia primarily synthesize quinolinic acid and additional compounds that are toxic. This sequence of events exemplifies the association between inflammation in the nervous system and the degeneration of neurons via activation of the kynurenine pathway [318].

Studies in human and animal models have postulated that pathogenesis of depression may be mediated by NMDA receptors, as well [319–321]. Data from other studies have indicated that disruption of glutamate neurotransmission might be involved in the pathogenesis of depressive symptomatology [322, 323]. A case-control study demonstrated considerably increased levels of glutamate in the occipital cortex of the participants who had depression and who did not receive any medications. These participants underwent comparison with healthy controls who were matched according to gender and age [324]. In fact, a postmortem study that assessed the differences in glutamate receptors in the frontal cortices of human suicide victims versus those of controls who died of sudden death due to causes other than suicide, reported that dysfunction of glutamate receptors may also be implicated in the pathophysiology of suicide [321]. A study performed by Paul et al. (1994) reported that mice that received 17 distinct antidepressant medications/treatments [including citalopram, electroconvulsive shock, imipramine, monoamine oxidase inhibitors, and selective serotonin reuptake inhibitors (SSRI)] over two weeks, as opposed to those that received the medication/treatment for only one day, had adaptive alterations in the NMDA receptor-radioligand binding. These effects were mostly limited to the cerebral cortex [325]. Such constancy of effects from different antidepressant medications and treatments points towards the fact that all antidepressants might act via a similar mechanism that involves adaptive alterations in NMDA receptors [319]. Studies even have shown that Riluzole and Lamotrigine, both of which inhibit release of glutamate, also have effects similar to antidepressants [326, 327]. Also, studies have reported that patients with major depression who had received sub-anesthetic doses of intravenous ketamine, which has antagonistic activity on NMDA receptors, had rapid improvement in their symptoms of depression [328, 329] and suicidal ideation in the setting of major depression [330]. Moreover, Mackay et al. (2006) reported a sustained increase in activation of the kynurenine pathway for a least 1 year or longer after TBI [331]. In essence, TBI may set forth an inflammatory cascade, which upregulates the levels of free radicals and quinolinic acid in the brain, leading to neuronal damage and potentially, to suicidal behavior.

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STRATEGIES FOR REDUCING POST-TBI INCREASED RISK FOR SUICIDAL BEHAVIOR

Since TBI has been recognized as a risk factor for death by suicide, it becomes vital not only to modify the clinical protocols accordingly to incorporate suicide risk assessment in patients who pursue services for rehabilitation, but also to screen for TBI in people who seek psychiatric care. Nationwide suicide rates are increasing, despite the efforts to manage SSDV [332]. Likewise, many treatments for TBI were not effective in clinical studies, despite showing promise in preclinical studies. This discrepancy might have been due to the fact that therapeutic approaches in both SSDV and TBI did not consider the combination of risk factors for distinct subgroups in need of targeted prevention and treatment and also did not address treatment targets based on their pathophysiology.

Managing risk for suicidal behavior in general with/without TBI

Clinical trials for managing suicide risk acutely have led to emergence of new treatments. Previously, suicidal patients with severe or treatment-resistant depression usually received a course of electroconvulsive therapy that had a short-term life-saving effect [333]. Acute anti-suicidal effects of a subanesthetic dose of ketamine, lasting for at least 1 week, were reported less than 10 years ago [330, 334, 335]. As most antidepressant medications augment the noradrenergic and/or serotonergic functions, and since disturbances in both these neurotransmitter systems have been associated with suicidal behaviors [336–339], it is reasonable to expect an anti-suicidal effect with these medications. However, there is a dearth of direct evidence for such an effect. In a pilot double-blind, randomized, clinical trial, treatment with paroxetine (an SSRI) was reported to be associated with a greater reduction in suicidal ideation and a better antidepressant effect than bupropion (a noradrenergic–dopaminergic drug), with the patients having the most severe suicidal ideation achieving the greatest therapeutic advantage [340]. According to a systematic review [341], antidepressants decrease suicidal ideation, as well as suicidal behavior in depressed patients, and various research strategies together provide reasonable evidence to support this claim. In this review, it was further stated that well-designed epidemiological studies have provided evidence for the prophylactic effect of antidepressants on suicidal behavior, in particular, death by suicide [341]. Randomized control group studies, with some utilizing a placebo arm, provide evidence for the beneficial effects of antidepressants on suicidal ideation [341]. Similarly, Rihmer and Akiskal (2006) reported that, in most countries with traditionally high suicide rates, the widespread use of antidepressants appeared to lead to a highly significant decline in suicide rates, with the decline being more prominent in women compared to men [342].

Counterintuitively, in children and adolescents, a pro-suicidal effect of antidepressant medications has been reported [343]. However, only scarce direct evidence is available that supports a pro-suicidal effect of antidepressants, and later studies have pointed out that this effect is actually less likely [344–346]. Effects of lithium, antipsychotic drugs, and mood stabilizers on suicidal behavior have also been debated by researchers [347]. It is likely that there has been an overestimation of harmful effects on suicide risk by antiepileptic drugs [347]. Reduction of the risk for fatal and non-fatal suicidal behavior by lithium is supported by a sizeable amount of evidence [348]. Protective effect of lithium on suicide may be related to its anti-apoptotic effects [349], serotonin-enhancing properties [350], and its potential to increase the volume of gray matter after its long-term use [351]. In patients with schizophrenia, compared to olanzapine, clozapine has a protective effect on suicidal behavior [352]. Anti-suicidal effects of both clozapine [353] and lithium [354] are independent of their effectiveness as an anti-psychotic and as a mood-stabilizer respectively. These drugs' specific efficacy profiles possibly indicate that both these medications alter the diathesis, at least partially, to decrease suicide-risk [120].

Managing risk for suicidal behavior specifically in individuals with TBI

It is now well established that individuals with TBI have both an increased risk of death by suicide and greater prevalence rates for suicide attempt and suicidal ideation. Consequently, efforts to screen and prevent suicide should begin at the time of the intake procedure by the rehabilitation clinicians who should also continue with regular follow-ups, especially during phases of transition or stress. Additionally, to identify possible risk factors related to death by suicide, the clinicians engaged in providing psychiatric care should acquire information related to the lifetime

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history of TBI in their patients [355]. The Rocky Mountain Mental Illness Research Education and Clinical Center TBI Toolkit [356] not only delineates assessment procedures that should be followed by mental health and rehabilitation clinicians, but it also highlights the clinical and research potential of the evidence-based screening tools included in this kit.

Pharmacological approaches

Limited evidence is available regarding the effectiveness of pharmacological agents that can mitigate certain risk factors associated with an increased risk of death by suicide in victims of TBI. In a double blind, parallel-group trial, patients with mild to moderate TBI matched for gender, education, and age were randomly assigned to receive either methylphenidate, sertraline, or placebo, in order to compare their effects on the neuropsychiatric sequelae in TBI [357]. Results indicated that both methylphenidate (5–20 mg/day) and sertraline (25–100 mg/day) had significant effects on reducing symptoms of depression; however, methylphenidate appeared to be more useful in improving the daytime sleepiness and cognitive function [357]. Similarly, Zhang and Wang [358] reported that compared to placebo, methylphenidate (5–20 mg/day) significantly improved cognitive deficits and depressive symptoms in patients with TBI. In fact, long-term treatment with methylphenidate (short-acting methylphenidate: 15–90 mg/day, and long-acting methylphenidate: 70 mg/day) has also been reported to significantly improve cognitive functions in individuals with TBI [359]. As discussed in the sections above, cognitive dysfunction, in turn, has been linked to death by suicide. Hence, methylphenidate may, at least indirectly, reduce the risk of death by suicide in patients with TBI who also have cognitive deficits. Methylphenidate (single-dose of 30 mg/day) also has been reported to partially restore the response inhibition in patients with TBI [360], which could reduce both impulsive behavior and the risk for death by suicide. On the other hand, methylphenidate, as well as other stimulants, may also precipitate or exacerbate insomnia [361, 362], which, as described above, is also risk factor for death by suicide.

Evidence from a retrospective study supported improvement, post-administration of 200–400 mg of amantadine, in cognitive and/or motor functions in children during the acute post-TBI phase [363]. A non-blinded, randomized controlled trial demonstrated that a 12-week course of 150–200 mg of amantadine improved the behavioral symptoms in pediatric study subjects and that the most gain in cognitive performance occurred in individuals who had sustained TBI in the period of $\leq$2 years from the administration of amantadine [364]. More recently, in a randomized, placebo-controlled, parallel-group and double-blind study, chronic problems with irritability and aggression secondary to TBI were also reported to have improved with administration of amantadine 100 mg by mouth twice daily (morning and noon time) [365]. However, in a larger sample, upon doing multiple comparisons, this treatment effect was not statistically significant compared to placebo [366]. The authors speculated that observer bias masked the treatment effect [366].

In a 6-week open label pilot study, researchers demonstrated that quetiapine, at dosages between 25 to 300 mg daily, was well-tolerated and effective in decreasing aggression (an endophenotype of death by suicide) and irritability, secondary to TBI [367]. Cognitive functioning also improved after administration of quetiapine in patients who developed aggressive symptoms post-injury [367].

Sertraline has been the most common SSRI studied for the efficacy of treating post-TBI depressive symptoms. Results have been mixed, with studies showing significant improvement in depressive symptoms [368, 369] to no significant improvement in depressive symptoms [370] compared to placebo intervention. Interestingly, in a randomized control trial, Jorge et al. [371] reported that, in patients following TBI, sertraline may be effective in preventing the onset of depressive disorders. In an open label study, moclobemide was reported to rapidly reduce the symptoms of depression and anxiety in patients with TBI [372].

Following TBI, sleep/wake disorders can develop frequently [373]. In a single-blind, prospective, longitudinal study, treatment for sleep-wake disorders was reported to be associated with statistically significant improvements in depressive symptoms, speed of language processing, and language skills [374], indicating improvement in cognitive domains as well as mood, thereby possibly lowering the risk for death by suicide. Melatonin [375] has shown promise in treating sleep disturbance in TBI patients.

Non-pharmacological approaches

Certain non-pharmacological measures have also shown promise in reducing the risk factors for death by suicide in individuals with TBI. Bahraini et al. [135] identified only one randomized clinical trial that utilized a suicide prevention intervention,

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based on application of cognitive behavioral therapy in a group setting, targeting individuals with a history of TBI. Significantly decreased hopelessness scores were reported post-treatment ($p = 0.03$) with maintenance of treatment effects at a 3-month follow-up period [135]. This group-based intervention was named Window to Hope [376] and was recently adapted for use with US veterans [377, 378]. However, more research is needed to determine the effectiveness and efficacy of Window to Hope in preventing suicide.

In individuals with TBI, various forms of exercise or physical activity have been shown to improve mood/depressive symptoms [379–382], cognitive functions [383, 384], perceived stress [382] and sleep quality [384], all of which may contribute to reducing the risk for death by suicide. Notably, improvement of depressive symptoms with exercise in TBI patients probably has a dose-dependent relationship with less depressive symptoms in individuals who exercise for greater duration per week [379, 380]. Cognitive behavioral therapy (CBT) [385] and acupuncture [386] have shown promise in treating sleep disturbance in TBI patients, which can also help lower their risk for death by suicide.

Preliminary evidence suggests that low pressure (1.5 atmospheres absolute) hyperbaric oxygen therapy (HBOT) in military participants having mild to moderate chronic blast-induced TBI was associated with significant improvements in cognitive functions, impulsivity, depression, anxiety and in quality of life [387]. These effects were consistent with single photon emission computed tomography (SPECT) brain imaging in the same participants [387], as demonstrated by 1) significant post-HBOT improvement in SPECT statistical parametric mapping analysis (diffuse improvements in regional cerebral blood flow after 1 and 40 HBOT sessions), 2) SPECT coefficient of variation in all white matter and some gray matter regions of interest (ROIs) after the first HBOT, and 3) half of white matter ROIs after 40 HBOT sessions.

Fairly robust evidence from a number of studies supports the therapeutic benefits of CBT for mitigating symptoms of depression after TBI when combined/not-combined with other psychotherapeutic interventions, including motivational interviewing and non-directive counseling and cognitive remediation [388–390]. Especially in veteran populations, a psychotherapeutic intervention called Cognitive Symptom Management and Rehabilitation Therapy (CogSMART), in combination with supported employment, has been reported to improve prospective memory functioning ($d = 0.72$; i.e., moderate-large effect size), post-concussive symptoms ($d = 0.97$; i.e., large to very-large effect size), and severity of depressive symptoms ($d = 0.35$–0.49; i.e., small to medium effect size) [391]. CBT, which can be administered in person or by telephone [388], has not been shown to be more efficacious than supportive psychotherapy for treating depressive symptoms secondary to TBI [392]. However, Potter et al. [393] reported that depressive symptoms did not improve with CBT in TBI patients. However, improvements in persistent post-concussion symptoms, including anxiety, fatigue and quality of life, were reported in TBI patients who had completed CBT sessions over a shorter period of time [393].

Social peer-mentoring intervention in patients with TBI was reported to be associated with a significant improvement in perceived social support and increased satisfaction with social life, but counterintuitively, it was associated with an increase in depressive symptoms [394]. Other psychotherapeutic interventions, such as positive psychology interventions [395], scheduled telephone-based intervention [396], self-awareness group therapy [397], and Strategic Memory Advanced Reasoning Training [398], have also shown promise in treating mood/depressive symptoms and cognitive deficits following TBI.

Motto and Bostrom [399] conducted a randomized control trial with individuals who had refused to comply with ongoing care after hospitalization for a depressive or a suicidal state and who were known to be at risk of subsequent death by suicide. The outreach intervention involved mailing letters to the participants in the treatment group. Controls did not receive any follow-up contact. A statistically significant lower risk of death by suicide was reported in the treatment group but was limited to the first 2 years of the five year-long study [399]. Investigators hypothesized that this outreach intervention prevented deaths by suicide by increasing belongingness or reducing thwarted belongingness in the treatment group [400].

Thus, in order to enhance the wellbeing and health of individuals with a history of TBI and to reduce their risk of death by suicide, collaborative efforts between the health-care professionals are necessary, while using both pharmacological and non-pharmacological approaches. Experimental research in common pathways involved in TBI, including the role of neuroinflammation in suicidal behavior, may lead to designing future treatment targets for clinical trials, with individualization of

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treatment approaches. Future research should investigate strategies to boost utilization of mental health care services in high-risk groups (such as veterans), identify alleviating factors that could decrease risk for suicide, and examine post-TBI-related suicidal behavior and the mental health care services that are needed subsequently. Specific targeting of certain promising mechanisms, such as inflammation and the kynurenine pathway, requires significant investment in animal modeling suicide endophenotypes and in randomized experimental interventional paradigms in patients with TBI.

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Elevated Plasma MCP-1 Concentration Following Traumatic Brain Injury as a Potential "Predisposition" Factor Associated with an Increased Risk for Subsequent Development of Alzheimer's Disease

Lap Ho[a,1], Wei Zhao[a,1], Kristen Dams-O'Connor[b], Cheuk Y. Tang[c], Wayne Gordon[b], Elaine R. Peskind[e], Shrishailam Yemul[a], Vahram Haroutunian[c] and Giulio Maria Pasinetti[a,d,*]
[a]*Department of Neurology, Mount Sinai School of Medicine, New York, NY, USA*
[b]*Department of Rehabilitation Medicine, Mount Sinai School of Medicine, New York, NY, USA*
[c]*Department of Psychiatry, Mount Sinai School of Medicine, New York, NY, USA*
[d]*GRECC, James J. Peters Veterans Affairs Medical Center, Bronx, NY, USA*
[e]*VA Northwest Network Mental Illness Research, Education, and Clinical Center and Department of Psychiatry and Behavioral Sciences, University of Washington School of Medicine, Seattle, WA, USA*

Abstract. We explored whether changes in the expression profile of peripheral blood plasma proteins may provide a clinical, readily accessible "window" into the brain, reflecting molecular alterations following traumatic brain injury (TBI) that might contribute to TBI complications. We recruited fourteen TBI and ten control civilian participants for the study, and also analyzed banked plasma specimens from 20 veterans with TBI and 20 control cases. Using antibody arrays and ELISA assays, we explored differentially-regulated protein species in the plasma of TBI compared to healthy controls from the two independent cohorts. We found three protein biomarker species, monocyte chemotactic protein-1 (MCP-1), insulin-like growth factor-binding protein-3, and epidermal growth factor receptor, that are differentially regulated in plasma specimens of the TBI cases. A three-biomarker panel using all three proteins provides the best potential criterion for separating TBI and control cases. Plasma MCP-1 contents are correlated with the severity of TBI and the index of compromised axonal fiber integrity in the frontal cortex. Based on these findings, we evaluated postmortem brain specimens from 7 mild cognitive impairment (MCI) and 7 neurologically normal cases. We found elevated MCP-1 expression in the frontal cortex of MCI cases that are at high risk for developing Alzheimer's disease. Our findings suggest that additional application of the three-biomarker panel to current diagnostic criteria may lead to improved TBI detection and more sensitive outcome measures for clinical trials. Induction of MCP-1 in response to TBI might be a potential predisposing factor that may increase the risk for development of Alzheimer's disease.

Keywords: Alzheimer's disease, biomarker, long-term clinical TBI phenotypes, mild cognitive impairment, monocyte chemotactic protein-1, plasma, traumatic brain injury

[1]These authors contributed equally to this work.

*Correspondence to: Giulio Maria Pasinetti, MD, PhD, Department of Neurology, Mount Sinai School of Medicine, 1468 Madison Avenue, Annenberg Building, Room 20-02, New York, NY 10029, USA. Tel.: +1 212 241 7938 or +1 212 241 5563; Fax: +1 212 876 9042; E-mail: Giulio.Pasinetti@mssm.edu.

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INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of death and disability in the United States [1]. Somatic and neuropsychiatric complaints, including headache, dizziness, sleep disturbances, hyperarousal, mood disturbances, tinnitus, photosensitivity, oculomotor dysfunction, and pain, are common following TBI. Neurocognitive impairment including impaired memory, attention, concentration, and executive function are observed in individuals with TBI at all levels of severity [2, 3]. While the primary mechanisms of injury are bleeding and diffuse axonal injury [4–6], the pathophysiology of TBI is complex and not well understood.

TBI in the civilian population is typically associated with direct, closed impact mechanical trauma to the brain due to falls, motor vehicle accidents, assault, and sports-related injuries [7]. Among military personnel, particularly among veterans returning from the conflicts in Iraq and Afghanistan, TBI commonly results from exposure to blast pressure waves stemming from explosive devices, leading to symptom profiles similar to those seen in civilians [7–9]. In both military and civilian populations, these symptoms can have a minimal to profound impact on their daily functioning.

Several studies suggest that exposure to moderate-severe TBI is a risk factor for subsequent development of Alzheimer's disease (AD) [10–13], although the association between mild TBI and risk for AD is not yet well established. Neuropathological mechanisms that underlie AD may also be present after TBI, and these mechanisms may contribute to lasting and/or worsening neurological dysfunction. Evidence from human [14–16] and animal models [17] reveals abnormal accumulation of AD pathological hallmarks (e.g., amyloid-β peptides and tau proteins) in the brain and in cerebrospinal fluid following TBI. Moreover, elevation of plasma tau levels has been associated with worse TBI outcomes [18]. Thus, neurological dysfunction in TBI and in AD may share common neuropathological/neurodegenerative mechanisms.

Accumulating evidence has highlighted the association of TBI with defects in neural circuits and synapses, and the plasticity processes controlling these functions [19–24]. While genes relevant to these processes are expressed in the brain, some of these genes are also expressed in peripheral blood [25–28]. Thus, peripheral blood biomarkers may provide insights into the pathogenesis of neurological disorders and be used to monitor disease diagnosis and progression [29–31]. The brain controls many body functions via the release of signaling proteins, and both central and peripheral immune and inflammatory mechanisms are implicated in post TBI impairments [32–36]. Using high throughput antibody arrays and quantitative ELISA assays in the analysis of a civilian and a veteran TBI study cohort, we explored the feasibility of characterizing surrogate biological indices (biomarkers) from circulating blood plasma that are associated with specific impairments secondary to TBI.

MATERIALS AND METHODS

Subject recruitment and assessments

Civilian TBI and control subjects

Fourteen TBI and ten age-, gender-, and education-matched healthy control civilian participants were recruited from the Brain Injury Research Center at Mount Sinai (BIRC-MS). TBI participants sustained a blow to the head followed by loss of consciousness or a period of being dazed and confused. Informed consent was obtained from all subjects in accordance with the Institutional Review Board policies and procedures of the Mount Sinai School of Medicine (MSSM). Demographic information about the TBI and control participants is presented in Table 1.

All participants completed the Brain Injury Screening Questionnaire (BISQ) [37, 38] to comprehensively assess TBI history, i.e., the number and severity of impacts to the head sustained throughout their lifespan. Injury severity of the civilian TBI participants ranged from mild to severe, as classified using a 7-point scale ranging from 1 (no loss of consciousness, no confusion; i.e., no TBI) to 7 (loss of consciousness greater than 4 weeks in duration) [39]. The BISQ also solicits participants' self-report of functional difficulties and symptoms associated with brain injury. In addition, blood plasma was collected and banked for subsequent biomarker studies.

Veteran TBI and control subjects

Banked plasma specimens from 20 male Iraq and Afghanistan veterans with TBI and 20 male control cases were generously provided by the VA North-west Network Mental Illness Research, Education, and Clinical Center (MIRECC) at Veterans Affairs Puget Sound Health Care System (VAPSHCS) and the University of Washington

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Table 1
Characteristics of the civilian TBI study cohort. All TBI participants sustained a blow to the head followed by loss of consciousness or a period of being dazed and confused. Age-, gender-, and education-matched healthy civilians served as controls. Time post injury is the time frame between the occurrence of brain injury and volunteer's participation in this biomarker study. TBI group was characterized by a mean post injury interval of 13.2 ± 12.6 years. There were no significant differences between TBI and control group in terms of age ($p = 0.894$), years of education ($p = 0.831$), or gender distribution ($p = 1.0$). Ethnicity compositions of TBI and control groups were comparable

	Control	TBI
Number of cases	10*	14*
Gender	50% F	50% F
TBI severity		
Mean ± SD	1 ± 0	4.7 ± 1.9
Range	1	2 – 7
Age (mean yrs ± SD) at study participation	40.1 ± 11.5	40.8 ± 12.8
Post injury interval (mean yrs ± SD)	–	13.2 ± 12.6
Education (mean yrs ± SD)	15.6 ± 1.6	15.9 ± 3.4
Ethnicity composition		
White-Caucasian	60%	64%
Black-African American	10%	22%
Hispanic	20%	14%
Asian	10%	–

*The first 8 control and 12 TBI cases recruited were used in initial antibody array studies. Follow-up ELISA assessments of MCP-1 contents were conducted using all 10 Control and 14 TBI cases. Abbreviations: TBI, traumatic brain injury; yrs, years; F, female.

Table 2
Characteristics of the veteran TBI study cohort. TBI cases were comprised of male veterans with documented hazardous duty experience in Iraq and/or Afghanistan during Operation Iraqi Freedom/Operation Enduring Freedom, and all had reported experiencing at least one blast exposure in the war zone that resulted din mild TBI, as defined by American Congress of Rehabilitation Medicine criteria [39]. Age- and gender-matched healthy, cognitively normal male control cases were comprised of Iraq-deployed Veterans ($n = 9$) and community volunteers from the UWADRC ($n = 11$). The TBI group was characterized by a mean post injury interval of 3.9 ± 1.4 years. There were no significant differences between TBI and control group in terms of age ($p = 0.959$). As a group, the control subjects spent more time in education ($p = 0.003$). Ethnicity compositions of TBI and control groups were comparable

	Control	TBI
Number of cases	20	20
gender	100% M	100% M
Age (mean yrs ± SD) at study participation	29.7 ± 6.3	29.8 ± 5.9
Post injury interval (mean yrs ± SD)	–	3.9 ± 1.4
Education (mean yrs ± SD)	16.4 ± 3.3	13.8 ± 1.5
Ethnicity composition		
White	80%	70%
Black	5%	5%
White-Hispanic	5%	10%
Native Hawaiian/Pacific Islander	–	5%
Other or NA	10%	10%

Abbreviations: TBI, traumatic brain injury; M, male.

Alzheimer's Disease Research Center (UWADRC) in Seattle, WA. All Veteran participants recruited from the VAPSHCS had documented hazardous duty experience in Iraq and/or Afghanistan during Operation Iraqi Freedom/Operation Enduring Freedom, and all had reported experiencing at least one blast exposure in the war zone that resulted in mild TBI, as defined by American Congress of Rehabilitation Medicine criteria [39]. Control specimens were banked from age- and gender-matched cognitively-normal cases: nine cognitively-normal Iraq-deployed veterans plus eleven community volunteers recruited from the UWADRC, all of whom were medically healthy and had Mini-Mental State Examination scores of 29.4 ± 1.0 (mean ± standard deviation [range 27–30]), Clinical Dementia Rating (CDR) [40] scores of zero, no evidence or history of cognitive or functional decline (assessed with the National Alzheimer's Coordinating Center Uniform Data Set), and no history of blast exposure or impact head injury. Demographic information about the veteran TBI and control participants is presented in Table 2.

Plasma collection and antibody array

Freshly collected and banked plasma specimens were stored at —80°C. Antibody array studies were conducted using plasma specimens from the 20 cases (8 Control and 12 TBI cases) we recruited. We subsequently recruited an additional 2 control and 2 TBI cases for a total for a total of 24 (10 control and 14 TBI cases). All 24 cases were used in follow-up ELISA assessments of plasma MCP-1 contents.

Antibody array studies using the RayBio Cytokine Antibody Array 200 (Raybiotech Inc.) were conducted to measure relative levels of 120 known signaling proteins in plasma specimens [41]. Specific proteins assessed are presented in Table 3. Antibody array studies were conducted according to the manufacturer's instructions [42]. For each plasma sample, nitrocellulose membranes containing antibodies for each of the 120 proteins in duplicate spots were blocked, incubated with plasma, washed, and then incubated with a cocktail of biotin-conjugated antibodies specific for the different proteins. Membranes were developed with streptavidin-conjugated peroxidase and

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Table 3
A listing of the 120 proteins assessed using the antibody array. The proteins are listed by alphabetic order based on protein name

1	Adiponectin	31	CCL26/Eotaxin-3	61	ICAM-3	91	Leptin
2	AGRP	32	CCL27/CTACK	62	IFN-	92	LIGHT
3	Amphiregulin	33	CNTF	63	IGF1-R	93	M-CSF
4	ANG-2	34	CX3CL1/Fractalkine	64	IGFBP-1	94	MIF
5	Angiogenin	35	CXCL1,2,3/GRO-_	65	IGFBP-2	95	MSP_-chain
6	AXL	36	CXCL1,2,3/GRO-_	66	IGFBP-3	96	NGF-_
7	basic FGF	37	CXCL1,2,3/GRO-_	67	IGFBP-4	97	NT-3
8	BDNF	38	CXCL5/ENA-78	68	IGFBP-6	98	NT-4/5
9	BMP-4	39	CXCL6/GCP-2	69	IGF-I	99	Oncostatin M
10	BMP-6	40	CXCL7/NAP-2	70	IL-1 R-like 1	100	Osteoprotegerin
11	BTC	41	CXCL8/IL-8	71	IL-1 sRI	101	PDGF-BB
12	CCL1/I-309	42	CXCL9/MIG	72	IL-1ra	102	PLGF
13	CCL2/MCP-1	43	CXCL11/I-TAC	73	IL-1_	103	SCF
14	CCL3/MIP-1_	44	CXCL12/SDF-1	74	IL-1_	104	Sgp130
15	CCL4/MIP-1_	45	CXCL13	75	IL-2	105	TGF-J
16	CCL5/RANTES	46	EGF	76	IL-2 sRa	106	TGF-_3
17	CCL7/MCP-3	47	EGFR	77	IL-3	107	TIMP-1
18	CCL8/MCP-2	48	Fas	78	IL-4	108	TIMP-2
19	CCL11/Eotaxin	49	FGF-4	79	IL-5	109	TNFR-1
20	CCL13/MCP-4	50	FGF-6	80	IL-6	110	TNFR-2
21	CCL15/MIP-1d	51	FGF-7	81	IL-6 sR	111	TNF-_
22	CCL16/HCC-4	52	FGF-9	82	IL-7	112	TNF-_
23	CCL17/TARC	53	Fit-3L	83	IL-10	113	TPO
24	CCL18	54	G-CSF	84	IL-11	114	TRAIL R3
25	CCL19/MIP-3_	55	GDNF	85	IL-12p40	115	TRAIL R4
26	CCL20/MIP-3_	56	GITR	86	IL-12p70	116	TYRO3
27	CCL22/MDC	57	GITR-L	87	IL-13	117	uPAR
28	CCL23/CKb8-1	58	GM-CSF	88	IL-15	118	VEGF-B
29	CCL24/Eotaxin-2	59	HGF	89	IL-16	119	VEGF-D
30	CCL25/TECK	60	ICAM-1	90	IL-17	120	XCL1/Lymphotactin

ECL chemilu-minescense reagents and exposed to autoradiographic film (BioMax Lite, Kodak). Autoradiographic films were scanned and digitized spots were quantified with *Imagene 6.0* data extraction software (BioDiscovery Inc.). Local background intensities were subtracted from each spot, and the average of the duplicate spots for each protein was normalized to the average of six positive controls on each membrane. Expression data from the two filters per sample were normalized to the median expression of all 120 proteins followed by Z score transformation. Adjusted student t tests were used to test the significance of the protein expression differences between TBI and control cases using SPSS software. $p < 0.05$ was the cut off to choose proteins for further studies.

ELISA assay

Plasma MCP-1 levels were measured using the Quantikine human CCL2/MCP-1 ELISA Kit (R&D Systems) following the manufacturer's instructions. T-tests were used to determine group differences and correlations between MCP-1 contents and neuropsychological measures or brain imaging data (see below).

Brain imaging studies

Diffusion tensor imaging (DTI) was performed on the civilian study cohort only. Images were acquired on a Philips 3.0 T Achieva (Best, The Netherlands) scanner using a diffusion-weighted spin echo sequence (TR = 5682 ms, TE = 70, FOV = 21.0 cm, FA = 90°, 54 axial slices, thickness = 2.5, no skip, matrix size = 104 × 106, b-factor = 1200 s/mm^2, 32 gradient directions plus b0, 2 averages). A high-resolution 3D T1-weighted structural image with good grey/white matter differentiation was acquired using a fast-field echo sequence for co-registration and normalization purposes (TR = 7.5 ms, TE = 3.4 ms, FOV = 22.0 cm, FA = 8°, 172 sagittal slices, thickness = 1 mm, no skip, Matrix size = 220 × 204 × 172).

Diffusion tensor images were eddy-current-corrected. Fractional anisotropy (FA) and mean diffusivity maps were calculated using the FSL comprehensive library of analysis tools for brain imaging data (http://www.fmrib.ox.ac.uk/fsl). Whole

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brain group comparisons of the diffusion parameters were performed. First, FA images were spatially normalized to the International Consortium for Brain Mapping (ICBM) brain template using tract based spatial statistics [43]. The parameters used to warp the FA images to ICBM template and the white matter skeleton were applied to the diffusivity map images, followed by statistical comparisons using the FSL Randomize routine to test for group differences in FA and diffusivity maps (separately). Clusters were identified using Threshold-Free Cluster Enhancement [44]. FA values of the whole brain and several individual tracts were extracted for further statistical analysis using Statistica V9 (Statsoft Inc., Tulsa, OK). Correlations between FA and plasma MCP-1 contents were computed.

Brain specimens

Banked postmortem frontal cortex (BM9) brain specimens from 7 mild cognitive impairment (MCI) cases (characterizedby CDR 0.5) and 7 neurologically-normal cases (characterized by CDR 0) were obtained from the MSSM Alzheimer's Disease Brain Bank. CDR assignment was based on cognitive and functional assessments collected during the last 6 months of life [40, 45].

There were no significant differences between MCI and neurologically-normal control cases with respect to the age of death (mean age of death for the MCI and the control groups was 85.4 ± 2.1 years and 85.9 ± 2.1 years, respectively; $p = 0.648$) or the postmortem interval (mean age of death for the MCI and the control groups was 7.5 ± 5.1 h and 4.5 ± 1.2 h, respectively; $p = 0.156$). A slightly higher proportion (86%) of the MCI cases was female compared to control cases (57% females), but the difference is not significant ($p = 1.0$).

Assessment of MCP-1 mRNA in postmortem brain specimens

Total RNA was isolated from approximately 50 mg of postmortem brain specimens using the RNeasy kit (Qiagen) following the manufacturer's instructions. First strand cDNA was synthesized from 1 (xg of total RNA using Superscript III Supermix for qRT-PCR (Invitrogen). Quantitative RT-PCR was performed using Maxima SYBR Green master mix (Fermentas) in ABI Prism 7900HT in 4 replicates. Human TATA-binding protein (TBP) expression level was used as an internal control. Data were normalized using the $2^{-\Delta\Delta Ct}$ method [46]. *T*-tests were used to determine group differences.

RESULTS

Identification of plasma TBI biomarkers in a civilian TBI study cohort

Plasma protein expression profile analysis, performed using the RayBio Cytokine antibody array platform in a subset of twelve TBI and eight healthy control participants from the civilian study cohort (Table 1), led to the identification of three candidate TBI biomarkers. In particular, TBI cases in the civilian study cohort were characterized byelevated plasma MCP-1 contents (Fig. 1A) and reduced plasma insulinlike growth factor-binding protein-3 (IGFBP-3) (Fig. 1B) and epidermal growth factor receptor (EGFR) (Fig. 1C) contents, relative to controls.

We explored the sensitivity and specificity of individual biomarkers or a combination of the three candidate biomarkers in distinguishing TBI from healthy control cases. Using unsupervised clustering analyses in the assessment of plasma biomarker content data from the antibody array studies, we found that a three-biomarker panel including all three candidate biomarker proteins best segregates TBI and control cases with 85% accuracy, 91% sensitivity, and 78% specificity (Fig. 1D, E).

Validation of MCP-1 as a plasma TBI biomarker

Elevated expression of MCP-1 in the brain has been observed following TBI [47, 48]. It is possible that MCP-1 may have a role in the development of chronic post-TBI clinical complications. We therefore continued to explore the differential regulation of MCP-1 in plasma specimens from TBI cases. Using an independent ELISA we confirmed significantly elevated contents of MCP-1 in plasma of TBI compared to control cases from the civilian study cohort ($p = 0.046$) (Fig. 2A).

Consistent with observations that mechanical- and blast-related TBI may share important pathophysiological features [7–9], our ELISA studies also demonstrated that blast-induced mild TBI cases in a Veteran population are also characterized by significantly higher plasma concentrations of MCP-1 ($p = 0.041$) (Fig. 2B).

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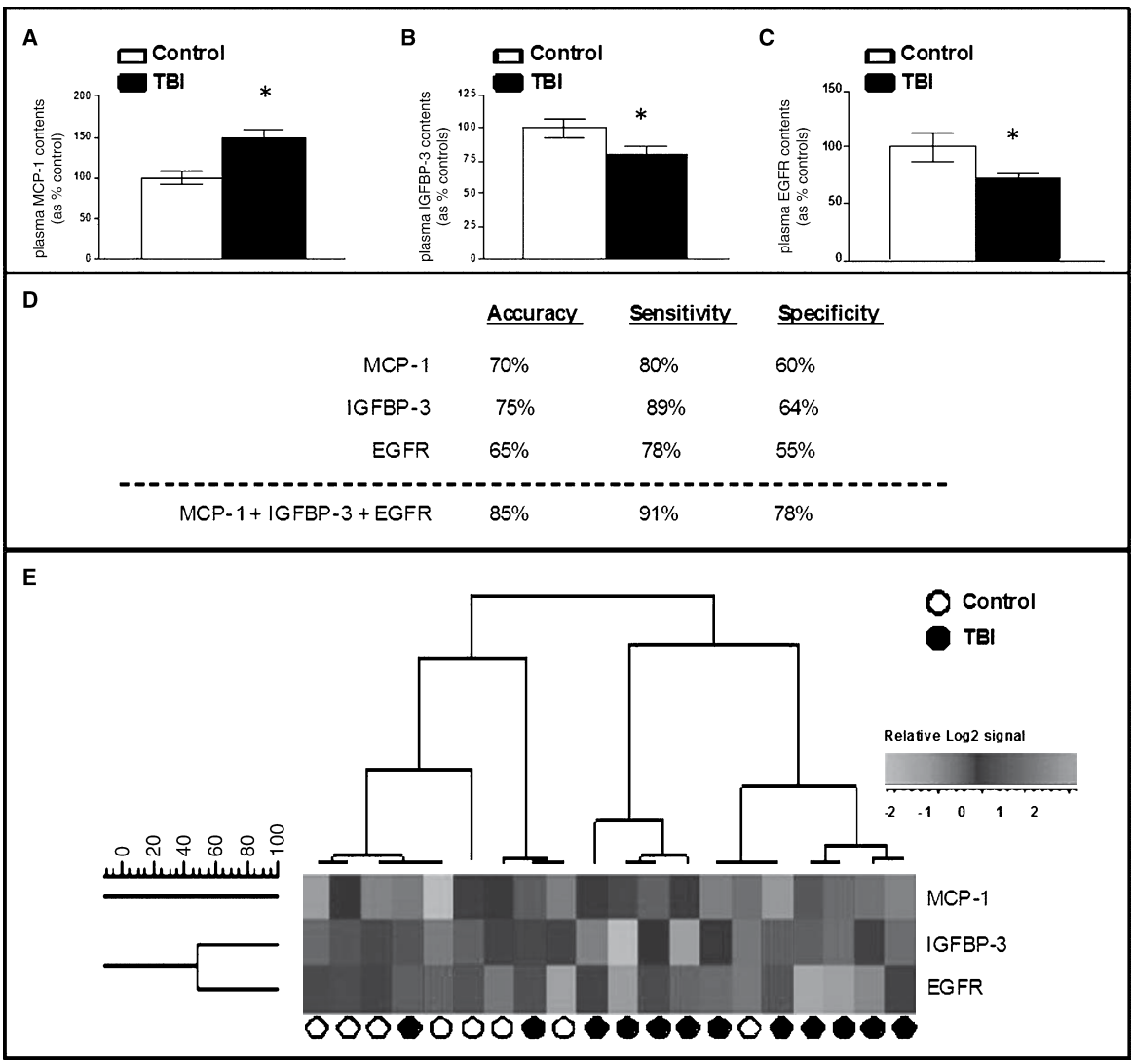

Fig. 1. Candidate plasma biomarker contents provide a sensitive and specific criterion for distinguishing TBI from control cases. Relative expression of 120 known signaling proteins in plasma specimens from a subset of 12 TBI and 8 control cases from the civilian study cohort (Table 1) were assessed by antibody array platforms (RayBio Cytokine Antibody Array 2000; Raybiotech Inc.). The student *t*-test was used to test the significance of the protein expression differences between TBI and control cases. (A–C) Outcomes of the antibody array study led to the identification of three candidate TBI biomarkers: MCP-1, IGFBP-3, and EGFR. Bar graphs represent mean ± SD of plasma biomarker contents for MCP-1 (A), IGFBP-3 (B), and EGFR (C) among TBI and control cases. *2-tailed student *t*-test, $p < 0.05$, TBI compared to the control group. (D, E) Relative plasma biomarker contents assessed by antibody arrays were tested by unsupervised clustering analysis using the UPGMA algorithm with cosine correlation as the similarity metric. (D) Summary table of analysis results using individual MCP-1, IGFBP-3, and EGFR or using a combination of all three-protein species (the "three-protein" model). Accuracy represents the percentage of all 20 TBI and normal healthy controls in the antibody array profile analysis study that were correctly diagnosed by the test, calculated as the number of correctly identified TBI and normal healthy controls divided by the total number of patients in this study. Sensitivity (true positive [TP]/[TP + false negative (FN)]) is the probability that a patient who was predicted to have TBI actually has it, whereas the specificity (true negative [TN]/[false positive (FP) + TN]) measures the probability that a patient predicted not to have TBI will, in fact, not have it. (E) A heat map graphically depicting the efficacy of using a three biomarker panel to distinguish TBI and control cases by unsupervised clustering analysis.

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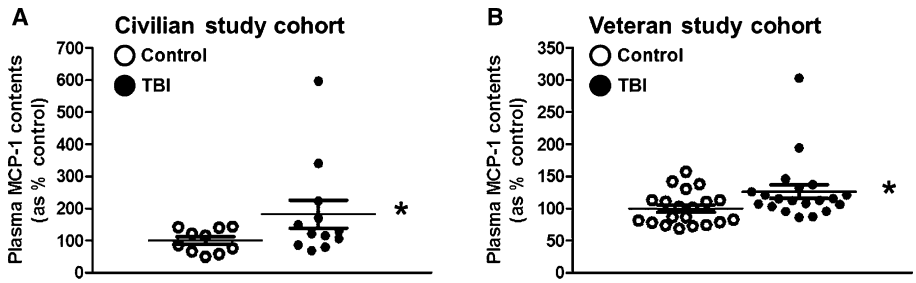

Fig. 2. Validation of plasma MCP-1 content as a clinically accessible TBI biomarker in two demographically distinct TBI study cohorts. An independent, quantitative ELISA assay was used to assess plasma MCP-1 contents in TBI and control cases from a civilian and a military veteran study cohort. (A) Plasma MCP-1 contents in TBI ($n = 14$) and control ($n = 10$) cases from the civilian study cohort (Table 1). (B) Plasma MCP-1 contents among veteran TBI ($n = 20$) and control ($n = 20$) cases (Table 2). Scatter graphs represent values for individual cases and group mean values ± SEM of plasma biomarker contents, expressed as % of controls. $^{*}p < 0.05$ by student *t*-test, TBI compared to the control group.

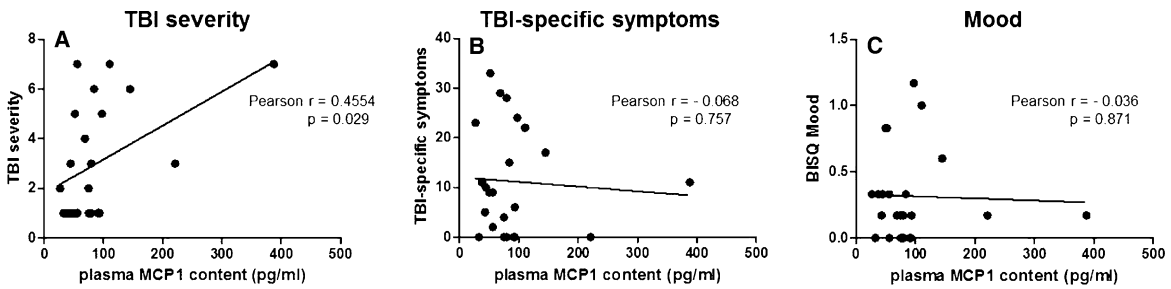

Fig. 3. Correlations of plasma MCP-1 content with self-reported indices of TBI complications. Correlation analyses were conducted using self-reported indices of TBI (assessed by BISQ [37, 38]) that were available for the civilian study cohort of 14 TBI and 10 control cases (Table I). Correlations of plasma MCP-1 contents (pg/ml) with (A) TBI severity ($p = 0.029$), (B) a summation of 25 cognitive symptoms that are sensitive and specific to TBI [38] ($p = 0.757$), and (C) self-assessments of mood ($p = 0.871$).

Association of plasma MCP-1 contents with TBI clinical symptoms

Based on the availability of self-reported clinical information for the civilian study cohort, we continued to explore potential associations between plasma MCP-1 contents and measures of TBI symptoms. We found that elevated plasma MCP-1 contents correlated significantly with severity of head injury ($p = 0.029$) (Fig. 3A). In contrast, we found no correlation between plasma MCP-1 contents and self-reported clinical symptoms as measured by the BISQ, including a subset of 25 cognitive symptoms that are sensitive and specific to TBI [38] ($p = 0.757$) (Fig. 3B) or mood ($p = 0.871$) (Fig. 3C).

Association of plasma MCP-1 contents with index of compromised axonal fiber integrity

Evidence suggests that increased brain contents of MCP-1 might promote degradation of the basic myelin protein, which is a major constituent of the axonal myelin sheath in the nervous system [47, 48]. Thus, we explored whether elevated plasma MCP-1 contents following TBI might reflect changes in axonal integrity of the brain.

DTI is an ideal technique to study white matter integrity using the FA index, a measure of axonal integrity and coherence. Based on the availability of DTI information for female TBI and control cases from the civilian study cohort (Table 1), we examined the correlation between plasma MCP-1 contents and FA measurements in the brain. Among the subset of female civilian cases examined, we found a non-significant trend ($p = 0.087$) associating increased plasma MCP-1 concentration with reduced whole brain FA values (Fig. 4A).

Further analyses evaluated axonal integrity in individual brain regions. We found a significant inverse correlation ($p = 0.021$) between plasma MCP-1 concentration and frontal cortex FA values ($p = 0.041$) (Fig. 4B): higher concentrations of plasma MCP-1 are associated with decreased FA, indicating white matter damage in the frontal cortex. The inverse relationship between plasma MCP-1 and FA is primarily localized in frontal white matter and the genu of the corpus cal-

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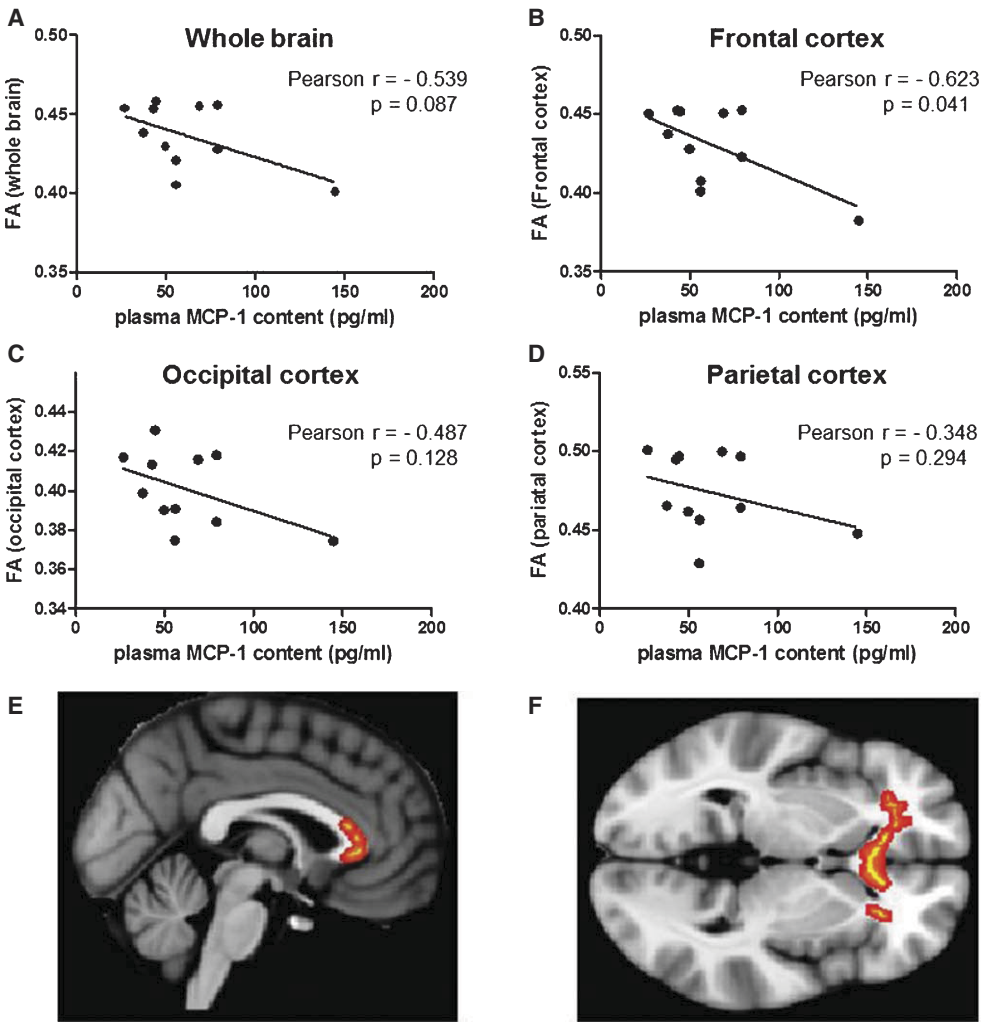

Fig. 4. Plasma MCP-1 contents are inversely correlated with brain FA measures. Correlational analyses were conducted using DTI information that was available for female (7 TBI and 5 control) cases from the civilian study cohort (Table 1). FA, a measure of white matter integrity [72], was calculated using the FSL comprehensive library of analysis tools for brain imaging data (http://www.fmrib.ox.ac.uk/fsl). Correlation of plasma MCP-1 contents (pg/ml) with whole brain FA (A), frontal cortex FA (B), occipital cortex FA (C), and parietal cortex FA (D). Representative sagittal (E) and transverse (F) sections of the brain illustrating significant correlations of FA with plasma MCP-1 contents (indicated by red-yellow) in frontal white matter and the genu of the corpus callosum.

losum (Fig. 4E, F). Outside of the frontal cortex, we observed no significant correlation between plasma MCP-1 concentrations and FA values in the occipital cortex ($p = 0.128$) (Fig. 4C) or parietal cortex ($p = 0.294$) (4D).

Elevated MCP-1 gene expression in the frontal cortex of cases characterized by MCI

TBI may be a risk factor for AD, and elevated levels of MCP-1 have been observed in brains of AD patients [49, 50] and AD transgenic mice [12, 51]. Based on this and the role of MCP-1 in promoting inflammatory responses and myelin degradation, it is possible that overexpression of MCP-1 in the brain may contribute to the initiation and/or progression of AD. We explored the regulation of MCP-1 in the brain of MCI cases who, similar to TBI cases, may be at higher risk for AD dementia. We found significantly higher contents of MCP-1 mRNA in postmortem frontal cortex (BM9) specimens of MCI compared to control cases (Fig. 5A). In control cases, we found no correlation between MCP-1 mRNA content in frontal cortex tissues and postmortem interval (Fig. 5B), age at death (Fig. 5C) or neuritic plaque density (Fig. 5D).

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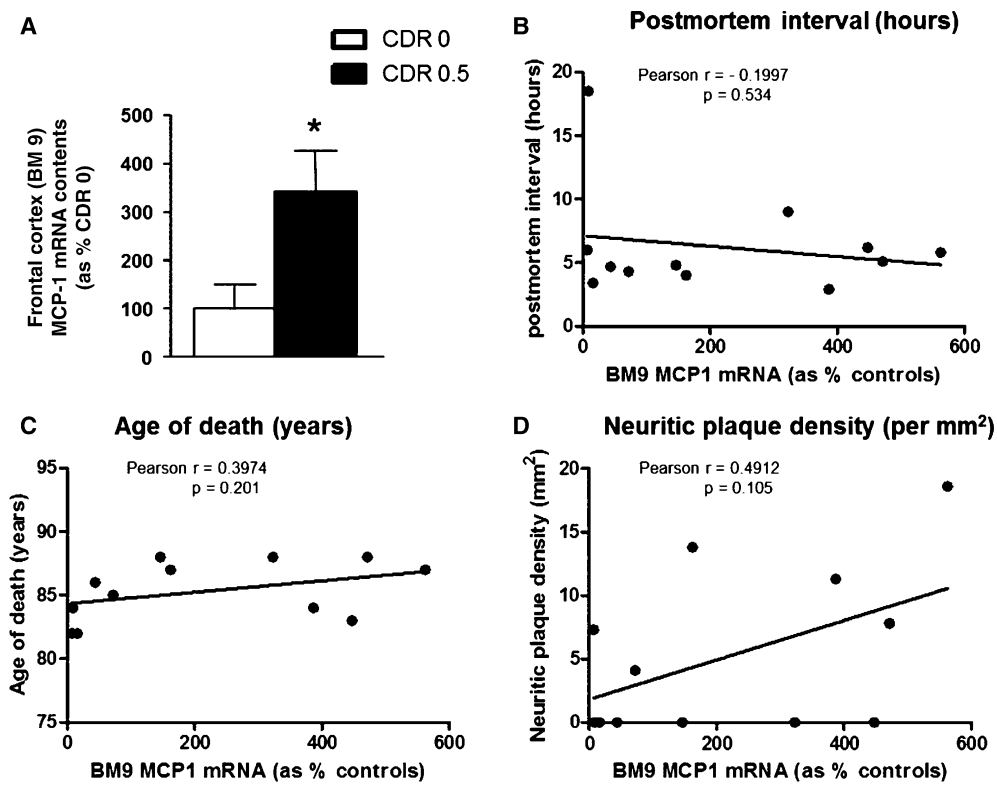

Fig. 5. Elevated MCP-1 mRNA in frontal cortex (BM9) brain specimens of cases characterized by mild cognitive impairment. (A) Contents of MCP-1 mRNA were assessed by qPCR and normalized to brain contents of human TATA-binding protein mRNA, used as an internal control. Bar graphs represent mean (±SEM) normalized MCP-1 mRNA contents in BM9 of CDR 0 cases ($n = 6$), expressed as % of MCP-1 mRNA contents in control cases ($n = 6$). *Student t-test, $p < 0.05$. B–D) Correlation analysis of BM9 MCP-1 mRNA contents with (B) postmortem interval, (C) age of death, and (D) neuritic plaque density (per mm^2). There was no significant correlation between brain MCP-1 mRNA contents and postmortem interval ($p = 0.534$), age of death ($p = 0.201$), or neuritic plaque density ($p = 0.105$).

DISCUSSION

Evidence suggests that appropriate rehabilitative interventions can reduce functional impairment after TBI [52–54]. In order to demonstrate the efficacy of clinical interventions, research must identify the biological, clinical, and neurological indices that are sensitive to the detection of functional impairments after TBI [55]. We explored the feasibility of identifying plasma protein species that might be useful as clinically accessible surrogate biological indices of TBI. Our antibody array studies identified three candidate TBI protein biomarkers: MCP-1, present at higher levels, as well as IGFBP-3 and EGFR, present at lower levels, in plasma of chronic TBI cases. Moreover, we demonstrated that a three-biomarker panel using all three biomarkers provides a potential criterion for segregating TBI and control cases with 85% accuracy, 91% sensitivity, and 78% specificity. Our evidence tentatively implicates the value of this three-biomarker panel as a biological surrogate for improved TBI detection and more sensitive outcome measures for clinical trials.

Consistent with previous research, our findings suggest that MCP-1 plays a role in post-TBI clinical impairments. Elevated expression of MCP-1 in the brain has been observed following TBI [47, 48] and in response to neurodegenerative disorders [56–63]. Moreover, MCP-1 is known to promote inflammatory responses and processes leading to degradation of the axonal myelin sheath [64–67]. Using an independent ELISA assay, we confirmed elevated plasma MCP-1 concentrations in TBI cases from both civilian and veteran cohorts.

TBI cases in both the civilian and veteran study cohort were characterized by a long interval between injury and participation in the study (mean ± SD interval of 13.2 ± 12.7 years and 3.9 ± 1.4 years for the civilian and the veteran cohorts, respectively) (Tables 1 and 2). Thus, upregulation of plasma

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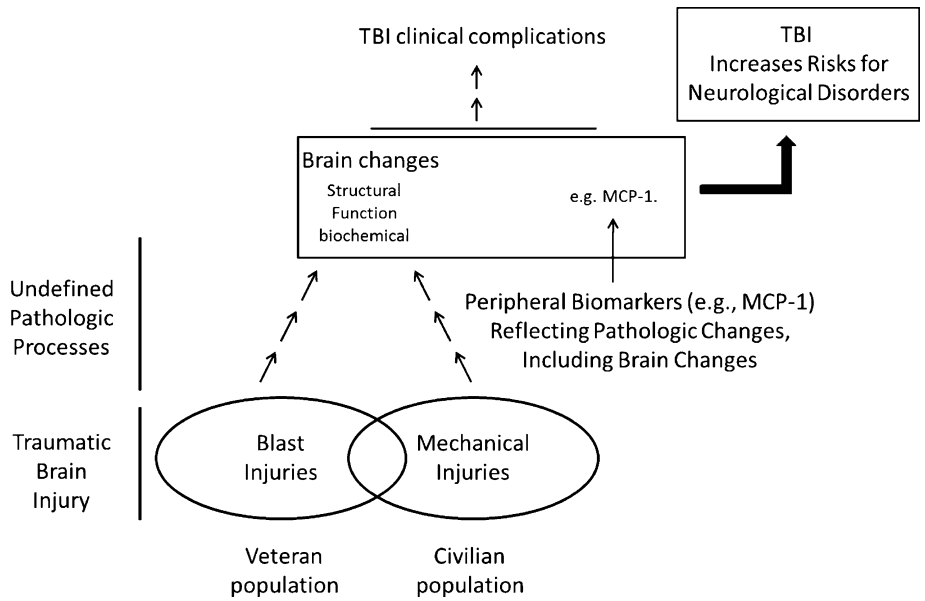

Fig. 6. Proposed mechanisms by which TBI may increase risk for Alzheimer's disease and other neurological disorders. Schematic represents an overview of a proposed model by which TBI exposure may mechanistically increase subsequent risk for Alzheimer's disease and other neurological disorders. Accordingly, TBI resulting from either mechanical injuries or blast injuries, which are predominant, respectively, in the civilian or the Veteran population, induces biological responses that lead to aberrant biochemical, structural and/or functional changes in the brain. For example, long-term induction of MCP-1 following TBI may contribute to demyelination processes that, ultimately, may reduce resilience of the brain to subsequent neurodegenerative insults.

MCP-1 contents among TBI cases is not an acute post-injury response, but more likely reflects a long-term consequence of TBI.

Our observation that plasma MCP-1 contents correlated with the severity of head injury but not measures of TBI symptoms suggests that plasma MCP-1 is a marker of injury *per se* rather than a marker of a specific injury-related impairments. Recent reports from our group [68] and from others [69–71] have demonstrated reduced FA, implicating impaired axonal pathways in the brains of TBI patients. In our present study, we found a significant correlation between elevated plasma MCP-1 contents and increasing white matter damage in the frontal cortex, as assessed by DTI FA. Our evidence suggests that elevated plasma MCP-1 concentration following TBI may provide a "window into the brain" that is associated with changes in axonal integrity in the brain, particularly in the frontal cortex.

Both TBI and MCI subjects may be at elevated risk for AD dementia. We found significantly elevated MCP-1 expression in the postmortem frontal cortex of MCI cases, suggesting that induction of MCP-1 following TBI might be a potential "predisposition" factor that imposes higher risk for AD or accelerated aging. As we schematically illustrated in Fig. 6, we propose that TBI resulting from either mechanical injuries or blast injuries results in a cascade of biological responses that lead to aberrant biochemical, structural, and/or functional changes in the brain that remain many years post-injury. While mechanisms underlying these pathological modifications are not well defined, it is possible that these changes in the brain may contribute to a chronic clinical course of TBI and/or increased risk for neurodegenerative disorders. Our data presented here suggest that chronic elevation of MCP-1 following TBI may contribute to demyelination processes that, over time, may reduce resilience of the brain to subsequent neurodegenerative insults leading to AD or other neurodegenerative disorders.

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Handbook of Traumatic Brain Injury and Neurodegeneration
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Decreased Level of Olfactory Receptors in Blood Cells Following Traumatic Brain Injury and Potential Association with Tauopathy

Wei Zhao[a,1], Lap Ho[a,1], Merina Varghese[a], Shrishailam Yemul[a,d], Kristen Dams-O'Connor[b], Wayne Gordon[b], Lindsay Knable[a], Daniel Freire[a], Vahram Haroutunian[c] and Giulio Maria Pasinetti[a,c,d,*]

[a]*Department of Neurology, Mount Sinai School of Medicine, New York, NY, USA*
[b]*Department of Rehabilitation, Mount Sinai School of Medicine, New York, NY, USA*
[c]*Department of Psychiatry, Mount Sinai School of Medicine, New York, NY, USA*
[d]*GRECC, James J. Peters Veterans Affairs Medical Center, Bronx, NY, USA*

Abstract. Traumatic brain injury (TBI) is a leading cause of death and disability among children and young adults in the United States. In this study, we explored whether changes in the gene expression profile of peripheral blood mononuclear cells (PBMC) may provide a clinically assessable "window" into the brain, reflecting molecular alterations following TBI that might contribute to the onset and progression of TBI clinical complications. We identified three olfactory receptor (OR) TBI biomarkers that are aberrantly down-regulated in PBMC specimens from TBI subjects. Down-regulation of these OR biomarkers in PBMC was correlated with the severity of brain injury and TBI-specific symptoms. A two- biomarker panel comprised of OR11H1 and OR4M1 provided the best criterion for segregating the TBI and control cases with 90% accuracy, 83.3% sensitivity, and 100% specificity. We found that the OR biomarkers are ectopically expressed in multiple brain regions, including the entorhinal-hippocampus system known to play an important role in memory formation and consolidation. Activation of OR4M1 led to attenuation of abnormal tau phosphorylation, possibly through JNK signaling pathway. Our results suggested that addition of the two-OR biomarker model to current diagnostic criteria may lead to improved TBI detection for clinical trials, and decreased expression of OR TBI biomarkers might be associated with TBI-induced tauopathy. Future studies exploring the physiological relevance of OR TBI biomarkers in the normal brain and in the brain following TBI will provide a better understanding of the biological mechanisms underlying TBI and insights into novel therapeutic targets for TBI.

Keywords: Biomarker, olfactory receptor, peripheral blood mononuclear cell, tauopathy, traumatic brain injury

INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of death and disability among children and young adults in the United States [1]. TBI is an acquired injury caused by a sudden trauma to the head that disrupts normal brain functioning, which leads to either transient or chronic impairments in physical,

[1]These authors contributed equally to this study.

*Correspondence to: Giulio Maria Pasinetti, M.D., Ph.D., Department of Neurology, Mount Sinai School of Medicine, 1468 Madison Avenue, Annenberg Building, Room 20-02, New York, NY 10029, USA. Tel.: +1 212 241 7938 or +1 212 241 5563; Fax: +1 212 876 9042; E-mail: Giulio.Pasinetti@mssm.edu.

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cognitive, emotional, and/or behavioral functions. In the civilian population, TBI is typically associated with direct, closed impact mechanical trauma to the brain due to falls, motor vehicle accidents, sports, etc. [2]. In contrast, TBI among military personnel, particularly among veterans returning from the Persian Gulf region, is primarily due to exposure to blast pressure waves stemming from blast-producing weaponry, leading to prototypical cognitive deficits including impairments in attention, memory, processing speed, and executive functioning [2–4]. Both civilians and veterans who suffer from TBI exhibit symptoms that range in severity from mild to very severe, with a minimal to profound impact on daily functioning. The reasons why TBI induces different clinical symptoms among affected individuals are not yet known.

New evidence has highlighted defects in neural circuit and synapses, and the plastic processes controlling these functions, in TBI [5–11]. While genes relevant to these processes are expressed in the brain, some of these genes are also expressed in circulating blood cells, such as peripheral blood mononuclear cells (PBMCs) [12–15]. Consistent with this, recent studies have illustrated that PBMC-associated biomarkers may provide insights into the pathogenesis of neurological disorders such as Alzheimer's disease and can be used to monitor disease diagnosis and progression [16, 17]. Thus, PBMC may also provide an ideal, clinically assessable "window" into the brain, reflecting molecular alterations following TBI which might contribute to the onset and progression of clinical TBI phenotypes.

Evidence suggests that appropriate interventions can reduce functional impairment after TBI [18–20]. In order to demonstrate the efficacy of clinical interventions, research must identify the biological, clinical, and neurological indexes that are sensitive to the detection of functional impairments after TBI. Therefore, in this study we explored the feasibility of identifying clinically assessable TBI biomarkers and the potential function of identified TBI biomarkers in TBI neuropathology.

MATERIALS AND METHODS

Civilian TBI and control subjects

Eleven individuals with TBI (five male and six female), with TBI severity ranging from mild to severe, and nine control participants (four male and five female) were recruited from the Brain Injury Research Center at the Mount Sinai School of Medicine (MSSM). Medical documentation of TBI was reviewed for each participant. The age- and education-matched controls were recruited using advertisements placed in local media and flyers, and through word of mouth. Following a capacity screening to assess the individual's ability to comprehend the purpose and procedures of the study and provide informed consent, the informed consent process was performed in accordance with MSSM IRB policies and procedures.

All participants were screened using the Brain Injury Screening Questionnaire (BISQ) [21, 22] to assess the kinds of situations in which a brain injury might have occurred, and the number and severity of hits to the head sustained throughout the lifespan. Using information gained from the BISQ and from individual interviews conducted by the authors, injury severity was classified using a 7-point scale ranging from 1 (no loss of consciousness, no confusion (i.e., no TBI)) to 7 (loss of consciousness greater than 4 weeks in duration) [21, 22]. For those who have been injured, questionnaires in the BISQ solicit participants' self-reported measures of functional difficulties and symptoms associated with brain injury and events, conditions other than brain injury that might lead to symptoms similar to those seen in brain injury, as well as the occurrence of persistent, highly disruptive symptoms (functional changes) that do not "go away", which TBI participants may continue to experience following the blow to the head.

Veteran TBI and control subjects

For this biomarker validation study, we obtained banked PBMCs from a cohort of five OEF/OIF veterans and a control cohort of seven age- and gender-matched veterans without TBI (non-TBI veterans). TBI diagnosis is based on confirmation according to Defense and Veterans Brain Injury Center (DVBIC) criteria of sustained injury to the head plus subsequent alteration of consciousness, and Repeatable Battery for Neuropsychological Testing (RBANS) scores of one standard deviation below the norms established for age and education of individuals in question. Non-TBI control classification is based on DVBIC confirmation of no sustained injury to the head and RBANS scores less than one standard deviation below the norms established. Most of the veteran TBI cases exhibit comorbid post-traumatic stress disorder (PTSD). Thus, our non-TBI control cases are also matched for PTSD diagnosis. In this

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study, diagnosis of PSTD is based on a score of 50 or more in the PTSD Checklist – Civilian Version.

Postmortem brain specimens

Human postmortem brain samples from 4 neurologically normal cases (characterized by a Clinical Dementia Rating (CDR) of 0) were obtained from the Alzheimer's Disease Brain Bank of the Mount Sinai School of Medicine. A multistep approach based on cognitive and functional status during the last 6 months of life was applied to the assignment of CDR [23] as previously reported [24, 25].

PBMC and RNA isolation

Blood specimens were collected into BD Vacutainer CPT Cell Preparation Tubes. PBMC were isolated following manufacturer's instructions (Becton, Dickinson and Company) and were stored at –80°C until use. Total RNA was isolated from approximately 10–50 mg of PBMC using RNA STAT-60 (Tel-Test) according to the manufacturer's instructions. The purity and concentration of RNA samples were determined from OD260/280 readings using a dual beam UV spectrophotometer and RNA integrity was determined by capillary electrophoresis using the RNA 6000 Nano Lab-on-a-Chip kit and the Bioanalyzer 2100 (Agilent Technologies).

Microarray study and analysis

Total RNA was directly labeled using the FlashTag™ HSR Biotin RNA Labeling Kit according to the manufacturer's instructions (Genisphere). Verification of biotin labeling was obtained by an enzyme-linked oligoabsorbant assay (ELOSA) using Immobilizer™ Amino – 8 well strips (Nunc/Thermo Fisher Scientific) according to instructions supplied by Genisphere. Labeled RNA (1.0 μg) was hybridized for 16 h at 48°C to Affymetrix HuGene 1.0 ST arrays containing probe sets for 28,869 genes. Arrays were washed and stained on a Fluidics Station 450 (Affymetrix) according to the manufacturer's recommended procedures. The arrays were stained with phycoerythrein-conjugated streptavidin (Life Technologies) and the fluorescence intensities were determined using a GCS 3000 7 G high-resolution confocal laser scanner and AGCC software (Affymetrix). The scanned images were analyzed with the RNA QC tool (Affymetrix) using RMA global background correction, quantile normalization and median polish summarization to generate quantified data (as recommended by Genisphere). Quality control metrics for arrays included normalized signal values > 1000 for five spike-in control oligo probe sets (Genisphere).

RNA probe sets exhibiting significant differential expression (SDE) were identified using the following steps in GeneMaths XT (Applied Maths): 1) Probe sets with array detection p-values ≤ 0.05 for all samples in at least one experimental group were selected for further analysis; 2) Performed Discriminant Analysis (DA) and determined the largest percentage of remaining probe sets that permitted correct group assignment of samples in unsupervised hierarchical clustering by the Unweighted Pair-Group Method using Arithmetic averages (UPGMA) based on cosine correlation of row mean centered log2 signal values; this was the top 50 percentile; and 3) in the DA top 50 percentile, selected probe sets with absolute signal log2 fold changes ≥ 1.0 and independent t-test p-values ≤ 0.05 adjusted for multiple testing error by the Benjamini-Hochberg FDR correction method. Unsupervised hierarchical clustering of probe sets and heat map generation were performed in GeneMaths XT following row mean centering of log2 transformed MAS5.0 signal values; probe set clustering was performed by the UPGMA method using Cosine correlation as the similarity metric. For comparative purposes, clustered heat maps included probe sets for spike-in controls (Genisphere), or endogenous small RNAs exhibiting: 1) Array detection p-values ≤ 0.05, and 2) either a) a log2 signal value standard deviation ≤ 0.025 for all samples or b) in the DA top 50 percentile with a FC > 1.3 in the opposite direction of the selected SDE profile.

Confirmatory quantitative PCR

First strand cDNA was synthesized from 1 μg of total RNA using Superscript III Super-mix for qRT-PCR (Invitrogen). Quantitative RT-PCR was performed using Maxima SYBR Green master mix (Fermentas) in ABI Prism 7900HT in four replicates with primers amplifying the seven olfactory receptor candidates: OR4Q3 (Forward: CACCTGCTCCAATCTCCTATG, Reverse: TCCCCTAACATCTTTGGCAC); OR51L1 (Forward: TTCCCACACCTTTGCTACTG, Reverse: AATACTGTTGGTCCTGGCATC); OR4D10 (Forward: CCATCTCTGTCACCTTCACTG, Reverse: ATGGCTGACTTCATCTCATGG); OR4M1 (Forward: TCTGTTAATGTCCTATGC-CTTCC, Reverse: AATGTGGGAATAGCAGGTGG);

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OR52N5 (Forward: ATGCTACCACACTCACCA AC, Reverse: CATCAGCAATACACCCCTCAG); OR2J3 (Forward: CCTCTCATCCTCATTCTCACT TC, Reverse: CAAACACTTTCTGAAGCCCAG); OR11H1 (Forward: AACTGGTCATACTGTGCT GG, Reverse: GGGCAGAAACACAATCCAATG). Human TATA-binding protein (TBP, Forward: TGCACAGGAGCC AAGAGTGAA, Reverse: CTGGAACGGTGAAG-GTGACA) expression level was used as an internal control. Data were normalized using the $2^{-\Delta\Delta Ct}$ method [26]. Levels of olfactory receptor mRNAs were expressed relative to those in control groups and plotted in GraphPad Prism.

Lentiviral plasmid construction and lentivirus packaging

We obtained pCMV6-XL5-OR4M1 cDNA clone from Origne and subcloned the OR4M1 ORF into lentivial plasmid pLVX-IRES-ZsGreen (Clontech). The inserted OR4M1 sequence was verified by sequencing. For lentivirus packaging, we transfected Lenti-X 293T cells with either pLVX-OR4M1-IRES-ZsGreen or pLVX-IRES-ZsGreen using Lentiviral Packaging System (Clontech). Medium was collected 48 h after transfection and a ten-fold concentration step was performed using Lenti-X Concentrator (Clontech).

Primary neuron culture, lentiviral transduction, and cAMP assay

Embryonic day 15 cortico-hippocampal neuronal cultures were prepared from C57BL6 mouse (Jackson Laboratory) as previously described [27]. Cells were seeded onto poly-D-lysine-coated 12-well plates at 5×10^5 cells per well and cultured in Neurobasal medium supplemented with 2% B27, 0.5 mM L-glutamine, and 1% penicillin-streptomycin (Life Technologies). On Day 5 of culture, primary corticohippocampal neuron cultures were transduced with lentiviral particles overexpressing OR4M1 or control lentiviral particles by spin infection (800 g × 90 min at 30°C). Transduction efficiency was monitored by ZsGreen expression. 72 h after lentiviral transduction, cells were pretreated with 3-isobutyl-l-methylxanthine (IBMX), an inhibitor of cAMP phosphodiesterase, for 10 min followed by ligand treatment (10 μM) for 10 min. cAMP assay was performed using a colorimetric cAMP ELISA assay kit (Cell Biolabs).

Luminex multiplex assay and western blot analysis

Primary cortico-hippocampal neurons were infected with OR4M1 lentiviral particles and stimulated with acetophenone (10 μM) for 1 h. Multiplex luminex assay was performed using the Milliplex xMAP 8-plex multipathway signaling-phosphoprotein kit (Millipore) as previously described [28]. Protein concentrations were determined using the BCA protein assay kit (Thermo Scientific). 30 μg of total protein were loaded onto 12% SDS-PAGE and subjected to western blot analysis with antibody recognizing phospho-JNK (T183/Y185) (Cell Signaling), phospho-ERK (T185/Y187) (Cell Signaling), and PHF-1 (phospho-tau (S396/S404), a generous gift from Peter Davies at Albert Einstein College of Medicine). Blots were quantified in ImageJ (NIH), normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnologies) and then plotted in GraphPad Prism.

RESULTS

Identification of select olfactory receptors as clinically accessible candidate TBI biomarkers

We analyzed the gene expression profile in PBMC derived from subjects with TBI and age-, gender-, and education-matched healthy controls. Our data showed that female subjects had a more robust profile than males probably due to a rather smaller number of male subjects in the study cohort. Taking this into consideration, further analysis and confirmatory studies were all performed in a subset of the study cohort which only contains female subjects. Demographic information for the female TBI and control participants is summarized in Table 1. Using microarray gene expression profile analysis, we identified a panel of 102 candidate biomarker genes (36 annotated and 66 non-annotated genes) that are differentially regulated (t-test $p < 0.05$) following Benjamini-Hochberg false discovery rate correction [29] by > 1.5-fold in PBMC specimens of TBI cases compared to control cases (Fig. 1A). More importantly, we were able to correctly separate all cases into TBI and non-TBI groupings using this panel of 102 genes in an unsupervised hierarchical clustering analysis (Fig. 1A). Interestingly, among the panel of 102 candidate TBI biomarkers that we identified from microarray data, 7 were olfactory receptors (Fig. 1B). Our microarray studies revealed that each of the 7 ORs were down-

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Table 1
Demographic information for the civilian study cohort. TBI severity was classified using a 7-point scale ranging from 1 (no loss of consciousness, no confusion (i.e., no TBI)) to 7 (loss of consciousness greater than 4 weeks in duration) [23]. Time post injury is the time frame between the occurrence of brain injury and volunteer's participation in this biomarker study

	Control	TBI
Number of cases	5	6
Gender	100% female	100% female
TBI severity		
Mean ± SD	1 ± 0	4 ± 1.67
Range	1	2–6
Age (mean $y \pm$ SD) at study participation	34.80 ± 14.48	35.33 ± 13.32
Post injury interval (mean $y \pm$ SD)	—	5.42 ± 5.30
Education (mean $y \pm$ SD)	16.80 ± 1.10	14.50 ± 2.35
Ethnicity composition		
White-Caucasian	80%	66.7%
Black-African American	—	33.3%
Hispanic	20%	—

regulated in PBMC of TBI cases compared to those of control cases in this study cohort (Figs. 1B, 2A).

Confirmatory qPCR studies

Using independent qPCR assays, we confirmed that three of the ORs (OR11H1, OR4M1, and OR52N5) were significantly down-regulated in PBMC of TBI cases (Fig. 2B). For the remaining four ORs (OR4D10, OR2J3, OR4Q3, OR51L1), our qPCR evidence showed lower levels of these ORs in PBMC of TBI compared to control cases, but these differences did not reach statistical significance (Fig. 2B). This particular result is conceivably due to a high degree of gene expression variability for these 4 ORs among healthy control cases and the relatively small sample sizes that we used in our exploratory study.

We continued to explore the sensitivity and specificity of an individual or combined role of OR11H1, OR4M1, and OR52N5 in distinguishing TBI cases from normal healthy controls in our study cohort (Fig. 2C). Using unsupervised clustering analyses, we found that a two- biomarker panel comprised of OR11-H1 and OR4M1 provides the best crite-

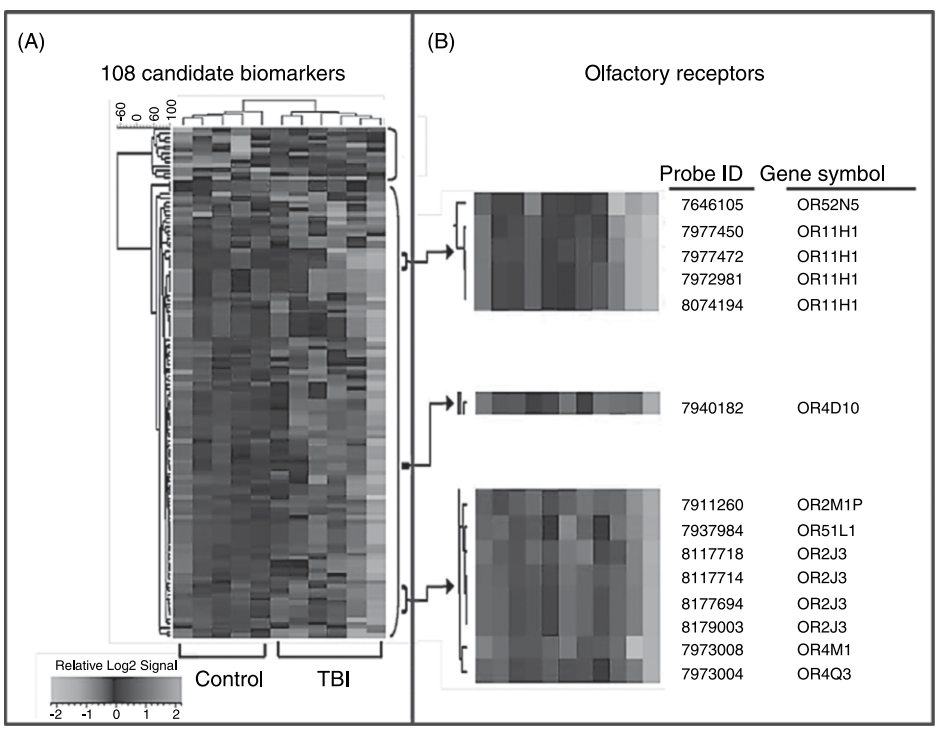

Fig. 1. Development of blood biological indices (biomarkers) capable of correctly segregating TBI and control cases. Gene expression profile analysis of PBMC specimens from TBI and 5 age-, gender-, and education-matched control cases using a microarray platform (Affymetrix) led to the identification of a panel of 102 candidate biomarker genes. A) The 108 differentially-regulated genes identified were subjected to unsupervised hierarchical clustering analysis using the UPGMA algorithm with cosine correlation as the similarity metric. Results are presented as a heat map (left panel) demonstrating that the content of the 108 biomarker panel is able to correctly segregate TBI from control cases. B) 7 ORs and one OR pseudogene that are down-regulated in PBMC of TBI cases.

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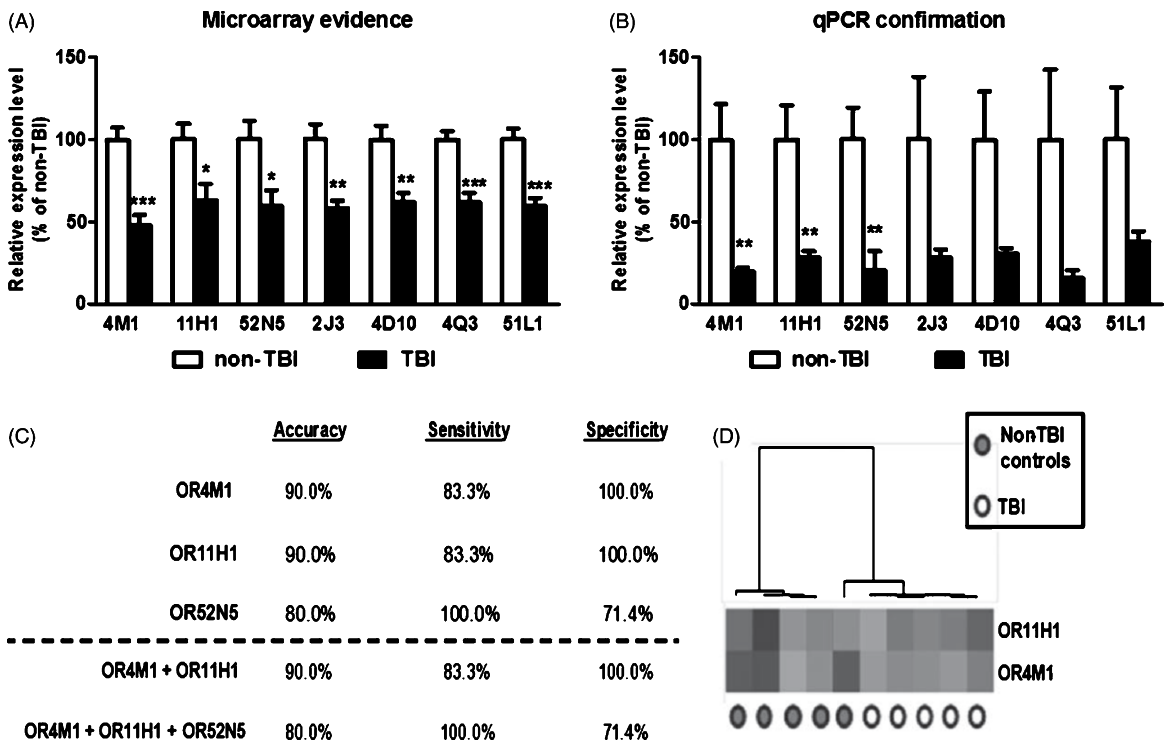

Fig. 2. Down-regulation of OR genes in PBMC of TBI cases provide a sensitive and specific criterion for distinguishing TBI from control cases. mRNA contents for each of the 7 candidate OR biomarker genes in TBI and control cases were analyzed by microarray (A) or independent qPCR (B). Bar graphs represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by student *t*-test, TBI versus control. The efficacy of using biomarker contents from PBMC as a criterion to correctly segregate TBI and control cases was tested by unsupervised clustering analysis using the UPGMA algorithm with cosine correlation as the similarity metric. C) The accuracy, sensitivity, and specificity of OR11H1, OR4M1, OR52N5, or panels of ORs to distinguish TBI from control cases. D) A heat map graphically depicting the efficacy of using the two biomarker panel to distinguish TBI cases and control cases by unsupervised clustering analysis.

rion for segregating the TBI and control cases with 90% accuracy, 83.3% sensitivity, and 100% specificity (Fig. 2C, D).

TBI biomarker validation studies: Testing the validity of OR11H1, OR4M1, and OR52N5 to distinguish TBI from non-TBI control cases from a study cohort of OEF/OIF veterans

The pathophysiological mechanisms underlying mechanical and blast-related TBI may differ in some ways, but they share important pathophysiological features. Similarities between the pathophysiologies of mechanical and blast-related TBI [2] suggested that information gathered from TBI cases in the civilian population may also be relevant to combat-related TBI. Based on this, we tested the ability of OR11H1, OR4M1, and OR52N5 to distinguish TBI cases from non-TBI controls from a study cohort of OEF/OIF veterans. For this biomarker validation study, we obtained banked PBMCs from a cohort of five OEF/OIF veterans and a control cohort of seven age-and gender-matched veterans without TBI (non-TBI veterans) (Table 2). Similar to our above-mentioned observations regarding OR biomarkers from the civilian study cohort, we found that OR4M1 (Fig. 3A), OR11H1 (Fig. 3B), and OR52N5 (Fig. 3C) are also significantly down-regulated in PBMC specimens of veteran TBI cases compared to that of non-TBI control cases. The two biomarker panel of OR11H1 and OR4M1 we identified from the civilian TBI study cohort is also capable of distinguishing veteran TBI from control veteran cases with 83% accuracy, 80% sensitivity, and 86% specificity (Fig. 3D & E).

OR biomarker contents in PBMCs are correlated with TBI severity & long-term clinical neuropsychological complications

Continuing to explore the potential relevance of OR11H1, OR4M1, and OR52N5 to TBI clinical

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Table 2
Demographic information for the veteran study cohort. TBI diagnosis is based on confirmation according to Defense and Veterans Brain Injury Center (DVBIC) criteria of sustained injury to the head plus subsequent alteration of consciousness, and Repeatable Battery for Neuropsychological Testing (RBANS) scores of one standard deviation below the norms established for age and education of individuals in question. Non-TBI control classification is based on DVBIC confirmation of no sustained injury to the head and RBANS scores less than one standard deviation below the norms established. Most of the veteran TBI cases exhibit co-morbid post-traumatic stress disorder (PTSD). Thus, our non-TBI control cases are also matched for PTSD diagnosis. In this study, diagnosis of PSTD is based on a score of 50 or more in the PTSD Checklist – Civilian Version

	Control	TBI
Number of cases	7	5
Gender	57% male	80% male
Age (mean $y \pm$ SD)	30.1 ± 9.4	31.0 ± 7.1
Interval since last deployment (mean $y \pm$ SD)	2.8 ± 2.3	4.0 ± 3.8
Education (mean $y \pm$ SD)	13.3 ± 2.8	13.8 ± 1.5
Percentage of cases with co-morbid PTSD	85.7%	100%
Ethnicity composition		
Black-African American	14.3%	40%
Hispanic	85.7%	60%

symptoms, we found that the contents of the three OR biomarkers in PBMC was significantly and inversely correlated with TBI severity: lower OR mRNA contents in PBMCs are associated with exposure to more severe head injury (Fig. 4A–C). We also found that OR TBI biomarker contents in PBMC were significantly correlated with select aspects of self-reported clinical TBI symptoms. In particular, PBMC contents of OR11H1 or OR4M1 were significantly associated in an inverse fashion with TBI-specific symptoms, a summation of 25 cognitive symptoms that are sensitive and specific to TBI [22]. OR11H1 or OR4M1 content within PBMC were not associated with self-assessments of mood (Fig. 4D). Expression level of the third OR biomarker, OR52N5, was not correlated with TBI-specific symptoms or self-assessment of mood (Fig. 4D).

Ectopic expression of OR TBI biomarkers in multiple brain regions outside of the olfactory bulb

The brain represents a key target tissue for understanding TBI clinical complications and for the development of clinical interventions. In order to explore the potential physiological relevance of OR11H1, OR4M1, and OR52N5 in the brain, we assessed the RNA contents of OR biomarkers in the brain. We found that the three TBI biomarker ORs were expressed in multiple regions of the brain from

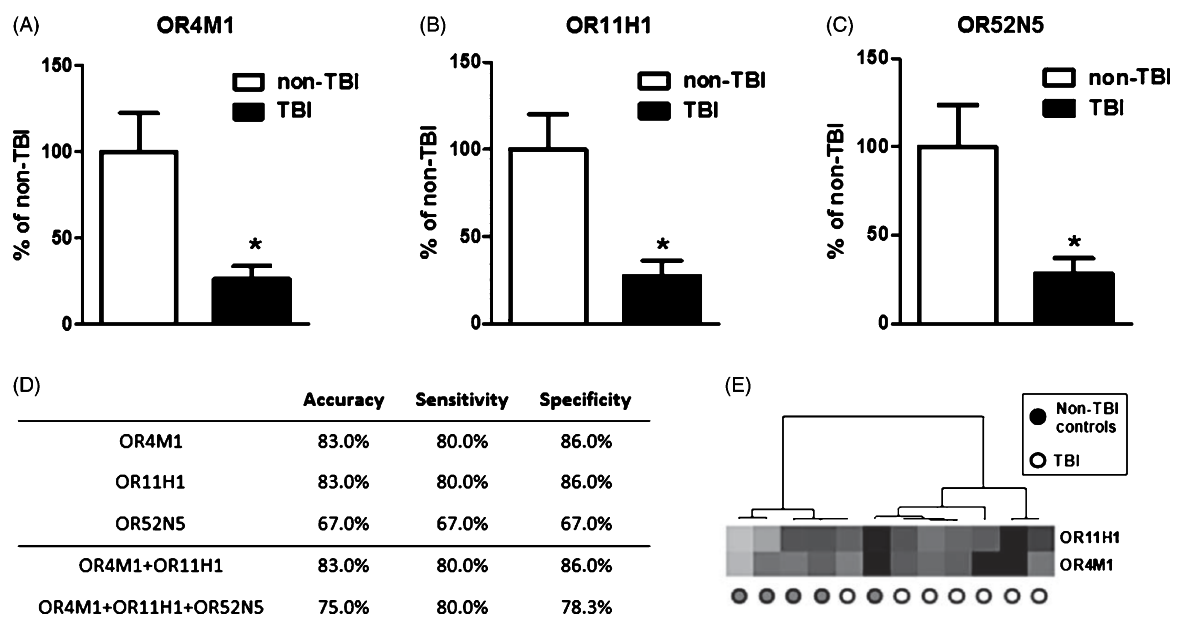

Fig. 3. Validation of OR TBI biomarkers in a veteran study cohort. The mRNA contents for (A) OR4M1, (B) OR11H1, and (C) OR52N5 in TBI and control cases from a veteran study cohort were analyzed by qPCR. Bar graphs represent mean + SEM. *$p < 0.05$ by student t-test, TBI versus control. D) The accuracy, sensitivity, and specificity of OR11H1, OR4M1, OR52N5, or panels of ORs to distinguish veteran TBI from control veteran cases. E) A heat map graphically depicting the efficacy of using the two biomarker panel to distinguish veteran TBI cases and control veteran cases by unsupervised clustering analysis.

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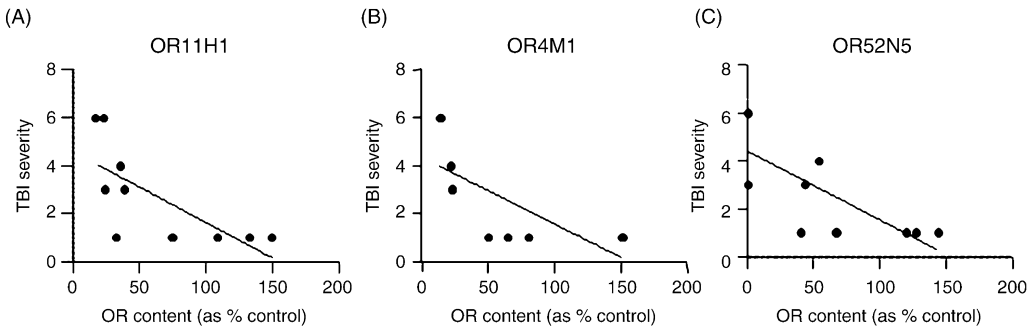

(D)	TBI severity		TBI-specific symptoms		Mood	
	Correlation coefficient	p-value	correlation coefficient	p-value	correlation coefficient	p-value
Olfactory Receptor 11H-1	-0.709	0.022	-0.711	0.021	-0.449	NS
Olfactory Receptor 4M-1	-0.72	0.019	-0.733	0.016	-0.26	NS
Olfactory Receptor 52N-5	-0.757	0.011	-0.518	NS	-0.251	NS

Fig. 4. Olfactory receptor TBI biomarker contents in PBMCs are inversely correlated with TBI severity and long-term neuropsychological complications. Correlation analysis was used to test the potential association between PBMC OR11H1, OR4M1, and OR52N5 mRNA content and the severity of TBI injury or self-reported measures of TBI complications. OR11H1 (A), OR4M1 (B), and OR52N5 (C) content in PBMCs are inversely correlated with TBI severity. D) Correlation coefficients and *p*-values of associations between individual OR biomarker content in PBMCs and TBI severity, TBI-specific symptoms (a summation of 25 cognitive symptoms that are sensitive and specific to TBI) and self-assessment of mood.

postmortem brain specimens, including temporal gyrus (BM22), entorhinal cortex (BM36), occipital cortex (BM17), and the hippocampal formation (Fig. 5A & B). In agreement with our RT-PCR data, we also found the expression of OR TBI biomarkers in the hippocampal formation region from the human brain genome-wide microarray database [30] (Fig. 5C–E). Our observation that the three OR biomarkers were ectopically expressed in multiple brain regions outside of the olfactory bulb suggested potential function(s) of these ORs in the brain unrelated to the detection and processing of olfactory information.

Activation of OR TBI biomarker modulates tau neuropathology-related phenotypes in vitro

Olfactory receptors are members of the class A rhodopsin-like family of G protein-coupled receptors (GPCRs). Once the odorant has bound to the odor receptor, the receptor undergoes structural changes and it binds and activates the olfactory-type G protein on the inside of the olfactory receptor neuron. The G protein (Golf and/or Gs) in turn activates adenylate cyclase, which converts ATP into cyclic AMP (cAMP). So we first examined the expression level of Golf and β-arrestin in the PBMC of TBI and control subject and no change was detected (Supplementary Figure 1; available online: http://www.j-alz.com/issues/34/vol342.html#supplementarydata04).

Considering that tau neuropathology is a main pathological feature following TBI, we continued to explore whether OR4M1 activation might influence tau processing mechanisms. We transduced primary cortical-hippocampal neuron culture with OR4M1 lentiviral particles and screened several odorants for their ability to activate OR4M1 using cAMP ELISA assay. In Fig. 6A, we presented some representative odorants that were capable or not capable of activating OR4M1. Positive ligands were used to stimulate OR4M1-overexpressing primary cortico-hippocampal neurons and the potential impact of activation of OR4M1 on signaling pathways and aberrant tau phosphorylation were assessed. Using

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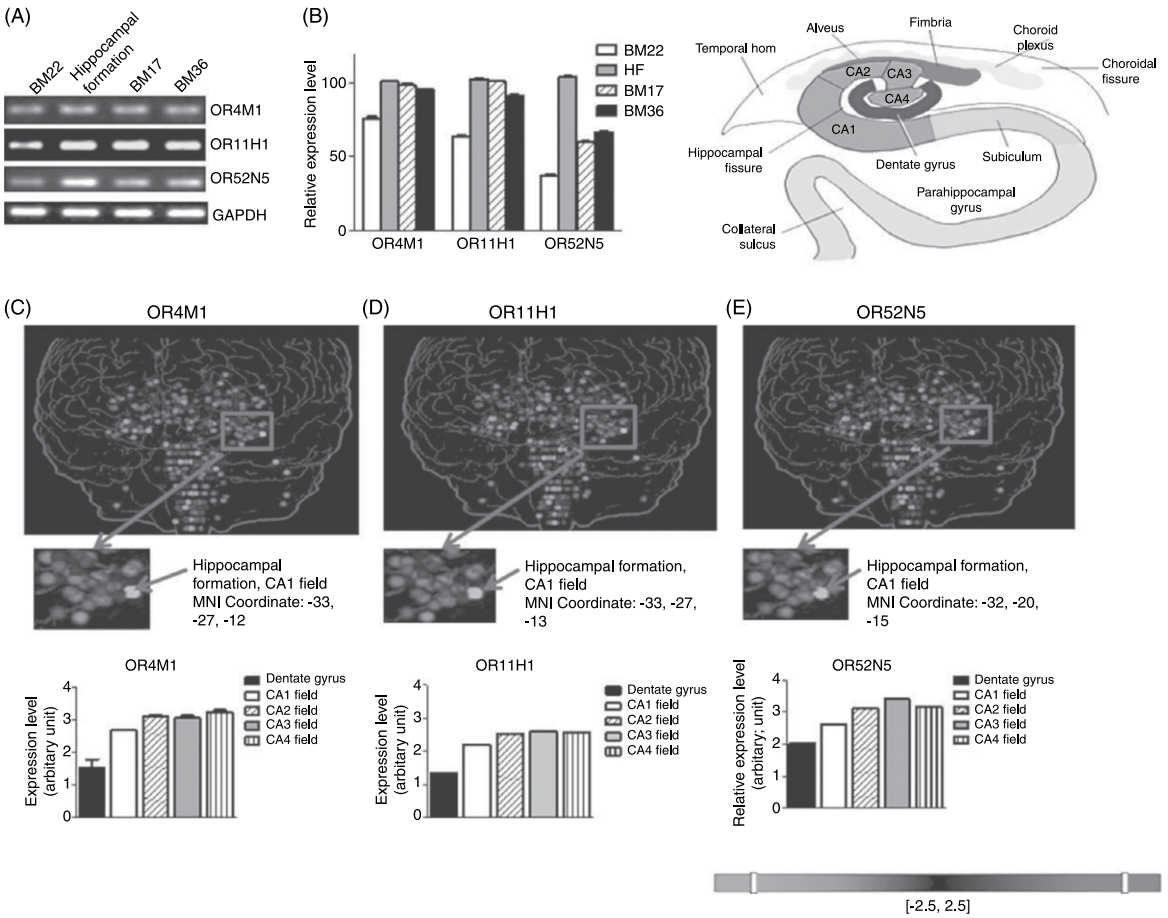

Fig. 5. Olfactory receptor TBI biomarkers expression in the brain. OR11H1, OR4M1, and OR52N5 mRNA expression by (A) RT-PCR and (B) relative expression level in postmortem superior temporal gyrus (BM22), hippocampal formation (HF), occipital cortex (BM17), and entorhinal cortex (BM36) specimens from neurologically normal cases. Expression pattern of (C) OR4M1, (D) OR11H1, and (E) OR52N5 in the brain by genome-wide microarray from the Allen Human Brain Atlas [30].

luminex multiplex technology, we found that upon acetophenone stimulation, phosphorylation of c-Jun N-terminal kinase (JNK) at Thr183/Tyr185 was significantly reduced in neurons overexpressing OR4M1 (Fig. 6B), indicating the activation of OR4M1 might influence JNK signaling pathway. The phosphorylation of ERK1/2 (Fig. 6B), among others (STAT3, MEK, p70S6, IKBa, and CREB, see Supplemental Figure 2), did not change upon acetophenone stimulation, suggesting that this inhibition of JNK signaling is rather specific. The stress-activated kinase JNK belongs to the mitogen-activated protein kinase family and takes part in signaling cascades initiated by various forms of stress. Its targets include the microtubule-associated protein tau [31]. Hyperphosphorylation of tau at Ser396/Ser404 is a known feature associated with tau neuropathology in neurodegenerative disorders such as Alzheimer's disease. Encouragingly, we observed that overexpression and activation of OR4M1 in primary neurons significantly reduced cellular content of abnormally phosphorylated tau at Ser396/Ser404 (PHF1 epitope) (Fig. 6C), suggesting that activation of OR4M1 might result in protection against abnormal tau processing.

Collectively, our data demonstrated that select olfactory receptors (e.g., OR11H1, OR4M1, and OR52N5) were down-regulated in the PBMC of TBI cases and could serve as TBI biomarkers. Activation of OR4M1 resulted in protection against tau neuropathological features possibly through the JNK signaling pathway (Fig. 7).

DISCUSSION

Consistent with accumulation evidence suggesting that PBMC-associated biomarkers may provide

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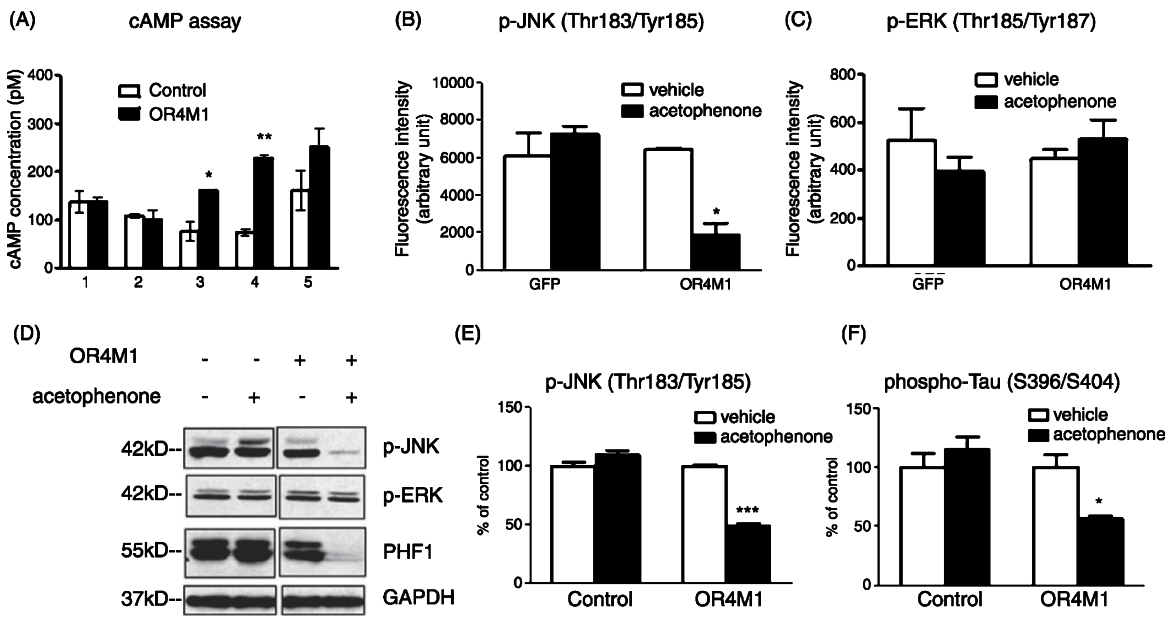

Fig. 6. Activation of OR4M1 resulted in reduced tau phosphorylation via JNK signaling pathway. Primary cortico-hippocampal neuron culture was transduced with lentiviral particles overexpressin OR4M1 or control lentiviral particles by spin infection (250 g × 90min at 30°C). A) Transduced cells were treated by (1) hedione, (2) (+) citronellal, (3) acetophenone, (4) pyrazine, and (5) 2-isobutyl-3-methopyrazine (all at 10 μM, Sigma-Aldrich) for 10min and cAMP assay was performed. Transduced neurons were also treated with acetophenone for 1 h, and multiplex luminex assay was performed using the Milliplex xMAP 8-plex multipathway signaling-phosphoprotein kit (Millipore) according to manufacturer's protocol: (B) Change of JNK phosphorylation on Thr183/Tyr185 and (C) ERK1/2 phosphorylation on Thr185/Tyr187. JNK and tau phosphorylation were also measured by (D) western blot analysis using antibodies recognizing phospho-JNK, phospho-ERK, or phospho-tau (PHF-1 epitope) and quantified (E, F) using GAPDH as a loading control. $*p<0.05$, $**p<0.01$, $***p<0.001$ by two-tailed student *t*-test.

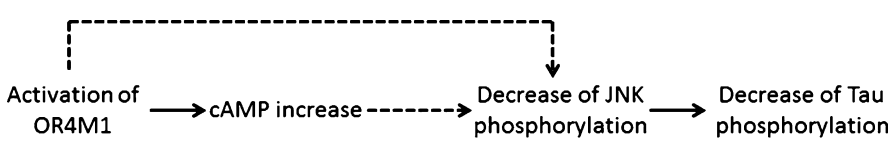

Fig. 7. Scheme of working hypothesis.

insights into the pathogenesis of neurological disorders, results from our studies revealed that expression of select ORs in PBMC may serve as clinically assessable surrogate biological indices of TBI. ORs are G protein-coupled receptors known to be expressed in nasal epithelium olfactory neurons, where they are responsible for the detection of odorants [32]. However, ectopic expression of select ORs, defined as a biological event or process that occurs in an abnormal location or position in the brain or other tissues, has also been described [33]. Our high throughput microar-ray evidence, followed by data from independent qPCR confirmatory studies, led to the identification of three OR TBI biomarkers (OR11H1, OR4M1, and OR52N5) that are ectopically expressed in PBMC and are aberrantly down-regulated in PBMC specimens from subjects with a history of TBI. Among the TBI cases in our study cohort, there was a significant time lag between the occurrence of brain injury and the volunteers' participation in this biomarker study; the average post-injury interval among the TBI cases was 5.4 ± 5.3 years (Table 1). Thus, down-regulation of the three ORs in PBMC among TBI cases was reflections of long-term physiological consequences of TBI.

We found that down-regulation of the three OR biomarkers in PBMC were directly correlated with the severity of brain injury in our TBI participants. Among the three OR biomarkers, we found that a two-biomarker panel, comprised of OR11H1 and OR4M1, provides the best criterion for segregating the TBI and control cases with 90% accuracy, 83.3% sensitivity, and 100% specificity. Interestingly, we found that PBMC contents of OR11H1

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and OR4M1 were inversely correlated with cognitive-related symptoms.

While additional studies will be required to clarify the mechanisms underlying the inter-relationships between changes in OR biomarker contents in PBMC, the initial severity of brain injury, and long-term clinical consequences of TBI, outcomes from our studies suggest that additional applications of this two-OR biomarker panel to current diagnostic criteria may lead to improved TBI detection and more sensitive outcome measures for clinical trials.

Initial and persistent cognitive deficits are the most common complaints after TBI [34, 35]. The entorhinal-hippocampus system is known to play an important role in the formation and consolidation of memories, particularly spatial memories [36–38]. Among the brain regions surveyed, we found the highest content of OR TBI biomarkers in the hippocampal formation, with relatively lower levels of OR TBI expression in the entorhinal cortex. Based on this and on our observation that the down-regulation of OR11H1 and OR4M1 in the PBMC are inversely correlated with self-reported indexes of cognitive functions, it is likely that ectopic expression of OR biomarkers in the entorhinal-hippocampus circuitry might be relevant to long-term deficits in cognitive functions following TBI.

It is well known that TBI may be a risk factor for dementia [39], but recent evidence suggests that Alzheimer's disease-related neuropathological mechanisms may contribute to cognitive dysfunction in TBI. The two characteristic neuropathologies of Alzheimer's disease are the abnormal accumulation and deposition of amyloid-β peptides and tau proteins in the brain. Evidence from humans [40–42] and experimental animal models [43] has also revealed abnormal accumulations of amyloid-β peptides and tau proteins in the brain and in cerebrospinal fluid following TBI. Interestingly, elevation of plasma tau levels has been associated with increasingly severe outcomes of TBI [44]. Multiple signaling pathways have been demonstrated to be involved in TBI pathology, including the AKT signaling pathway [45], glycogen synthase kinase-3 signaling pathway [46], STAT3 signaling pathway [47], ERK signaling pathway [48], and JNK signaling pathway [49]. We found that activation of OR4M1 could lead to attenuation of abnormal tau phosphorylation on S396/404 via JNK signaling pathway, suggesting a possible link between OR4M1 and TBI-related tauopathy. Further studies will be needed to investigate abnormal tau phosphorylation level in the serum/cerebrospinal fluid of TBI subjects, especially the phosphorylation of Thr181, which has been implicated as a fluid-based marker in dementia [50]. Studies will also be needed to dissect in detail the signaling pathways that are associated with the attenuation of tau neuropathology through OR activation.

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Authors' disclosures available online (http://www.jalz.com/disclosures/view.php?id = 1571).

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The Influence of the Val66Met Polymorphism of Brain-Derived Neurotrophic Factor on Neurological Function after Traumatic Brain Injury

John D. Finan*, Shreya V. Udani, Vimal Patel and Julian E. Bailes
Department of Neurosurgery, NorthShore University Health System, Evanston, IL, USA

Abstract. Functional outcomes after traumatic brain injury (TBI) vary widely across patients with apparently similar injuries. This variability hinders prognosis, therapy, and clinical innovation. Recently, single nucleotide polymorphism (SNPs) that influence outcome after TBI have been identified. These discoveries create opportunities to personalize therapy and stratify clinical trials. Both of these changes would propel clinical innovation in the field. This review focuses on one of most well-characterized of these SNPs, the Val66Met SNP in the brain-derived neurotrophic factor (BDNF) gene. This SNP influences neurological function in healthy subjects as well as TBI patients and patients with similar acute insults to the central nervous system. A host of other patient-specific factors including ethnicity, age, gender, injury severity, and post-injury time point modulate this influence. These interactions confound efforts to define a simple relationship between this SNP and TBI outcomes. The opportunities and challenges associated with personalizing TBI therapy around this SNP and other similar SNPs are discussed in light of these results.

Keywords: Brain-derived neurotrophic factor, precision medicine, single nucleotide polymorphism, traumatic brain injury

INTRODUCTION

Traumatic brain injury (TBI) causes approximately 56,000 deaths, 282,000 hospitalizations, and 2.5 million emergency room visits in the United States every year [1]. Survivors of TBI may have prolonged or permanent deficits in social, emotional, cognitive, and motor function, depending on the severity of the injury and the location of the lesion. Functional outcomes vary widely across patients with TBI. This is true for both mild and severe TBI at both acute and chronic time scales. Several demographic and environmental factors are known to influence functional TBI outcomes, including age, gender, and history of TBI [2–4]. In addition, evidence is accumulating that genetic factors influence TBI outcomes [5–8]. Understanding these genetic factors is perhaps the most important challenge in modern TBI research, with ramifications for prognosis, therapy, and clinical trial design. Prognosis is exceptionally difficult in TBI [9]. Some patients continue to make functional gains steadily for years after a severe TBI while others do not. Also, some patients decline several years after exposure to mild head impacts while others do not [10]. A genetic factor that partially explained these differences would be valuable

*Correspondence to: John D. Finan, NorthShore University Health System Research Institute, 1001 University Place, Evanston, IL 60201, USA. Tel.: +1 224 364 7578; E-mail: jfinan@northshore.org.

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to patients and clinicians attempting to plan around likely future neurological function. On the therapeutic front, genetic factors that influence pathology may also influence how that pathology responds to therapy. An understanding of these influences may enable more effective personalized therapy. On a related note, it is increasingly clear that future trials of TBI therapies must be stratified to have any chance of success. More than 30 phase III clinical trials of candidate therapeutics for TBI have been conducted and all have failed [11]. The consensus in the research community is that TBI trials fail in part because of the heterogeneity of the patient population [12]. This heterogeneity creates a signal-to-noise problem: the more outcomes vary within treatment groups, the more treatment effect is required to achieve statistical significance. The solution is to stratify patients according to likely outcome and a genetic outcome predictor would be invaluable in this regard. In order to be useful, the genetic predictor must be common and must exert a powerful influence on TBI outcomes. The Val66Met single nucleotide polymorphism (SNP) of brain-derived neurotrophic factor (BDNF) occurs in about 30% of the U.S. population [13]. It has been shown to influence neurological function in healthy subjects [14], TBI patients [15, 16], and stroke patients [17, 18]. In this review, we summarize what is known about how this SNP influences TBI outcomes and explore its potential to inform prognosis, therapy, and clinical trial design.

BDNF AND THE VAL66MET SNP

The complex architecture of the human nervous system is established and maintained by local interactions between specialized neurons and their targets. Neurons require chemical signals called neurotrophins supplied by their targets for survival [19]. This simple mechanism organizes the nervous system during development and maintains this organization in adulthood. Neurons are generated in great excess during embryogenesis. However, only those neurons that enervate an appropriate target receive the neurotrophins they need to survive [20] so supernumerary neurons apoptote. In an analogous way, neurotrophins regulate the branching of surviving neurons and the formation of synapses so that neural circuits adapt to the requirements of their targets [21]. There are four neurotrophins: neural growth factor (NGF), BDNF, neurotrophin 3 (NT-3), and neurotrophin-4/5 (NT-4/5). Certain classes of neuron depend on certain neurotrophins for survival while remaining indifferent to others. This selectivity dynamically organizes neurons around their targets. BDNF plays a prominent role in hippocampal functions including learning and memory [22] because it regulates long term potentiation (LTP) [23]. Suppressing BDNF in mice by genetic knock out [24] or RNA interference [25] impairs performance in learning and spatial memory tasks. BDNF is well suited to its role in LTP because it is released in an activity-dependent fashion while all other neurotrophins are released at a constant rate [26]. Electrical stimulation triggers secretion of BDNF which then promotes synapse formation and enhances synaptic transmission. These changes are the cellular basis of LTP.

When translated, the BDNF molecule contains three important domains: the signal peptide, the pro domain, and the functional BDNF molecule. The signal peptide sequesters the molecule in the endoplasmic reticulum and is then removed [27]. Subsequently removing the pro-domain produces mature BDNF. The pro-domain must interact with the sortilin receptor in the Golgi wall to allow proper folding of the mature domain. Proper folding allows a motif in the mature domain to connect to carboxypeptidase E, and then BDNF is sorted into large dense core vesicles to be released via the regulated secretory pathway [28]. The pro-domain may be removed intracellularly in the trans-Golgi network by enzymes such as furin [29] or extracellularly by enzymes such as plasmin [30] or matrix metalloproteinase 7 [30]. Mature BDNF and pro-BDNF function antagonistically to regulate neuroplasticity [30]. Mature BDNF binds to the tyrosine related kinase receptor (TrkB) to activate pathways involved in LTP [31], cell survival [32], and dendrite formation [33]. ProBDNF acts via the p75 neurotrophin receptor (p75NTR) and sortilin to activate pathways involved in long term depression [34], apoptosis [35], and reducing dendritic complexity [34].

There are several hundred known polymorphisms of the BDNF gene [36]. Of these, Val66Met (also known as rs6265) is the most widely studied. It is a SNP at nucleotide 196 that causes the substitution of the typical valine with methionine at codon 66 (Val66Met). About 30% of the population is at least heterozygous for this SNP in the United States [13], but this proportion varies widely across global populations [13, 37]. Val66Met occurs in the portion of the BDNF gene that encodes the pro-domain. Therefore, it does not alter the function of the mature

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protein. For example, the Val66Met allele does not modulate the neurotrophic effect of mature BDNF on PC12 cells in culture [14]. However, it does alter intracellular sorting and secretion of the protein. Specifically, the SNP occurs in a portion of the prodomain that interacts with sortilin [38]. BDNF proteins with methionine at codon 66 (mBDNF) do not sort from the Golgi into secretory vesicles as well as BDNF proteins with valine at codon 66 (vBDNF) [14]. As a result, mBDNF tends to accumulate in the soma while vBDNF accumulates in punctate vesicles in the dendrites. Neurons secrete vBDNF but not mBDNF in response to activity. Activity-dependent release of BDNF drives LTP [21] so Met carriers have impaired neuroplasticity [14]. Hippocampal volume has been reported to be reduced in Met carriers by several investigators [39–41], although this conclusion remains controversial [42]. The Met allele is also associated with reduced grey matter volume in other brain structures including the amygdala [43] and dorsolateral prefrontal cortex [40]. The Val66Met SNP has functional consequences in healthy subjects. Human Met carriers generally have worse episodic memory than matched Val carriers [14] and show less activation of the hippocampus during memory tasks as measured using magnetic resonance imaging techniques [44]. Met carriers have inferior motor function and motor learning compared to Val/Val subjects [45]. While the SNP is generally deleterious in healthy subjects, benefits emerge in some disease states, including TBI.

VAL66MET IN TBI

The Met allele partially protects patients with severe TBI. In a study of male Vietnam veterans with penetrating TBI, TBI impaired executive function among Val/Val individuals but not among Met carriers [16]. In the same cohort, Val/Met individuals had better general intelligence, working memory, and processing speed than Val/Val individuals after TBI, although there was no difference between these groups before TBI [15]. It is worth noting that brain function was assessed in these individuals at very long-term time points, up to 40 years post injury. Met carriers secrete less of both mature BDNF and proBDNF in response to activity. The Met allele is protective after severe TBI, suggesting that the apoptotic action of proBDNF overcomes the survival signaling of mature BDNF in this context. Under these circumstances, impaired secretion would become neuroprotective [15, 16]. The Val allele may also aggravate the deleterious effects of proBDNF. *In vitro*, the Val form of proBDNF inhibits LTP and promotes tau hyperphosphorylation while the Met form does not [46]. Another possibility is that the Met allele improves long term outcomes because it shifts the excitatory / inhibitory balance of the brain toward excitation. The mouse model of the Val66Met SNP [47] has not been subjected to experimental TBI, to our knowledge, but it has been subjected to experimental stroke. The Met allele led to worse short term motor outcomes after stroke in this mouse model [48], due to inferior neuroplasticity and angiogenesis, but better long term outcomes [49], due to higher excitability in the striatum opposite the lesion.

The protective effect of the Met allele in severe TBI depends on age at chronic timepoints. A study of BDNF SNPs in severe TBI tested the hypothesis that the Met allele of Val66Met in conjunction with another putative high-risk polymorphism of BDNF, rs7124442, would increase the risk of death. In fact, the putative high-risk alleles reduced the risk of death at short time points (<7 days). At long time points (8–365 days), an interaction with age emerged. The Met allele along with the other hypothetical risk allele did indeed increase risk of death for patients under 45 years of age. However, for those over 45, these alleles were again protective [50]. Note that long term death rates were higher overall in the over 45 age group. This age dependence may arise because the balance of receptors that mediate pro-survival and pro-apoptotic effects of BDNF changes with age [51, 52]. Severe TBI uncouples the autonomic and cardiovascular systems [53] so the role of the Val66Met SNP in autonomic function is also important. Healthy Met/Met individuals have altered sympathovagal balance and less parasympathetic activity compared to Val/Val individuals [54]. Also, healthy Met/Met individuals had less cardiovascular reactivity to acute psychosocial stress than Val/Val individuals [55]. However, the effect of the Val66Met SNP on autonomic function after TBI is currently unknown.

The influence of the Val66Met SNP in mild TBI depends on gender and the post-injury time point. Female concussed athletes carrying a Met allele performed significantly better on assessments of olfactory function than their Val/Val counterparts [56]. Participants in this study were assessed an average of 27 months after their concussion. On the other hand, tests of mood conducted at 1 and 6 weeks post-injury in another cohort of patients with mild TBI found more depression and anxiety in Met carriers.

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Table 1
Influence of the Val66Met SNP on mild TBI pathology

	Acute	Chronic
Female	Deleterious [57]	Protective [56] Neutral [57]
Male	Deleterious [57]	Deleterious [57]

Note that different studies use different outcomes and different definitions of acute and chronic time periods.

The effect on depression was modulated by gender, being significant at both time points in males but only at the first time point in females [57]. Mild TBI increased depressive rumination and reduced cognitive flexibility among Met carriers but not among Val/Val homozygotes. Unfortunately, no data about the time of injury was collected in this study [58]. In this context, it is worth noting that Met allele carriers are more prone to rumination than Val/Val homozygotes in the absence of TBI [59] and that this trend becomes more pronounced under stress in Met homozygotes [60]. The Val66Met SNP lowered performance among both injured subjects and uninjured controls in a study of reaction times after mild to moderate TBI (Glasgow Coma Score 9–15) but this effect did not interact with TBI. However, several other polymorphisms of BDNF did influence the effect of TBI in a gender dependent fashion with more influence in males [61]. Table 1 summarizes what is known about the influence of the SNP on acute and chronic TBI pathology in males and females for mild TBI.

In addition to modulating the pathology of TBI, the Val66Met SNP modulates the risk of sustaining a TBI according to two consistent but small studies in human subjects [62, 63]. The reasons for this trend may be behavioral or physiological or both. Behaviors modulated by this SNP include addiction [64, 65], aggression [66, 67], participation in high risk sports [68], and hyper-active impulsivity (in people with attention deficit hyperactivity disorder) [69]. Met carriers have greater hostility and aggression, supporting a behavioral explanation for this trend [63]. However, larger human studies are needed to fully explain this phenomenon [70].

VAL66MET AND NEURODEGENERATION

One of the most important and mysterious questions in modern TBI research is how does TBI influence the likelihood of subsequent dementia. Striking evidence of early-life tauopathy has been discovered in athletes [10] and military veterans [71] with a history of repeated mild TBI. The unique spatial distribution of this tauopathy defines it as a distinct neuropathological entity: chronic traumatic encephalopathy [72]. Unfortunately, the prevalence of this condition is currently unknown. TBI also increases the risk of Alzheimer's disease (AD) [73, 74]. A recent study of a large population of U.S. military veterans found that all forms of TBI increased the risk of dementia with hazard ratios ranging from 2.36 for mild TBI without loss of consciousness to 3.77 for moderate to severe TBI [75]. The apolipoprotein E (APOE) gene exists in 3 isoforms, one of which, APOE $\varepsilon 4$, increases the risk of AD [76]. TBI amplifies the impact of APOE $\varepsilon 4$ on AD risk. One study reported that APOE $\varepsilon 4$ doubled the risk of AD absent a history of TBI but that the combination of APOE $\varepsilon 4$ with TBI history increased the risk of AD 10-fold [77]. Therefore, it is possible that other genetic risk factors for AD also synergize with TBI. The Val66Met SNP has been investigated as a genetic risk factor for AD. Early studies found that Met carriers had lower incidence of the disease [78] and, in the case of Met homozygotes, better reasoning skills in late life [79]. However, subsequent studies failed to replicate these findings [80, 81]. This inconsistency may arise in part from interactions of the Val66Met SNP with other factors. For example, the effect of the Val66Met SNP on phenotypes associated with AD was age dependent, with Met carriers being protected in late life but more susceptible in early life [82]. Gender also moderates the effect. The Met allele increased risk of AD in women but not in men in a Japanese cohort [83]. While both TBI and the Val66Met SNP influence the risk of dementia, there is as yet no published evidence demonstrating synergy between them. Nevertheless, the possibility of such a synergy merits investigation.

VAL66MET AND THERAPY

Determined efforts to discover a therapy that improves outcomes for all TBI patients have not yet succeeded. Therefore, the more modest goal of discovering a therapy that benefits a subset of TBI patients deserves attention. The Val66Met SNP may define such a subset because it has been shown to modulate the effectiveness of neurological drugs in a number of settings. For example, olanzapine for the treatment of schizophrenia is less effective in Met carriers [84]. Memantine was more effective as an add-on therapy for depressive symptoms in heterozygous bipolar disorder patients than

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in Val/Val homozygotes [85]. However, most of the pharmacogenetic investigation of the Val66Met SNP has addressed its influence on selective serotonin reuptake inhibitors (SSRIs), a popular class of anti-depressants. Two meta-analyses concluded that heterozygotes were more responsive to SSRIs than either Val or Met homozygotes. This effect was modulated by race, being stronger among Asians [86, 87]. However, TBI patients with depression were most likely to respond to citalopram (an SSRI) if they were Val homozygotes [88]. This discrepancy may indicate that post-TBI depression requires a different strategy for therapy personalization than other types of depression or it may reflect the fact that Val66Met was combined with another genetic predictor in this study (a polymorphism in the methylene tetrahydrofolate reductase gene). These results have immediate clinical relevance. Up to 53% of TBI patients experience depression in the first year after their injury [89] and SSRIs are a proven, first line therapy for this post-TBI depression [90].

The Val66Met SNP may also modulate the effectiveness of non-pharmaceutical therapies for TBI. Exercise improves emotional and cognitive outcomes after TBI [91, 92]. This effect depended on expression of BDNF [93]. Positive neurobiological effects of exercise in mice were diminished by the Met allele of the Val66Met SNP [94]. Since the Met allele impairs activity-dependent release of BDNF [14], it is reasonable to hypothesize that physical exercise does not promote recovery after TBI as readily in these individuals. This hypothesis has not as yet been tested directly in the literature. In a small study of spinal cord injury patients, Met carriers increased circulating BDNF less in response to physical exercise than Val/Val patients [95]. This trend approached but did not meet the threshold for statistical significance. Low levels of physical activity increased the risk of cognitive decline and dementia in an elderly Korean cohort and this effect was modulated by the Val66Met SNP, with Met alleles increasing risk [96]. In a study of healthy young adults, physical exercise improved performance on a memory task in Val/Val individuals but not in Met carriers [97]. A short period of motor training increased corticospinal output and motor map area in Val/Val human subjects but not in Met carriers, implying impaired experience-dependent plasticity in the motor cortex among Met carriers [98]. Val/Val subjects performed better on a motor learning task and retained more of what they learned. The Val66Met allele also caused differences in brain activation during motor activity as measured by functional magnetic resonance imaging [45]. However, persistent training overcame these deficits [99], suggesting that patience in motor rehabilitation of Met carriers will be rewarded.

CONCLUSION

TBI has been described as "the most heterogeneous of all neurological disorders" [11]. Several different pathologies, including edema, ischemia, hemorrhage, and diffuse axonal injury may or may not occur [100]. The distribution of TBI patients by age has three widely separated peaks at 0–4, 15–19, and >65 years of age [101]. In addition to Val66Met, there are several other common polymorphisms in genes such as DRD2, COMT, and ANKK1 that modulate outcomes [5–8, 102]. This heterogeneity implies that precision medicine could have many benefits in TBI. Clinical trial stratification would make expensive trials more likely to detect treatment effects in spite of confounding factors. Personalized therapy would allow new medicines to benefit some patients even if they cannot benefit all patients. However, daunting challenges must be overcome to realize the potential of personalized medicine in TBI. Not only do multiple patient-specific factors influence outcome, but these factors interact with each other in ways that cannot be ignored. This review took a reductionist approach, focusing on one SNP known to influence outcomes in TBI. However, it proved impossible to fully describe the role of this SNP without also considering how it interacts with ethnicity, age, injury severity, post injury time point, and gender. New interactions are likely to emerge over time as investigations continue, including interactions with other genetic polymorphisms. These interactions create confusion in the field because they lead to conflicting results in human studies conducted in different populations [103]. It is very difficult to eliminate all potentially confounding interactions from a human study but human *in vitro* models can be used to reduce confounds in a human system. In these models, induced pluripotent stem cell (iPSC) technology is used to create neurons or other neural cell types from a patient. These cells retain the genetic identity of the donor patient. Furthermore, gene editing technology can be used to introduce genetic variants one at a time against a uniform genetic background [104]. In these experiments, genetic risk factors can be eliminated or introduced individually to test hypotheses definitively. While this approach eliminates some confounds associated with

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conventional human studies, it has its own limitations. As in any *in vitro* system, the normal architecture of the tissue is lost. Also, human iPSC-derived neurons are typically immature, resembling fetal or neonatal neurons more than adult neurons [105]. However, rapid progress is being made to address these limitations. Brain organoids have been developed that juxtapose several neural cell types derived from human iPSCs in biofidelic configurations [106]. Aging of iPSC-derived neurons can be accelerated by expressing progerin, a truncated form of lamin A associated with premature aging disorders [107], or by telomerase manipulation [108]. Human *in vitro* models have already made significant contributions in other neurological disorders, including amyotrophic lateral sclerosis [109, 110]. They have recently been introduced in neurotrauma [111, 112] where they may eventually provide a powerful complement to traditional pre-clinical tools. The challenges associated with personalizing TBI medicine around patient-specific traits such as the Val66Met SNP are great. However, the potential rewards are greater still and this type of personalization will be a vital frontier for innovation in the field in the near future.

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Traumatic Brain Injury, Chronic Traumatic Encephalopathy, and Alzheimer's Disease: Common Pathologies Potentiated by Altered Zinc Homeostasis

Stuart D. Portbury and Paul A. Adlard*
The Florey Institute of Neuroscience and Mental Health, Kenneth Myer Building, The University of Melbourne, Parkville, VIC, Australia

Abstract. Alzheimer's disease, traumatic brain injury, and chronic traumatic encephalopathy represent conditions that have a profound socioeconomic impact for both the individual and the wider community. They are all characterized by specific protein aggregation that results in synaptic dysfunction, neuronal death, and consequent cognitive decline and memory loss. In this review, we present evidence to support the notion that the common pathologies found in all conditions, and indeed their associated cognitive deficits, may be linked by zinc (Zn^{2+}) ion dyshomeostasis. Elucidation of this hypothesis may present new therapeutic avenues for these devastating conditions.

Keywords: Alzheimer's disease, amyloid-β, brain injury, cognition, tau protein, TDP-43, zinc

INTRODUCTION

Traumatic brain injury (TBI) and chronic traumatic encephalopathy (CTE) are associated with a number of neurochemical, neurophysiological, and behavioral consequences that are also typically associated with neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease, and amyotrophic lateral sclerosis (ALS) [1]. Moreover, head trauma has been implicated as a primary risk factor for the development of a variety of neurodegenerative disorders, although the principal focus in the literature has been on understanding the link to AD. In this respect, a common feature underlying the development of age-related diseases such as AD, as well as to the development of the neurobiological sequelae following TBI and CTE, may be alterations in brain levels of zinc (Zn^{2+}). This is a theory first postulated by Frederickson and colleagues [2], and in this review, we will re-examine this notion in light of the current literature.

*Correspondence to: Associate Professor Paul Adlard, The Florey Institute of Neuroscience and Mental Health, Kenneth Myer Building, The University of Melbourne, 30 Royal Parade, Parkville, VIC 3052 Australia. Tel.: +61 3 90356775; Fax: +61 3 90353103; E-mail: paul.adlard@florey.edu.au.

ALZHEIMER'S DISEASE

AD is a progressive neurodegenerative disorder and is both the major cause of dementia and a leading cause of death in the elderly [3]. It is both sporadic and familial in nature; impairing memory, thinking and behavior, while destroying quality of life, ultimately culminating in death. There is no cure, nor is there an effective therapeutic intervention currently available. There are two major hallmark pathologies in the AD brain, and both are considered to be detri-

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mental to neuronal health. The first results from the deposition of the ~4 kD amyloid-β peptide (Aβ), a cleavage product of the much larger amyloid-β protein precursor (AβPP). Deposition of the Aβ peptides result in the formation of potentially neurotoxic extracellular amyloid plaques, which together with other oligomeric forms of Aβ, subsequently impair synaptic function and long term potentiation (LTP) [4–6].

The second additional and equally important neuropathology is the formation of intracellular neurofibrillary tangles (NFTs), which consist of hyperphosphorylated microtubule associated protein tau. These abnormal tau deposits are believed to impair axonal transport along microtubules, affecting synaptic plasticity, learning, and memory [7].

TRAUMATIC BRAIN INJURY

TBI is a major health problem worldwide and is predicted to be the major cause of death and disability internationally by 2020 [8]. Ten million people are affected by TBI annually, due to a variety of causes including vehicular accidents (60%), falls (20–30%), violence (10%), and sporting/workplace injuries (10%) [8]. In the US alone, it is estimated that 1.7 million people sustain a TBI annually, contributing to over 30% of the injury-related deaths annually in the US [9].

Brain injury arising from head trauma typically falls into one of two categories, acute or chronic. Acute brain injury is the consequence of a trauma or concussion resulting from accidents or abuse, and is most commonly a product of subdural hematoma. Falls are the most common form of acute traumatic brain injury followed by vehicular accidents, the latter being the most common cause of TBI related death [9]. Chronic brain trauma is the result of repeated head trauma and can lead to neurodegenerative disorders. A common example of chronic head trauma is dementia puglistica, which typically can develop in professional boxers [10]. More recently, the term dementia puglistica has been used interchangeably with the now preferred title of chronic traumatic encephalopathy (CTE), and is associated with many contact sports athletes, particularly American footballers, as well as blast-exposed military veterans [11–15]. Large numbers of TBIs are closed head injuries falling into one of two categories. The first category is concerned with focal injuries consisting of intracranial hematomas, contusions, and epidural and subdural hematomas, while the second category concerns diffuse axonal injury (DAI) [16].

Pathologically, both TBI and CTE result in DAI, a consequence of axonal shearing during acceleration and rotational forces derived from head impact. Within hours after TBI, neuronal damage has been observed in the cerebral cortex, hippocampus, and dentate gyrus [16]. Hippocampal damage is considered to be the major contributor to the memory and learning disturbances associated with clinical and experimental TBI and CTE [17].

SIMILARITIES BETWEEN AD AND TBI

Epidemiology

There is an increasing body of evidence to indicate that TBI and AD are linked epidemiologically. Several studies have reported that TBI is one of the strongest risk factors for the development of AD [18–23], and that AD is accelerated by a history of TBI [24–27]. Furthermore, the greater the severity of injury, the greater is the risk of developing AD [28, 29]. More recently, Marklund and colleagues used cerebral microdialysis in humans to demonstrate that TBI may result in the generation of potentially toxic Aβ species within the brain interstitium, and that Aβ levels were higher in individuals with predominantly diffuse axonal injury [30].

In another study, Nordstrom and colleagues found strong associations between young onset dementia (dementia before 65 years of age) of non-AD forms and TBIs of different severities. They also found an association between TBI and the rate of AD progression, such that individuals with a history of TBI that occurred relatively close in time to the onset of dementia had a greater risk of functional decline [31]. Similarly, Gilbert and colleagues found that individuals that experienced a TBI within ten years of dementia onset showed a faster rate of functional decline [32].

In contrast, however, other epidemiological studies have arrived at contrary conclusions, perhaps due to their retrospective nature and reliance on the memories of TBI subjects and secondary informants [33–40]. Kawai and colleagues, for example, examined cerebral Aβ deposition in patients with neurological impairment following TBI and found an absence of Aβ deposition in many patients with chronic neuropsychological impairment after TBI. While this does not support the premise that Aβ

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pathology progresses over time following TBI, the study was not a time course, nor did it investigate early post-TBI Aβ deposition [41]. Indeed, it has even been suggested that individuals with a history of TBI may actually present a unique clinical phenotype that is distinct from known dementia subtypes [42]. Regardless, larger controlled studies [29], coupled with the observation that the AD-associated apolipoprotein E [43, 44] is implicated in influencing both amyloid pathology and behavioral outcome post-TBI [45–53], has led to the common acceptance of an epidemiological link between TBI and AD.

Amyloid-β

A potentially declarative indication of a pathological link between AD and TBI was the observation that Aβ could be found in brain tissue in up to 30% of patients that died acutely following TBI [54, 55]. Unlike the plaques in AD, however, which accumulate over decades in the elderly, TBI plaques appear swiftly after injury regardless of age [55, 56]. In addition, the predominant Aβ peptide in TBI plaques is $A\beta_{1\text{-}42}$, which is the most pathogenic form of the peptide that is most prone to aggregate and which is principally associated with AD [57, 58]. It should be noted, however, that the plaques associated with TBI are primarily located in the white matter tracts at sites of DAI, whereas in AD they are located in the grey matter. In contrast, however, a recent study [59] utilizing a tracer (Pittsburgh Compound B) that binds Aβ in AD patients was used to bind amyloid plaques in TBI patients, revealing cortical grey matter and striatal tracer binding up to one year post injury. Moreover, binding levels were comparable to people with mild cognitive impairment.

Aβ has also been identified in ∼40–50% of CTE cases [60], and its toxicity is demonstrably potentiated by tau pathology, one of the major pathological hallmarks of CTE [61, 62].

Aβ formation in mouse TBI models

After the observation of Aβ plaque formation in human TBI, investigations began in animal models of TBI to determine whether axonal damage and AβPP accumulation was the source of plaques post-injury. Non-transgenic rat TBI models reproduced intra-axonal AβPP accumulation, but no associated Aβ accumulation in axons or plaques [63, 64]. The absence of Aβ was attributed to differences in the Aβ peptide between species [65]. However, transgenic mouse strains selected for their predisposition to develop plaques at advanced ages, failed to produce plaques following TBI paradigms. APP-YAC (APP-Yeast Artificial Chromosome) mice overexpressing human AβPP produced increased concentrations of Aβ in tissue without plaque formation [66], and another study using PDAPP (Platelet Derived Growth Factor Promoter-APP) mice overexpressing mutant human AβPP similarly failed to produce plaques after TBI (although the increased Aβ concentration post-TBI did accelerate hippocampal neuron death to result in memory impairment) [67]. These apparent discrepancies with the human condition were ultimately determined to relate to methodological issues.

More recently, and despite the initial difficulties with the mouse TBI models, two separate groups have had success in inducing aggregation and oligomerization of Aβ in mouse models after TBI. Taijiri and colleagues [68] utilized young presymptomatic APP/PS1 (APP-Presenilin 1) mice to demonstrate that TBI accelerated cognitive impairment and also resulted in a significant increase in Aβ deposits within the brain (they also showed a significant increase in Aβ deposits in the non-transgenic group following TBI). Similarly, Washington and colleagues [69] utilized a triple transgenic mouse model of AD and demonstrated that TBI rapidly increased the levels of both soluble and insoluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ at one day post-injury, with damaged axons identified as the major site of Aβ accumulation. These studies provide support for the notion of an interaction between TBI and AD-like pathology, and also highlight the difficulty in the use of animal models to recapitulate a human disease.

In many cases, murine models of TBI [70] utilize invasive techniques including craniotomy and subsequent blow or tissue crush to a stable head fixed in a stereotaxic frame, eliminating the rotational acceleration/deceleration of the brain, typical of human injury circumstances. Mouse models of TBI mimicking rotational forces have been attempted [71, 72] but have proven difficult to replicate human-like pathology due to the rodents relatively small brains of low mass and limited white matter. Subsequently, a swine model of TBI was developed mimicking rotational acceleration forces. Like humans, swine have large gyrencephalic brains with extensive white matter tracts, and additionally have an Aβ sequence identical to humans. The model was successful in producing DAI resembling that of humans in clinical situations [73, 74] and more importantly replicated AβPP accumulation in damaged axons, co-accumulation of Aβ,

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and the formation of parenchymal Aβ diffuse plaques in both the grey and white matter, although somewhat reduced compared to those observed in human TBI [75].

It should also be noted that the upregulation and accumulation of AβPP in response to injury could potentially be a protective mechanism, as AβPP under normal non-amyloidogenic processing has been shown to be beneficial in regards to neuroprotection, neurite outgrowth, and synaptogenesis [76–82]. One study, for example, demonstrated a reduction in neuronal cell loss and improved functional outcome in an experimental rat TBI model following administration of soluble non-amyloidogenic α-secretase cleaved AβPP [83]. Moreover, Corrigan and colleagues utilized a controlled cortical impact model of TBI to demonstrate that the administration of soluble AβPP could prevent cognitive deficits in AβPP -/- mice [84]. In contrast, numerous studies on human brain tissue samples of TBI victims have shown accumulations of AβPP in neurons and axons [85–89] in addition to the intra-axonal accumulation of Aβ [90–92]. This mass accumulation of proteins within the axon, in conjunction with the ensuing degradation of the axon through Wallerian degeneration, could liberate "toxic" Aβ species in addition to high levels of its precursor, AβPP. In fact, it has been demonstrated that plaque formation around damaged axons in TBI tissue occurs after AβPP accumulation and Aβ production in damaged axons [54, 90, 93, 94]. Further studies as to the role of Aβ accumulation and its effects in TBI need to be undertaken to determine its significance.

Tau

Tau is a normal axonal protein aiding in microtubule assembly and stability. However, phosphorylated tau has a reduced microtubule binding capacity, resulting in destabilized microtubules leading to reduced synaptic function. The abnormal phosphorylation of tau ultimately causes it to aggregate in the cytoplasm of the neuron, then form the paired helical filaments that comprise the NFTs that are found in the AD brain [95]. Together, microtubule destabilization and tangle formation act in concert to compromise overall function of the neuron. In addition to Aβ accumulation, and analogous to AD, hyperphosphorylated tau and NFTs are a prominent feature of TBI and CTE. Since the 1970s, NFTs have been demonstrated in the neocortex of boxers [10], while more recently they have also been described in many contact sport athletes [60]. Furthermore, neuropathological studies of blast-exposed military veterans have demonstrated similar tau pathology and tangle formation [15].

In TBI, extensive postmortem analysis has demonstrated abnormal tau phosphorylation in addition to NFTs [96–99]. Recently, Johnson et al. demonstrated widespread tau pathology many years after a single TBI in humans [72]. Furthermore, controlled cortical impact TBI in 3xTg-AD mice accelerated the development of tau abnormalities [100]. A recent study also indicated that both single and multiple blast traumas in humans have the potential to cause CTE with prominent tau pathology and NFTs [15]. A repetitive mild TBI model has similarly been shown to result in tau pathology [101].

Tau pathology in TBI and CTE, which is largely distributed in the white matter at sites of axonal degeneration, appears in an irregular distribution pattern that may be a reflection of alternate shearing forces induced by varied trauma direction and severity. In contrast, there is a stereotyped spatio-temporal propagation of tau pathology and NFTs in the grey matter in AD [10, 97]. Importantly, tau in both AD and CTE are phosphorylated at the same amino acids (including tau phosphorylated at Threonine 231), and all six isoforms are present, leading to the observation that the NFTs associated with AD are indistinguishable from those in TBI [98]. The major implication here is that there may be a uniform mechanism of phosphorylation and tangle formation, and hence, potentially a common treatment. While this connection is yet to be clearly annunciated, the hypothesis that the tauopathy evident in TBI and CTE may predispose individuals to AD decades after injury is not unrealistic.

TDP-43

Another protein with an emerging role in the pathogenesis of TBI and CTE is the transactivation response region deoxyribonucleic acid-binding protein 43 (named TAR DNA-binding protein or TDP-43). TDP-43 was initially considered to be a disease specific protein, wherein its accumulation was noted in frontotemporal lobar degeneration with ubiquitin inclusions and ALS [102]. However, other studies have revealed the presence of TDP-43 pathology in other neurodegenerative diseases as well, specifically AD and dementia with Lewy bodies [103, 104]. Furthermore, recent evidence has also shown a widespread accumulation of TDP-43 protein in box-

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ers and American footballers suffering from CTE [104, 105].

The role of TDP-43 in AD has yet to be elucidated, however it is clear that it is a common pathological feature in AD [104, 106–108]. It is estimated that TDP-43 immunoreactivity is present in roughly 30% of sporadic AD cases, and in 14% of familial AD and Down's syndrome brains [109]. The clinical implication of this is an apparent exaggeration of the hippocampal atrophy in AD cases with TDP-43 pathology. More recently, the development of a transgenic mouse model of TDP-43 overexpression has revealed not only motor dysfunction and hippocampal atrophy, but a parallel impairment in learning and memory and a reduced induction of long-term potentiation, a widely accepted electrophysiological correlate of learning and memory [110], directly related to the overexpression of TDP-43 [111].

COGNITIVE DECLINE AND MEMORY LOSS IN AD, TBI, AND CTE

The clinical manifestation of AD is memory loss and cognitive decline. It is hypothesized to be a result of synaptic dysfunction [112] resulting in the inhibition of LTP. In AD, the synaptic dysfunction is believed to be the result of exposure to various Aβ species [112–115]. Furthermore, in animal models the synaptic dysfunction manifests as a reduction in LTP [6, 116, 117], and has also been demonstrated to be conducive to the formation of hyperphosphorylated tau and the establishment of NFTs [118, 119], further contributing to cognitive decline [5].

Memory loss and cognitive decline is also a clinical manifestation of TBI and CTE and has been recognized for some time. Moreover, a recent study indicated that US National Football League players, whom are subject to repetitive concussions or mild brain traumas, were up to four times more likely to die from a neurodegenerative disease (particularly AD and ALS) than the general population [120]. Similarly, memory loss and cognitive decline caused by TBI and CTE are a common occurrence in blast-exposed military veterans [15].

Mouse models of CTE and repetitive mild TBI are also associated with learning and memory deficits, with a recent study demonstrating that a single blast neurotrauma exposure impaired axonal conduction and reduced LTP in conjunction with long-term behavioral deficits [15]. A study of mild repetitive trauma also confirmed that memory loss and cognitive decline is associated with CTE in mice [121]. Of particular note was that while a single trauma was adequate to produce the histopathological criteria and cognitive decline characteristic of CTE, subsequent traumas dramatically enhanced the phenotype.

AD, TBI, AND CTE: ARE THEY LINKED THROUGH ALTERED ZN^{2+} ION HOMEOSTASIS?

In review so far we have demonstrated that Aβ, tau, and TDP-43 underlie pathologies that are present to varying degrees in AD, TBI, and CTE. In each of these progressive neurodegenerative conditions, a decline in memory, learning, and executive function is the major clinical manifestation in both humans and animal models. Similarly, a deficit in hippocampal LTP is evident in animal models where there is an increase in expression and/or an aggregation of each protein discussed thus far. Given the demonstrated role of Zn^{2+} in the aggregation of each of these proteins [122–124], together with the reported role of Zn^{2+} in learning and memory [125], we have hypothesized that the modulation of Zn^{2+} is the critical factor linking AD, TBI, and CTE.

Zn^{2+} in the brain

Zn^{2+}, a physiological element found in abundance in the brain, exerts a critical role in many physiological processes [126]. However, excess amounts of Zn^{2+} are potentially neurotoxic. Approximately 85% of Zn^{2+} ions in the brain are closely bound to proteins and enzymes, and the other 15% are isolated in pre-synaptic vesicles in the terminals of a subset of neurons suitably titled Zn^{2+} enriched neurons [127–132]. Zn^{2+} secreting neurons in the mammalian brain are exclusively glutamatergic [133–135] and are abundant in the hippocampus, amygdala, and neocortex [136]. Although Zn^{2+} is an integral part of normal brain function, when the brain is compromised through disease or injury there is a resulting change in the distribution of metal ions that may initiate or potentiate disease.

WHERE DOES ZN^{2+} FIT INTO THE BIOLOGY OF AD AND TBI?

The pathological aggregation and deposition of TDP-43, NFTs, and Aβ are all modulated by Zn^{2+} [122, 123, 137], and are present to varying degrees not just in AD, but TBI and CTE as well [138]. All

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of these conditions result in impairments in cognition, which itself is dependent upon normal Zn^{2+} homeostasis [125]. Given this, it is plausible to conclude that Zn^{2+} may be a critical factor in the onset and progression of both the neuropathology and the symptoms that characterize AD, TBI, and CTE.

ZN^{2+} ALTERATIONS IN AD AND TBI

Aβ and Zn^{2+}

Over the past two decades, one of the most popular theories proposed to account for the development of AD has been the "amyloid cascade hypothesis". The theory states that the excessive accumulation of Aβ causes AD pathology and subsequent cognitive decline [4, 139]. A number of discoveries have now been made that have identified metal ions as being key players in the cascade of events surrounding amyloid deposition in the brain [140]. With regard to Zn^{2+}, for example, Aβ precipitation is elicited by sub-stoichiometric concentrations of Zn^{2+} [122, 141–143], with specific Zn^{2+} binding sites identified within Aβ at Histidine 13 and Histidine 14 [144] that alter conformation and normal cellular processing through aggregation. Consistent with this, amyloid plaques are robustly enriched with Zn^{2+} [127, 145].

Of further relevance to the development of AD and TBI is the observation that Zn^{2+} released into the glutamatergic synapse, by exocytosis of Zn^{2+}-filled vesicles, is required to maintain memory and cognition in mice [146]. The ZnT3 transporter is responsible for the loading of the vesicles with Zn^{2+}, is expressed at sites of amyloid pathology, and has been linked not only to the release of Zn^{2+} (via exocytosis of vesicles that are loaded with Zn^{2+}) within the glutamatergic synapse, but also to the formation of Aβ plaques [147, 148]. Moreover, Deshpande and colleagues demonstrated a role for synaptic Zn^{2+} in activity-dependent Aβ oligomer formation and accumulation at excitatory synapses. The study demonstrated that the reduction of synaptic Zn^{2+} levels, by either pharmacological or genetic modulation, significantly reduced Aβ oligomer targeting to the synapse [149]. The activation of the glutamatergic N-methyl-D-Aspartate (NMDA) receptor, which is critical in synaptic plasticity and memory function [150], also appeared to regulate Aβ oligomer synaptic targeting. TBI increases extracellular levels of glutamate and of extracellular Zn^{2+} [151], and is a major regulatory component of excitotoxicity [152, 153]. As noted previously, there is also a co-release of Zn^{2+} and glutamate into the extracellular space that could lay the foundation for toxic accumulation and aggregation of other proteins such as TDP-43 and tau. Alternatively, it has been proposed that Zn^{2+} sequestration by Aβ may lead to reduced Zn^{2+} availability at synaptic terminals resulting in the loss of important Zn^{2+} modulatory activity [2].

Tau and Zn^{2+}

As discussed earlier, it is likely that the same mechanisms that modulate tau in AD are also relevant to the tau pathology present in TBI and CTE. In 2004, Pei et al. determined that excess Zn^{2+} could induce several molecular pathways leading to an increase in total tau, both unphosphorylated and phosphorylated, in SH-SY5Y cells [124]. Further studies demonstrated that the P70 S6 kinase directly phosphorylated tau, and in the presence of Zn^{2+} induced increased tau phosphorylation [154]

In another study, the effect of Zn^{2+} on the aggregation of human tau fragments was investigated. Results indicated that low micromolar concentrations of Zn^{2+} dramatically accelerated wild type tau fibril formation. Further, the fibrillization occurred at concentrations ten to thirty fold lower than Zn^{2+} concentrations present in synaptic vesicles in the brain. Higher concentrations of Zn^{2+} led to the formation of granular tau aggregates that rapidly assembled into mature tau fibrils upon Zn^{2+} chelation [137]. Synaptically-released Zn^{2+} has also been shown to promote tau hyperphosphorylation in rat hippocampal slices [155]. This finding is of particular significance, as the location of NFT formation greatly overlaps with Zn^{2+} containing glutamatergic neurons. Furthermore, the synaptic Zn^{2+}-mediated tau hyperphosphorylation can be abolished by the use of Zn^{2+} chelators or the blockade of synaptic Zn^{2+} release [155].

Hyperphosphorylated tau is a major pathological finding in AD, as well as in TBI and CTE. A theory has recently been proposed around the intersection of Zn^{2+}, tau, Aβ, and cognition [3]. The theory, which could equally apply to TBI and CTE, suggests that Zn^{2+} sequestration by Aβ (with a subsequent decrease in intracellular Zn^{2+} ion concentrations) affects the stability of polymerized microtubules and their subsequent binding to tau, leading to cognitive decline and neurodegeneration.

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TDP-43 and Zn^{2+}

A potential intersection between Zn^{2+} and TDP-43 was recently uncovered, with the presence of Zn^{2+} shown to be a requirement to induce the depletion and aggregation of endogenous TDP-43 [123]. As altered metal homeostasis and increased oxidative stress are hypothesized to be central features of many neurodegenerative disorders, this is clearly an area that requires further study to explore the role of TDP-43 in the development and progression of the brain pathology and subsequent clinical symptoms observed in multiple CNS disorders.

Oxidative stress and Zn^{2+}

Oxidative stress is a feature of the AD and TBI brain, and metals such as Zn^{2+} have been implicated in many different molecular mechanisms related to oxidative stress. Cuajungco and Lees [156] demonstrated that intra-hippocampal injections of nitric oxide generators produced an accumulation of chelatable zinc in hippocampal neuronal perikarya. It was hypothesized that the Zn^{2+} may have been released from axon terminals. Significantly, Frederickson et al. [157] reproduced these results, and demonstrated that the observed release of Zn^{2+} was from presynaptic boutons.

Additionally, metallothioneins, the metal regulating proteins, have been shown to be redox regulated [158], and thus can release harmful pools of Zn^{2+} [159] upon stimulation with reactive oxygen species (ROS) and exogenous oxidants [160, 161]. Moreover, Zn^{2+} accumulation can concomitantly induce mitochondrial dysfunction and further ROS generation [162]. Additionally, in neuronal cultures taken from a 3xTg mouse model of AD (expressing mutant AβPP, presenilin-1, and tau), ROS-dependent intraneuronal Zn^{2+} increases were significantly higher compared to control cultures [163]. While not definitive, these observations cumulatively reflect the potential importance of Zn^{2+} homeostasis in pathways that may contribute to the pathogenesis of AD, TBI, and CTE.

Cognition and Zn^{2+}

Long-term potentiation is believed to be a key mechanism underlying synaptic plasticity, and is an accepted *in vitro* correlate for learning and memory [164]. A recent study clearly highlighted the fact that vesicular Zn^{2+} is a requirement for LTP function and is critical for the proper functioning of hippocampal circuitry in health and disease [125]. Zn^{2+} was shown to modulate the plasticity of neuronal synapses, whereby elimination of Zn^{2+} prevented neuronal synaptic activity and increasing Zn^{2+} promoted it. Moreover, another study by Adlard and colleagues showed a distinct and pronounced age-dependent cognitive loss in ZnT3 knockout mice [146]. The ZnT3 protein controls synaptic vesicular Zn^{2+} levels and is required to regulate normal cognitive function. Thus, Zn^{2+} and cognition appear to be intricately linked, providing further support for the intersection of AD and TBI, where LTP and Zn^{2+} are both affected.

OTHER INTERACTIONS WITH ZN^{2+}

In this review, we have focused on a small number of pathways that are likely to be involved in the pathogenesis of AD and TBI, and which are modulated by Zn^{2+}. There are likely a number of other pathways, both Zn^{2+}-dependent and Zn^{2+}-independent, that we have not covered, but which may also be involved in the pathogenesis of AD and TBI. Similarly, this review has primarily focused on Zn^{2+}, however, there are potentially equally important roles for Cu and Fe in the pathogenesis of AD, and both may be important in features that overlap with TBI and CTE as well.

TBI AND ZN^{2+}

TBI and CTE can kill neurons via direct mechanical damage such as cellular membrane rupture and DAI, and also indirectly through ischemia [165]. It is ischemia associated with TBI and CTE that initiates a release of glutamate from axon terminals after injury [166] leading to excitotoxicity and cell death [167]. In conjunction with this observation, evidence indicates that the synaptic release of Zn^{2+} from presynaptic boutons can cause injury to post synaptic neurons under excitotoxic conditions. Studies in ischemia [168] and status epilepticus [169] clearly demonstrate that Zn^{2+} is translocated from Zn^{2+}-containing presynaptic boutons to dying postsynaptic somata.

Suh and colleagues confirmed that synaptically released Zn^{2+} contributed to neuronal injury after TBI [170], paralleling the release of glutamate evident after head trauma [152]. The study firmly denoted a link between the involvement of Zn^{2+} in TBI by demonstrating depletion of Zn^{2+} from presynaptic vesicles, appearance of Zn^{2+} in the somata

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of injured post synaptic neurons, and pointedly, protection against neuronal injury and neuronal death by extracellular chelation of Zn^{2+}. Moreover, protective effects of Zn^{2+} chelation in TBI were shown to correlate with upregulation of neuroprotective genes in rat brain [171], further emphasizing the toxic role of Zn^{2+} in TBI.

It is clear, therefore, that Zn^{2+} may have an important role in the neuronal death that characterizes the TBI brain. Perhaps equally important, however, is consideration of the role of Zn^{2+} in other pathways, pathologies, and symptoms that are shared between TBI, CTE, and AD. As described above, Zn^{2+} may be a critical factor underlying key features of these conditions and as such, targeting Zn^{2+} ion homeostasis may represent a novel therapeutic approach. Future studies aimed at clarifying the role of Zn^{2+} and its modulation in TBI could include high resolution spatial and temporal profiling of metal changes around the injury site using sophisticated techniques such as laser ablation-inductively coupled plasma mass spectrometry, or multi-isotope imaging mass spectroscopy. Either of these techniques utilizing time course studies to monitor metal changes could inform us as to the optimal timing of Zn^{2+} replacement/chelation, or other Zn^{2+}-targeted therapy.

THERAPEUTIC IMPLICATIONS

As the release of "toxic" Zn^{2+} from presynaptic vesicles in TBI, seizures, and ischemia has been suggested to mediate cellular damage and death, it would not be unrealistic to hypothesize that chelation of Zn^{2+} may be an effective therapeutic path in these disorders. One study, however, found that chemical blocking of Zn^{2+} ions in the CNS increased neuronal damage following TBI in mice [172]. Moreover, the study used ZnT3 Knockout mice, and when compared to their wild type littermate controls, exhibited elevated neuronal damage, a finding in direct contrast with the toxic release of synaptic Zn^{2+} theory. Nonetheless, Koh and colleagues demonstrated vivid rescue of ischemic neuronal excitotoxicity via intracerebral Zn^{2+} chelator administration [173]. Other studies have shown both positive and negative results for a number of different Zn^{2+} chelating agents in preventing Zn^{2+}-mediated neuronal loss [174–176]. More broadly speaking, dietary Zn^{2+} supplementation to TBI-injured rats has been suggested to improve performance in cognitive tasks and reduce depressive like behaviors (major depression represents one of the primary complications of TBI, occurring in ~40% of patients) [177, 178]. However, it is unclear as to whether dietary Zn^{2+} supplementation modifies synaptic Zn^{2+} levels, and zinc deficient diets have been suggested to have little effect on brain Zn^{2+} [179]. That being said, Yeiser and colleagues [180] have suggested that dietary Zn^{2+} levels are crucial in limiting cell death four weeks after brain injury.

These data point to the notion that the interaction of Zn^{2+} in the pathobiology of TBI is not a simple linear relationship solely reliant upon excess release of synaptic Zn^{2+}. It is possible that Zn^{2+} chelation or modulation may be optimal at critical periods of cellular change post-injury. The apparent disparity of results may at first appear daunting to dissect, however, it may simply demonstrate the importance of maintaining Zn^{2+} homeostasis throughout the cellular response to injury.

This is also true for other conditions, such as AD. Recent studies have indicated that the use of metal chaperones, compounds that reversibly bind metals and appear to correct metal ion imbalances, may be of therapeutic value, at least initially. The use of the copper and Zn^{2+} chaperone clioquinol, for example, was demonstrated to reduce plaque burden in the Tg2576 AD transgenic mouse model [181]. More recently, clioquinol and a second-generation 8-hydroxy quinolone (PBT2) were shown to rapidly restore cognition in AD transgenic mice, concurrent with decreased Aβ burden. Mechanistically, these compounds exhibited a pronounced chaperone-like activity specifically for zinc and copper [182]. A similar phenomenon has been shown in normal aged mice treated with PBT2, where an increase in hippocampal Zn^{2+} levels (to partially restore a deficit present in the aged animals) was associated with a restoration of cognitive performance and a host of other biochemical and anatomical changes consistent with improved synaptic plasticity and brain function [183]. This class of compound has demonstrated therapeutic advantages not only in AD mouse models [181, 182, 184], but has also successfully navigated clinical trial criteria for AD [184–186]. These results, taken together with both the published literature other unpublished data (Adlard et al.), suggest that Zn^{2+} modulates the pathologies discussed in this review, and are indeed sensitive to metal modulation. Importantly, however, it is the maintenance of metal ion homeostasis that is likely to be a more optimal therapeutic approach over chelation or supplementation paradigms.

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CONCLUSION

The precise role of Zn^{2+} in TBI (and CTE) is currently unclear, however, many studies as outlined in this review, coupled with the fact that TBI patients have Zn^{2+} deficiencies proportional to injury severity [187], indicate that Zn^{2+} may be intricately involved. The role of Zn^{2+} as a modulator of Aβ precipitation and subsequent amyloid plaque formation, as well as tau phosphorylation in AD, has been firmly established. Similarly, the evidence for a modulatory role of Zn^{2+} in TBI affecting tau, TDP-43, and Aβ is continually developing. The observation that Zn^{2+} can aggregate TDP-43, is associated with cognitive decline, and is present in many neurodegenerative diseases has also been recognized. Furthermore, the critical role of Zn^{2+} in cognition may be extremely relevant to all conditions where learning and memory are impacted. With the same pathological phenomena of AD also present in TBI and CTE, it is fair to postulate that Zn^{2+} dyshomeostasis may represent a common mechanistic link. Moving forward, it will be important to delineate the key interactions between Zn^{2+} and key proteins other than those more classical ones relevant just to AD (as discussed in this review), as Zn^{2+} is critically involved in a host of key cell signaling pathways. Furthermore, there are also other common phenotypes of AD, TBI, and CTE that may also involve Zn^{2+}, namely depression, and to a lesser degree irritability and aggression. Collectively, the data suggest that Zn^{2+} may be a common mediator of the pathology and the symptoms observed in AD, TBI, and CTE. Therefore, therapeutic agents that target the failure in metal ion homeostasis, to affect a reduction in Aβ precipitation and NFT formation and a consequent improvement in cognitive function, may be efficacious in improving outcomes in multiple CNS disorders.

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Proteomic Profiling of Mouse Brains Exposed to Blast-Induced Mild Traumatic Brain Injury Reveals Changes in Axonal Proteins and Phosphorylated Tau

Mei Chen[a,b,1], Hailong Song[c,1], Jiankun Cui[c,e], Catherine E. Johnson[d], Graham K. Hubler[f], Ralph G. DePalma[g], Zezong Gu[c,e,*] and Weiming Xia[a,h,*]
[a]*Geriatric Research Education and Clinical Center, Office of Research and Development, Edith Nourse Rogers Memorial Veterans Hospital, Bedford, MA, USA*
[b]*Department of Environmental Health, Harvard T.H. Chan School of Public Health, Boston, MA, USA*
[c]*Department of Pathology & Anatomical Sciences, University of Missouri School of Medicine, Columbia, MO, USA*
[d]*Department of Mining and Nuclear Engineering, Missouri University of Science and Technology, Rolla, MO, USA*
[e]*Truman VA Hospital Research Service, Columbia, MO, USA*
[f]*Sidney Kimmel Institute for Nuclear Renaissance, Department of Physics and Astronomy, University of Missouri, Columbia, MO USA*
[g]*Office of Research and Development, Department of Veterans Affairs, Washington, DC, USA; Department of Surgery, Uniformed University of the Health Science, Bethesda, MD, USA*
[h]*Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Boston, MA, USA*

Abstract. Alzheimer's disease (AD), the most prevalent form of dementia, is characterized by two pathological hallmarks: Tau-containing neurofibrillary tangles and amyloid-β protein (Aβ)-containing neuritic plaques. The goal of this study is to understand mild traumatic brain injury (mTBI)-related brain proteomic changes and tau-related biochemical adaptations that may contribute to AD-like neurodegeneration. We found that both phosphorylated tau (p-tau) and the ratio of p-tau/tau were significantly increased in brains of mice collected at 3 and 24 h after exposure to 82-kPa low-intensity open-field blast. Neurological deficits were observed in animals at 24 h and 7 days after the blast using Simple Neuroassessment of Asymmetric imPairment (SNAP) test, and axon/dendrite degeneration was revealed at 7 days by silver staining. Liquid chromatography-mass spectrometry (LC-MS/MS) was used to analyze brain tissue labeled with isobaric mass tags for relative protein quantification. The results from the proteomics and bioinformatic analysis illustrated the alterations of axonal and synaptic proteins in related pathways, including but not being limited to substantia nigra development, cortical cytoskeleton organization, and synaptic vesicle exocytosis, suggesting a potential axonal damage caused by blast-induced mTBI. Among altered proteins found in brains suffering blast, microtubule-associated protein 1B, stathmin, neurofilaments, actin binding

[1]These authors contributed equally to this work.

*Correspondence to: Zezong Gu, MD, PhD, Department of Pathology & Anatomical Sciences, Center for Translational Neuroscience, University of Missouri-Columbia, School of Medicine, Columbia, MO 65212, USA. Tel.: 573-884-3880; Fax: 573-884-4612; E-mail: guze@health.missouri.edu and Weiming Xia, PhD, Building 70, Room 202, Geriatric Research Education and Clinical Center, Edith Nourse Rogers Memorial Veterans Hospital, Bedford, MA 01730, USA. Tel.: +1 781 687 2852; Fax: +1 781 687 3463; E-mail: weiming.xia@va.gov.

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proteins, myelin basic protein, calcium/calmodulin-dependent protein kinase, and synaptotagmin I were representative ones involved in altered pathways elicited by mTBI. Therefore, TBI induces elevated phospho-tau, a pathological feature found in brains of AD, and altered a number of neurophysiological processes, supporting the notion that blast-induced mTBI as a risk factor contributes to AD pathogenesis. LC/MS-based profiling has presented candidate target/pathways that could be explored for future therapeutic development.

Keywords: Alzheimer's disease, bioinformatics, mild traumatic brain injury, open-field blast, proteomics

INTRODUCTION

Mild traumatic brain injury (mTBI) results in significant morbidity and mortality throughout the world. Overlapping neuropathological features of patients suffering Alzheimer's disease (AD) or traumatic brain injury (TBI) suggest that pathological proteins for these two conditions may interact [1, 2]. In AD, widespread neuronal loss is associated with the pathological hallmarks of neurofibrillary tangles composed of phosphorylated tau and neuritic plaques composed of amyloid-β protein (Aβ). Animal brains suffering TBI exhibit tau pathology upon following controlled cortical impact, and these transgenic mice exhibit dramatic increases in levels of tau [1]. Several studies have associated TBI with a significantly increased risk of the development of late neurodegenerative diseases, such as AD and Parkinson's disease [3]. Soldiers exposed to even a single blast can develop behavioral and psychiatric problems within a year following injury [4]. Even a single TBI may induce long-term neuropathological changes akin to those found in neurodegenerative disease and associated with the later development of syndromes of cognitive impairment such as AD [5]. In humans, repetitive impact-induced mTBI leads to chronic traumatic encephalopathy (CTE) with increased tau pathology and other neurodegenerative changes in the brain [6]. CTE has been observed in veterans exposed to explosive blast [7]. Clinical reports describe post-concussive stress and depression symptoms along with worse cognitive performance in subjects with blast-induced mTBI, both in short- (0–7 days) and long-term (6 months to 5 years) follow-up exams after the blast [8–10]. Additional reports confirm that mTBI is positively associated with depression and increased anxiety levels [11].

Among the several mechanisms that have been proposed as possible explanations for these associations, two prime events occurring in ongoing blast injury are neuroinflammation and deposition of abnormal microtubule proteins such as tau [12]. Mutations in the tau gene cause frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) [13]. Transgenic mice expressing mutant tau show close association with tau mutation-containing, neurofibrillary tangle (NFT) formation, and neurodegeneration [14–16]. Tau protein has been used as a marker for axonal damage, and tau levels in cerebrospinal fluid (CSF) reflect these changes in the central nervous system (CNS), especially, total tau protein levels (t-tau), phosphorylated tau (p-tau), and the ratio of $A\beta_{42}$/tau have been explored as potential markers for AD [17–19]. Other related proteins include neurofilament light chain (NfL), which was found to be increased in CSF and blood of patients with AD or tauopathies. In animals, blocking Aβ lesions attenuates the NfL increase [20]. These proteins may affect the formation of Aβ/tau pathologies.

Proteomic research [21, 22] using brain tissues or biofluids from either patients or animal models have made it feasible to identify a large number of proteins associated with mTBI [23, 24]. MS-based proteomics possess the advantage of having no requirement of prior knowledge of the proteins being identified, allowing for unbiased, hypothesis-free biomarker discovery in complex biological samples such as plasma and brain tissue extracts. Therefore, MS-based proteomic approach can be used to unravel novel biological processes underlying TBI, neurodegeneration and repair, or to represent complex protein–protein interactions during disease progression. However, MS-based proteomic analysis has not been performed on samples derived from animals exposed to open field blast-induced mTBI.

In this study, we use proteomic profiling of mouse brains suffering blast-induced mTBI to explore contribution of mTBI toward AD pathologic process. Tau associated biological processes were found to be differentially regulated in blast-induced mTBI mouse models, and levels of phospho-tau were increased in brains of mice collected at 3 and 24 h after the exposure to blast. Behavioral and pathological alterations were observed in blast exposed mice. We employed tandem mass tag (TMT)-labelling in conjunction with liquid chromatography-tandem mass spectrometry

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(LC-MS/MS) approach to investigate differentially expressed proteins in mouse brain samples after exposed to a single low-intensity open-field blast for the purpose of discovering candidate proteins associated with blast-induced mTBI and AD pathology. Differentially expressed proteins were analyzed using bioinformatics tools DAVID and Metacore to determine if functional relationships were altered in mice exposed to blast. This approach allows us to evaluate effect of blast on mice by understanding the proteomic profiles of brain tissue as a way of identifying potential molecular signatures of pathological alteration that may contribute to AD pathogenesis.

MATERIALS AND METHODS

Materials and reagents

Unless indicated, all reagents used for biochemical methods and cell culture preparation were purchased from Sigma-Aldrich (St Louis, MO, USA). Reagents for BCA protein assay and sample preparation for LC-MS/MS analysis were purchased from Thermo Scientific (Rockford, IL, USA). TMT reagent six-plex and ten-plex kits were available commercially (Thermo Scientific Pierce, Rockford, IL, USA).

Open-field primary blast injury in mice

Male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME), at 2 months old, were housed with a 12-h light/dark cycle and given unrestricted access to food and water. Open-field blast exposures were conducted at the Missouri University of Science & Technology and were recently reported [25, 26]. Briefly, for the experimental setting of open-field low-intensity blast injury with the modification as described here, mice were placed in animal holders in prone position at 2.15-meter (m) distance away from detonation of 350 g C4 explosive (both 1 m above ground) generating a magnitude of 82 kPa [e.g., 11.9 pounds per square inch (PSI)] peak overpressure and maximal impulse at 71 kPa (10.3 PSI) × ms. The Sham group underwent identical procedures as blast groups only without blast exposure. Following blast wave exposure, animals were removed from the platform and returned to the original cage. After recovery from anesthesia, mice were able to spontaneously move and continuously being monitored for at least 15–30 min. Animals were allowed access to food and water *ad libitum* before and after blast exposure. Mice were randomly assigned into experimental or sham control groups which were double-blinded to investigators analyzing experimental outcomes. All procedures were in accordance with the University of Missouri approved protocols for the Care and Use of Laboratory Animals and ARRIVE guidelines.

Behavioral test

The behavioral test, Simple Neuroassessment of Asymmetric imPairment (SNAP), was used after blast exposure to assess the neurological status of coordination, motor strength, proprioception, gait, and mental responses. The tests examined interaction, cage grasp, visual placing, pacing/circling, gait/posture, head tilt, visual field, and baton as we previously described [25, 27].

Brain collection and pathological examination

In total, there were thirty-seven mice in the present study, in which thirty-two mice were randomly divided into four groups at 3 h ($n = 4$), 24 h ($n = 9$), 7 days ($n = 11$), 30 days ($n = 4$), and 15 weeks ($n = 4$) after a single 82-kPa low-intensity blast exposure and five mice as the sham control group [25, 26, 28, 29]. Mice were euthanized using overdoing of isoflurane and cerebral cortex regions were dissected from the blast-exposed mouse brain. Silver staining and quantitative analyses were performed as previously described [25, 30]. The FD NeuroSilver Kit II was used for silver staining according to the manufacturer's instructions. Each batch of the experiments was included the brain sections from different groups to avoid variability of the staining intensity. Tiff images of sections with the defined coordinates for stereological analysis were captured containing the regions of interests of the corpus callosum and entorhinal cortex (bregma 0.14 to 0.02 mm), cerebral peduncle (bregma –1.94 to –2.06 mm), and fornix (bregma 0.14 to 0.02 mm), based on the mouse brain atlas. These images were exported and analyzed with ImageJ software (NIH, Bethesda, MD). The background (gold color) was subtracted from each image using the color splitter and image calculator functions, so that only the gray-black silver deposits were visible. Densitometric analysis of silver staining was then performed using ImageJ. Serial sections were averaged from each animal to produce a final silver staining value. The intensity of silver staining was expressed in arbitrary units, ranging from 0 to 255.

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Preparation of brain tissue for MS

Sample lysis buffer (2% SDS, 0.5M tetraethylammonium bicarbonate (TEAB), protease inhibitor cocktail) were added to each brain tissue and then homogenized by TissueLyser LT (Qiagen, Valencia, CA). Tissue homogenates were centrifuged at 17,000 × *g*, for 20 min at 4°C. The supernatant was transferred into a new vial for protein concentration measurement by BCA assay. Preparation of tryptic peptides for TMT 10-plex labelling was carried out according to the manufacturer's instructions. Briefly 100 μg protein from each sample was transferred into a new vial and adjusted to a final volume of 100 μL with TEAB and reduced with tris (2-carboxyethyl) phosphine (TCEP) at 55°C for 1 h, and then alkylated with iodoacetamide for 30 min in the dark. Methanol-chloroform precipitation was performed prior to protease digestion. In brief, four parts methanol was added to each sample and vortexed, then one part chloroform was added to the sample and vortexed, and three parts water was added to the sample and vortexed. The sample was centrifuged at 14,000 × *g* for 4 min at room temperature and the aqueous phase was removed. The organic phases with protein precipitate at the surface was subsequently washed twice with four parts methanol and centrifuged with supernatant being removed subsequently. After air-drying, precipitated protein pellets were re-suspend with 100 μL of 50 mM TEAB and digested with trypsin overnight at 37°C.

TMT-labeling and sample clean up

TMT enables relative quantitation of proteins present in multiple samples by labeling peptides with isobaric stable isotope tags that fragment upon collision-induced dissociation into reporter ions used for quantitation. In this study, TMT ten tags for ten samples (blast samples and controls) were analyzed simultaneously, avoiding run-to-run variation. Tryptic digested peptides from brain samples were labeled with TMT 10-plex reagents (Thermo Fisher) according to manufacturer's instructions. Briefly, the TMT reagents (0.8 mg) were dissolved in 41 μL of anhydrous acetonitrile. Aliquots of samples were incubated with TMT reagents for 1 h at room temperature. The reactions were quenched by 8 μL of 5% hydroxylamine solution and reacted for 15 min. The combined TMT labelled samples were dried under SpeedVac, and then reconstituted by dilute trifluoroacetic acid solution followed by desalting by Oasis HLB 96-well μElution plate (Waters) prior to LC-MS/MS analysis.

Phosphoprotein enrichment and analysis

For enrichment of phospho-peptides, samples were processed using the Fe-NTA phospho-peptide enrichment kit and followed by graphite spin columns according to the manufacturer's instructions (Thermo Scientific Pierce) before LC-MS/MS analysis.

LC-MS/MS analysis

LC MS/MS was performed on a Q Exactive Orbitrap Mass Spectrometer (Thermo Fisher Scientific) coupled with a Dionex ultimate 3000 HPLC system equipped with a nano-ES ion source. The TMT labelled peptides were separated on a C18 reverse-phase capillary column (PepMap, 75 μm × 150 mm, Thermo Fisher) with linear gradients of 2–35% acetonitrile in 0.1% formic acid, at a constant flow rate of 300 nL/min for 220 min. The instrument was operated in the positive-ion mode with the ESI spray voltage set at 1.8 kV. A full scan MS spectrum (300–1800 m/z) was acquired in the Orbitrap at a mass resolution of 70,000 with an automatic gain control target (AGC) of 3e6. Fifteen peptide ions showing the most intense signal from each scan were selected for higher energy collision-induced dissociation (HCD)-MS/MS analysis (normalized collision energy 32) in the Orbitrap at a mass resolution of 35,000 and AGC value of 1e5. Maximal filling times were 100 ms in full scans and 120 ms (HCD) for the MS/MS scans. Ions with unassigned charge states and singly charged species were rejected. The dynamic exclusion was set to 50 s and a relative mass window of 10 ppm. The data were acquired using Thermo Xcalibur 3.0.63. The phospho-peptides were analyzed under the same conditions as above, other than the separation which was done with linear gradients of 1–30% acetonitrile in 0.1% formic acid, and full scan MS ranging 300–2000 m/z with NCE at 25.

Protein identification and quantification

Raw data were processed using Proteome Discoverer (Version 2.1, Thermo Fisher Scientific). Data were searched against the *mus musculus* Universal Protein Resource sequence database (UniProt, August, 2013). The searches were performed with the following guidelines: Trypsin digestion with two missed cleavage allowed; fixed modification,

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carbamidomethyl of cysteine; variable modification, oxidation of methionine, TMT 10plex (peptide labeled) for N terminus and Lys; MS tolerance, 5 ppm; MS/MS tolerance, 0.02 Da; false discovery rate (FDR) at peptide and protein levels, < 0.01; and required peptide length, ≥6 amino acids. Phosphorylation of serine, threonine, and tyrosine was defined as variable modification. Protein grouping was enabled, meaning if one protein were equal to or completely contained within the set of peptides of another protein, these two proteins were put into the same protein group. At least one unique peptide per protein group was required for identifying proteins. The relative protein abundance ratios (fold changes) between blasted and shamed groups were calculated. The changes in protein levels were considered significant if fold change was > 1.2 (upregulated) or < 0.9 (downregulated), and the *p*-value is less than 0.05 in two independent experiments.

Quantification of tau and phospho-tau by ELISA

Tau and p-tau peptide at Thr residue 181 were quantified by ELISA [31]. The capture antibody for tau is BT-2. To capture specific phosphorylated tau at Thr residue 181, antibody AT270 was used. The detecting antibody is the biotinylated HT7 that recognizes residue 159–163 of tau. All three tau antibodies were purchased from Thermo Scientific (Rockford, IL). ELISA was performed using Meso Scale Discovery platform (MSD, Gaithersburg, MD). The internal calibrators of p-tau and tau were purchased from Meso Scale Discovery.

Data analysis

An enrichment analysis was performed by submitting the UniProt accession numbers containing the list of differentially regulated proteins to open-source software Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.7 [32, 33]. DAVID is able to extract biological features/meanings associated with large gene lists. The gene names of the proteins were entered to analyze GO biological process (GOBP), GO molecular function (GOMF), and GO cellular components (GOCC) and KEGG pathways. Statistically significant differences (p-value < 0.05) were identified using an EASE score (a modification of Fisher's exact test), which was provided by the DAVID gene bioinformatics online resource. The cellular and molecular process networks of the dysregulated proteins were also analyzed using Metacore. Metacore uses these proteins and their identifiers to navigate the curated literature database and extract the biologically relevant information among the candidate proteins. Associated biofunctions were generated, along with a score representing the log probability of a particular process network.

RESULTS

Increase of phospho-tau in brains of mice at 3 and 24 h after the blast

In this study, we analyzed the mouse brain samples at 3 h, 24 h, 30 days, and 15 weeks after the blast. We applied ELISA to quantify levels of tau and phospho-tau in the same brain tissue later used for LC-MS/MS analysis. We found a significant increase of phospho-tau in mouse brains collected at 3 and 24 h after the blast, while a reduction of tau was also observed in the same samples (Fig. 1). We also calculated the ratio of p-tau/tau and found that both levels of p-tau and the ratio of p-tau/tau were increased compared to those from brains of mice without exposure to blast (Sham). The impact of blast to mouse brains was manifested as early as 3 h, and remained after 24 h, when significant reduction of tau and increase of p-tau were observed. Total tau level was decreased ∼20% compared to that of sham mouse brains, and the p-tau level was increased 1.2 folds, and these changes were found statistically significant. Thirty days after the blast, tau levels bounced back and remained at similar levels to those of sham mice. Similarly, p-tau dropped a statistically significant 20% after 30 days and then remained at similar levels to those of sham mice after 15 weeks (Fig. 1). The ratios of p-tau/tau reflected similar changes to the levels of p-tau in mouse brains harvested at 30 days and 15 weeks after the blast.

Behavioral and pathological alteration in mice after the blast

We performed an explorative behavioral SNAP test on the mice exposed to 82-kPa low intensity blast (LIB). SNAP provides a sensitive, reliable, time-efficient, and cost-effective means of assessing neurological deficits in mice. Each individual test had a scoring range of 0-5 based on the assessment guidelines (Fig. 2). Using the current settings and 350 g of high-energy explosive C4, SNAP assessments revealed a significant difference in the blast group compared to sham controls at both 24 h and 7 days

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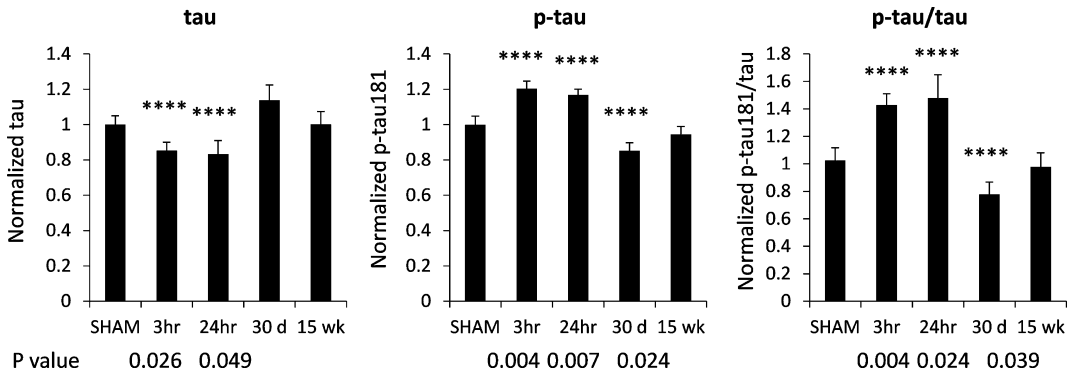

Fig. 1. Quantification of total tau and phosphorylated tau from mouse brain at different time points after the blast. Brain lysates were subjected to ELISA using Meso Scale Discovery platform, and same samples were quantified for the levels of tau and p-tau. The ratio of p-tau to tau (p-tau/tau) was calculated for each sample, and the levels of tau, p-tau, p-tau/tau in brains of control mice were normalized to 1. Relative levels of these analytes in brains of mice harvested at 3 h, 24 h, 30 days, and 15 weeks after the blast were illustrated. The bar represents standard error of means (SEM). Asterisks represent statistically significant differences.

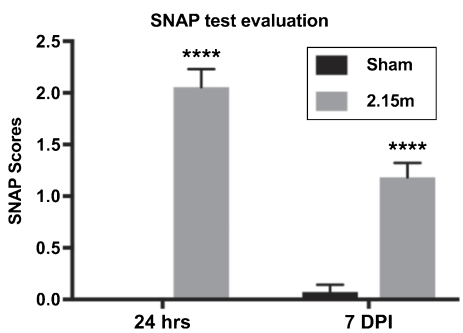

Fig. 2. Neurological deficits after low intensity blast exposure assessed by SNAP test. Neurological status was evaluated by SNAP tests at 24 h and 7 days post injury (DPI). All comparisons were performed between blast (2.15 m, distance between the animals and the blast) and sham group on the same DPI; $n = 5$ for sham, $n = 9$ at 24 h after blast, $n = 11$ at 7 DPI. Differences were considered significant at $p < 0.05$ for all analyses, with asterisks representing $p < 0.0001$.

post injury (DPI) (Fig. 2), indicating that neurological deficits affecting coordination, motor strength, proprioception, gait, and mental status occurred after LIB exposure.

Axonal injury in LIB-exposed animals occurred mainly at 7 DPI, as we previously described [25]. Degenerating axon-terminals and dendrites, with high affinity for silver (argyrophilia) were visualized on silver-stained tissue sections (Fig. 3A-C). Densitometric analyses by NIH ImageJ were examined on various white matter regions rich for axons, including corpus callosum and entorhinal cortex, cerebral peduncle, and fornix. The silver stain intensities in blast group were significantly higher than in sham controls at 7 DPI for all subregions (Fig. 3D-F). We have previously seen that the abnormalities at 7 DPI appeared to return to comparable levels of sham controls at 30 DPI (data now shown).

Global protein alteration in brains exposed to blast

To understand impact of blast to brain proteins that drive these phenotypes, analysis of both global proteomics and phospho-proteomics were conducted using mouse brains collected at 3 h, 24 h, 30 days, and 15 weeks after the blast. We specifically searched for significant changes in proteins from mouse brains harvested at the time points (3 h and 24 h) where we also observed significant changes in tau and p-tau proteins. A total of 1,692 proteins and 296 phosphoproteins were identified at global level and analyzed in this study. By comparing to the sham mouse brain, the fold of changes (ratio $\geq$1.2 or $\leq$0.9) of proteins observed in brains of mice at 3 h and 24 h after the blast was used for functional analysis by DAVID. At the global level, relative to the proteins identified from the sham controls, only a few proteins were downregulated after the blast, including Hemoglobin subunit α(Hba) and Hemoglobin subunit β-1(Hbb-b1). They are enriched in the cellular location of hemoglobin complex. Their primary molecular functions are oxygen transporter and oxygen/heme binding. On the other hand, 130 proteins were upregulated with the fold changes $\geq$1.2 after the blast. DAVID functional analysis in three categories, including cellular components (CC), biological process (BP), and molecular function (MF), of upregulated proteins at 3 h after the blast

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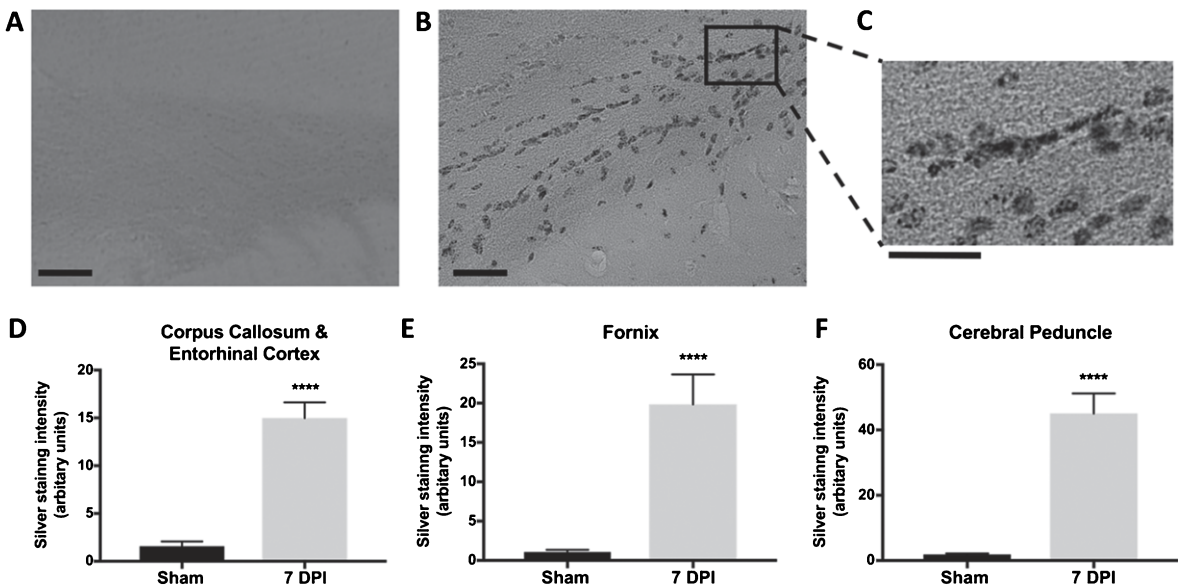

Fig. 3. Axonal injury detected and quantified after LIB exposure by silver staining. A) Representative images from the sham group showed no axonal injury (scale bar = 50 μm). B, C) Representative images from the blast group showed axonal injury at corpus callosum and entorhinal cortex (scale bar = 50 μm). Inset box represent the higher magnification of the indicated area (scale bar = 25 μm). D-F) Intensity of silver staining in corpus callosum and entorhinal cortex, fornix, and cerebral peduncle regions in blast group at 7 DPI were significantly higher than that in the sham group. All comparisons were performed between blast and sham groups with *t*-test; $n = 5$ for sham controls, and $n = 4$ for blast at 7 DPI. Differences were considered significant at $p < 0.05$ for all analyses, with asterisks representing $p < 0.0001$.

were conducted (Fig. 4A-C). The top two enriched CC are cytoplasm and neuronal cell body. Among CC, axon was also significantly enriched ($p = 1.2E-4$), followed by other neuron related components, such as postsynaptic dendritic spine and microtubule (Fig. 4A). Besides general BP such as transport and translation, most altered proteins were enriched in cell-cell adhesion and vesicle mediated transport. In addition, proteins enriched in axon guidance, axon extension and microtubule-based process were also illustrated (Fig. 4B). The top enriched MF (Fig. 4 C) include cadherin binding/cell-cell adhesion, hydrolase activity, nucleotide binding, microtubule binding, filamin binding, and β-tubulin binding activities ($p \leq 3.0E-2$).

We have examined proteins involved in each of the three top enriched categories BP, CC, and MF that are related to neuronal functions and listed upregulated proteins from animals harvested at 3 h post the blast (Table 1). The BP included positive regulation of axon extension and axon-dendritic transport. The upregulated proteins highly enriched in postsynaptic density and axon included huntingtin (Htt), which may play a role in microtubule-mediated transport or vesicle function. Proteins involved in microtubule-based process include Tubulin α-1A chain (Tubα1a) and Dynein light chain 1, cytoplasmic (Dynll1). GO enrichment analysis in each of the three categories (BP, CC, MF) revealed proteins related to those microtubule associated proteins, and network analysis of upregulated proteins at 3 h were illustrated by STRING (Fig. 5A). Proteins related to microtubule, such as Actr1b, Tubα1a, Tubβ2a, Dunll1, are among the proteins with the extensive network connections. These proteins are also observed in mouse brains harvested at 24 h (Fig. 5B), and less in mouse brains harvested at 30 days (Fig. 5C) and 15 weeks (Fig. 5D) after the blast exposure.

KEGG pathway analysis of proteins shows that synthesis and degradation of ketone bodies ($p = 4.1E-3$), dopaminergic synapse ($p = 6.8E-3$), and ribosome ($p = 4.0E-2$) were enriched. Those proteins involved in the dopaminergic synapse pathway include catechol-O-methyltransferase (Comt), guanine nucleotide binding protein (G protein) β4 (Gnb4), guanine nucleotide binding protein, αq polypeptide (Gnaq), guanine nucleotide binding protein, αstimulating, olfactory type (Gnal), kinesin family member 5 C (Kif5c) and protein phosphatase 1, catalytic subunit, α isoform (Ppp1ca). How these proteins connect to each other and respond to blast-induced protein alteration remain to be investigated.

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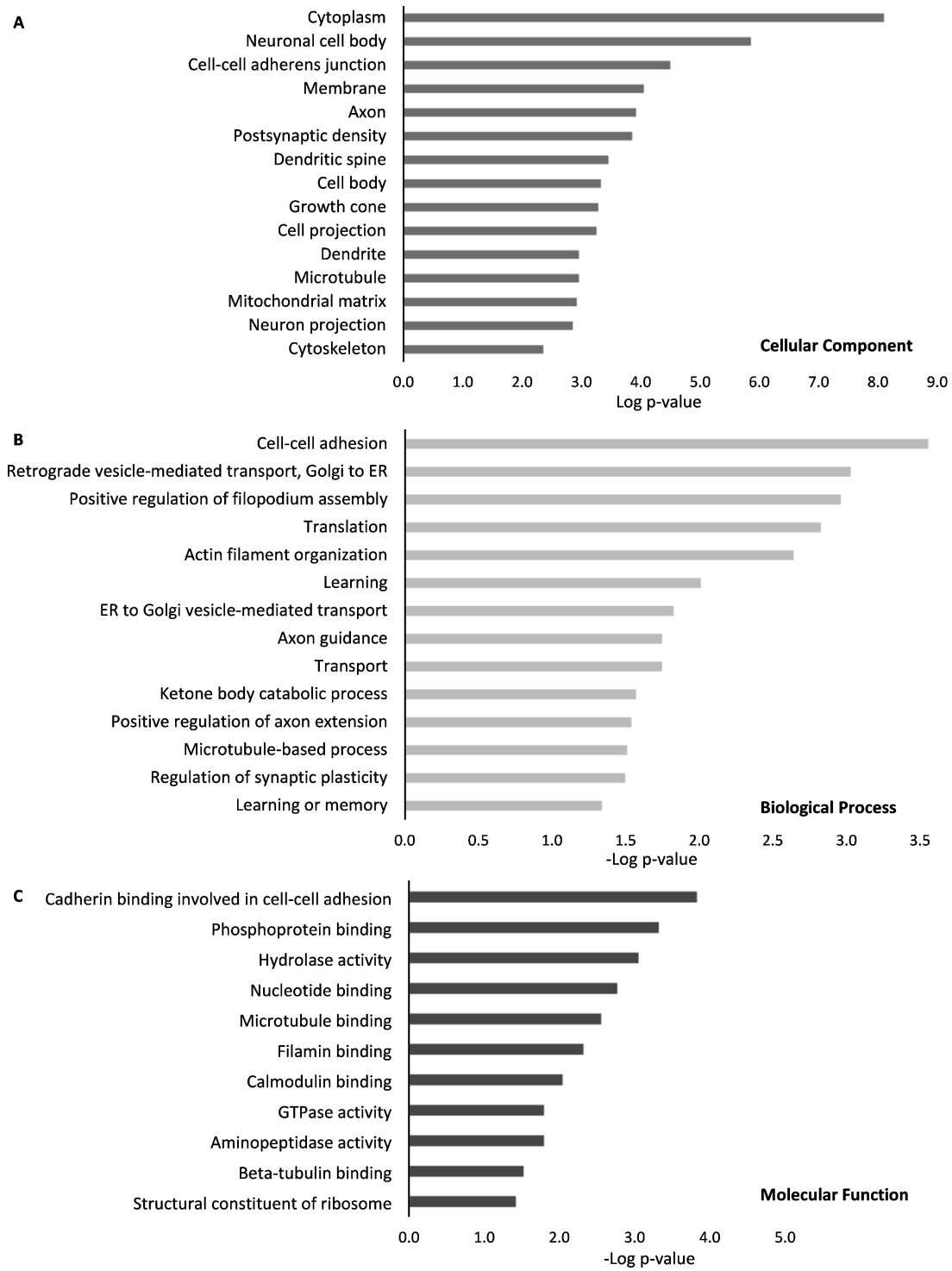

Fig. 4. GO ontology analysis of altered proteins at global level. Proteins from mouse brains harvested at 3 h after the blast were analyzed, and each of the three categories, cellular components (A), biological process (B) and molecular functions (C) were listed. Proteins from blast-exposed animals were compared to those from control/sham animals, and –Log of *p* values was plotted.

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Table 1
DAVID functional analysis of upregulated proteins at global level at 3 h after the blast and proteins enriched in each category

Categories	GO Terms	ACCESSION	GENE NAME
BP	Positive regulation of filopodium assembly		
		Q99JY9	ARP3 actin-related protein 3(Actr3)
		Q62188	Dihydropyrimidinase-like 3(Dpysl3)
		Q61553	Fascin actin-bundling protein 1(Fscn1)
		P35802	Glycoprotein m6a(Gpm6a)
	Actin filament organization		
		Q99JY9	ARP3 actin-related protein 3(Actr3)
		Q641P0	ARP3 actin-related protein 3B(Actr3b)
		Q9QXS6	Drebrin 1(Dbn1)
		Q61553	Fascin actin-bundling protein 1(Fscn1)
		Q60875	Rho/rac guanine nucleotide exchange factor (GEF) 2(Arhgef2)
	Learning		
		O88587	Catechol-O-methyltransferase(Comt)
		O35927	Catenin (cadherin associated protein), delta 2(Ctnnd2)
		P42859	Huntingtin(Htt)
		P31324	Protein kinase, cAMP dependent regulatory, type II beta(Prkar2b)
	Axon guidance		
		P97427	Collapsin response mediator protein 1(Crmp1)
		Q9EQF6	Dihydropyrimidinase-like 5(Dpysl5)
		O08917	Flotillin 1(Flot1)
		P06837	Growth associated protein 43(Gap43)
		P13595	Neural cell adhesion molecule 1(Ncam1)
	Positive regulation of axon extension		
		Q9QXS6	Drebrin 1(Dbn1)
		P10637	Microtubule-associated protein tau(Mapt)
		Q9D8E6	Ribosomal protein L4(Rpl4)
	Microtubule-based process		
		P63168	Dynein light chain LC8-type 1(Dynll1)
		P68369	Tubulin, alpha 1A(Tuba1a)
		Q7TMM9	Tubulin, beta 2A class IIA(Tubb2a)
	Regulation of synaptic plasticity		
		O35927	Catenin (cadherin associated protein), delta 2(Ctnnd2)
		P42859	Huntingtin(Htt)
		P13595	Neural cell adhesion molecule 1(Ncam1)
CC	Axon		
		P63054	Purkinje cell protein 4(Pcp4)
		O88587	Catechol-O-methyltransferase(Comt)
		P48320	Glutamic acid decarboxylase 2(Gad2)
		P06837	Growth associated protein 43(Gap43)
		P42859	Huntingtin(Htt)
		P33173	Kinesin family member 1A(Kif1a)
		Q9CQV6	Microtubule-associated protein 1 light chain 3 beta(Map1lc3b)
		P10637	Microtubule-associated protein tau(Mapt)
		Q9JM52	Misshapen-like kinase 1 (zebrafish)(Mink1)
		P13595	Neural cell adhesion molecule 1(Ncam1)
		O35136	Neural cell adhesion molecule 2(Ncam2)
	Postsynaptic density		
		O35927	Catenin (cadherin associated protein), delta 2(Ctnnd2)
		Q9JLM8	Doublecortin-like kinase 1(Dclk1)
		Q9QXS6	Drebrin 1(Dbn1)
		P06837	Growth associated protein 43(Gap43)
		P42859	Huntingtin(Htt)
		P10637	Microtubule-associated protein tau(Mapt)
		Q9JM52	Misshapen-like kinase 1 (zebrafish)(Mink1)
		Q60875	Rho/rac guanine nucleotide exchange factor (GEF) 2(Arhgef2)
	Dendritic spine		
		Q5SSM3	Rho GTPase activating protein 44(Arhgap44)
		P47757	Capping protein (actin filament) muscle Z-line, beta(Capzb)
		O88587	Catechol-O-methyltransferase(Comt)

(*Continued*)

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Table 1
(*Continued*)

Categories	GO Terms	ACCESSION	GENE NAME
		Q9QXS6	Ddrebrin 1(Dbn1)
		P35802	Glycoprotein m6a(Gpm6a)
		P31324	Protein kinase, cAMP dependent regulatory, type II beta(Prkar2b)
		P62137	Protein phosphatase 1, catalytic subunit, alpha isoform(Ppp1ca)
	Growth cone		
		Q3UNH4	G protein-regulated inducer of neurite outgrowth 1(Gprin1)
		P97427	Collapsin response mediator protein 1(Crmp1)
		Q62188	Dihydropyrimidinase-like 3(Dpysl3)
		Q9QXS6	Drebrin 1(Dbn1)
		Q61553	Fascin actin-bundling protein 1(Fscn1)
		P10637	Microtubule-associated protein tau(Mapt)
		P13595	Neural cell adhesion molecule 1(Ncam1)
	Microtubule		
		Q9Z0H8	CAP-GLY domain containing linker protein 2(Clip2)
		P63168	Dynein light chain LC8-type 1(Dynll1)
		P33173	Kinesin family member 1A(Kif1a)
		P28738	Kinesin family member 5 C(Kif5c)
		Q9CQV6	Microtubule-associated protein 1 light chain 3 beta(Map1lc3b)
		P10637	Microtubule-associated protein tau(Mapt)
		Q60875	Rho/rac guanine nucleotide exchange factor (GEF) 2(Arhgef2)
		P68369	Tubulin, alpha 1A(Tuba1a)
		Q7TMM9	Tubulin, beta 2A class IIA(Tubb2a)
MF	Microtubule binding		
		Q9Z0H8	CAP-GLY domain containing linker protein 2(Clip2)
		Q9EQF6	Dihydropyrimidinase-like 5(Dpysl5)
		P33173	Kinesin family member 1A(Kif1a)
		Q9CQV6	Microtubule-associated protein 1 light chain 3 beta(Map1lc3b)
		P10637	Microtubule-associated protein tau(Mapt)
		Q60875	Rho/rac guanine nucleotide exchange factor (GEF) 2(Arhgef2)
		Q9QY76	Vesicle-associated membrane protein, associated protein B and C(Vapb)
	Filamin binding		
		P97427	Collapsin response mediator protein 1(Crmp1)
		Q62188	Dihydropyrimidinase-like 3(Dpysl3)
		O35098	Dihydropyrimidinase-like 4(Dpysl4)
	Calmodulin binding		
		Q61656	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5(Ddx5)
		Q61545	Ewing sarcoma breakpoint region 1(Ewsr1)
		P63054	Purkinje cell protein 4(Pcp4)
		P08414	Calcium/calmodulin-dependent protein kinase IV(Camk4)
		P06837	Growth associated protein 43(Gap43)
		Q61481	Phosphodiesterase 1A, calmodulin-dependent(Pde1a)
	Beta-tubulin binding		
		P47757	Capping protein (actin filament) muscle Z-line, beta(Capzb)
		P42859	Huntingtin(Htt)
		Q9QY76	Vesicle-associated membrane protein, associated protein B and C(Vapb)

BP, biological process; CC, cellular components; MF, molecular functions.

Alteration of phospho-proteomics in brains of mice exposed to blast

We compared phosphoproteins from mouse brains and captured 296 phosphorylated proteins for quantification. Among the list of altered phosphoproteins at 3 h and 24 h after the blast (Table 2), 29 phosphoproteins were downregulated and only one was upregulated at 3 h, whereas four were downregulated and 17 proteins were upregulated at 24 h. Several clusters were enriched in these dysregulated proteins, such as synapse, cytoskeleton/actin binding, dendritic spine, microtubule, growth cone, and neural projection. Based on DAVID analysis, the top enriched protein domains at 24 h include Stathmin family, C2 calcium-dependent membrane targeting protein and Synaptotagmin. The phosphoproteins with largest increase include Add1, Camk2b, Syt1, and Stmn1. Ap3b2, Sgip1, Basp1, and Rph3a were among those significantly downregulated phosphoproteins (Table 2).

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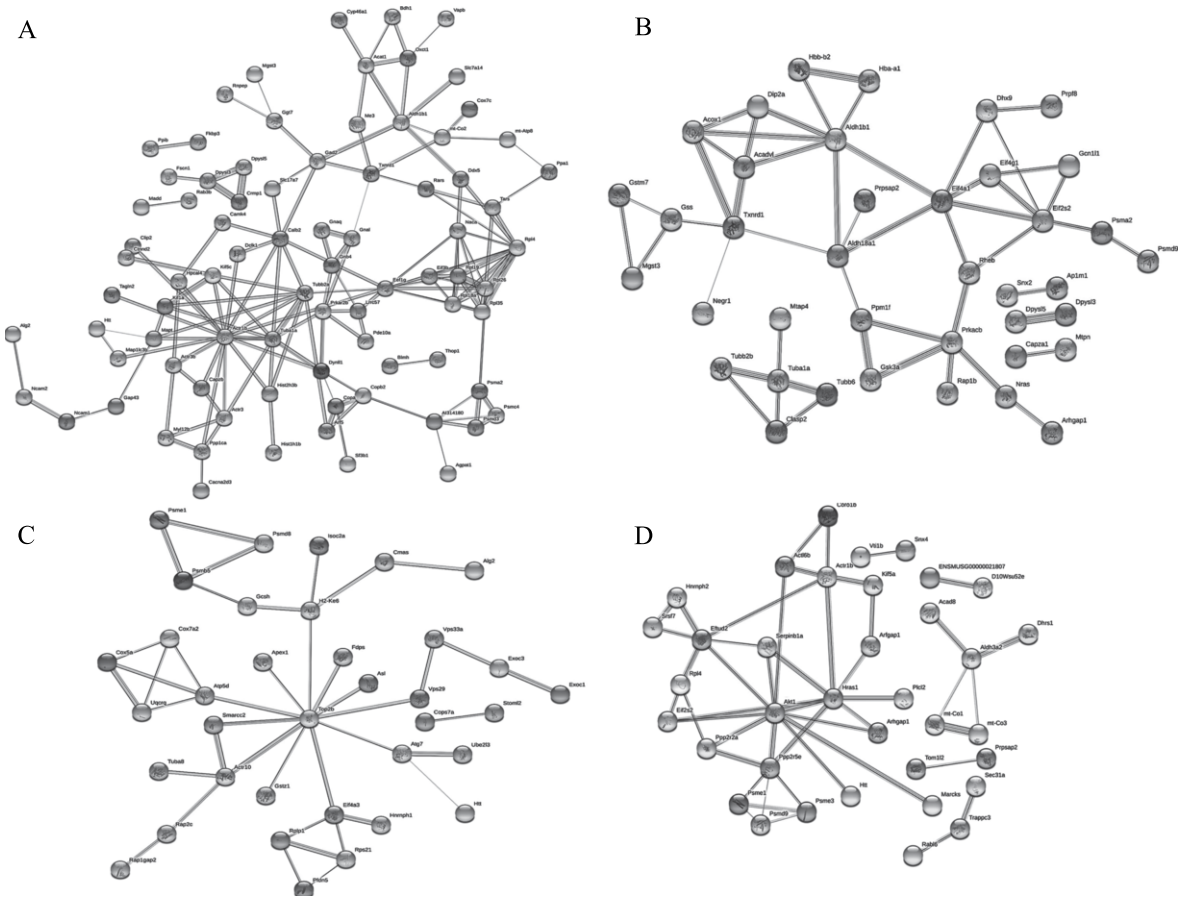

Fig. 5. Network analysis of proteins was illustrated using the web-based tool STRING 10.0. Proteins from mouse brains at 3 h (A), 24 h (B), 7 days (C), and 15 weeks (D) after the blast were compared to those from the control group using LC-MS/MS.

GO ontology analysis of CC of dysregulated phosphoproteins revealed alteration in synaptic proteins (Fig. 6A). At both time points, the downregulated phosphoproteins are highly enriched in postsynaptic density, synapse, cell projection, and cytoskeleton ($p \leq 4.40\text{E-}3$). Those dysregulated phosphoproteins were enriched in brains harvested at 3 h and 24 h (Table 2) after the blast, respectively. At 3 h post blast, nearly 50% of the downregulated phosphoproteins were enriched in cellular components related to synapse, postsynaptic density, myelin sheath and microtubule (Table 3). At 24 h post blast, 33% of the proteins are enriched at synapse, followed by cytoskeleton (33%), neuron projection (24%), dendrite (24%), and synaptic vesicle (14%) (Table 4).

The BP of dysregulated phosphoproteins was examined using GO ontology analysis (Fig. 6B). The only common enriched biological process at both time points is cytoskeleton organization. In addition, substantia nigra development and signal transduction at 3 h time point were also enriched. The top five enriched biological processes at 24 h are nervous system development, vesicle fusion, and cytoskeleton organization, microtubule depolymerization and synaptic vesicle exocytosis. MF of altered phosphoproteins from mouse brains at 3 h and 24 h after the blast were analyzed (Fig. 6C). Actin binding is the only common MF among the dysregulated phosphoproteins at both time points. Other cytoskeleton related MF, such as ankyrin binding and spectrin binding were also enriched at 3 h (Table 3). At 24 h, in additional to structural related MF, such as syntaxin binding and tubulin binding, calcium-dependent phospholipid is also enriched (Table 4).

The top three GO process network analysis of up- and downregulated proteins were revealed using Metacore (Fig. 6D). The networks related to cytoskeleton, such as Plectin 1, actin-binding Lim protein 1 (Ablim1) and neurofilament medium

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Table 2
Altered phosphoproteins in mouse brain at 3 h and 24 h after the blast

Time	Accession	Description	Score	Coverage	Ratio	MW [kDa]
3 h	Q9QYC0	Alpha-adducin (Add1)	114	17	1.96	80.6
	Q8BTI8	Serine/arginine repetitive matrix protein 2 (Srrm2)	54	6	0.77	294.7
	Q3UHJ0	AP2-associated protein kinase 1 (Aak1)	65	14	0.74	103.3
	Q61120	SHC-transforming protein 3 (Shc3)	12	5	0.71	52.1
	Q9QYG0	Protein NDRG2 (Ndrg2)	45	9	0.67	40.8
	Q8BGT8	Phytanoyl-CoA hydroxylase-interacting protein-like (Phyhipl)	14	7	0.67	42.3
	O55131	Septin-7 (Sept7)	29	12	0.66	50.5
	P04370	Myelin basic protein (Mbp)	24	23	0.65	27.2
	Q80XU3	Nuclear ubiquitous casein and cyclin-dependent kinase substrate (Nucks1)	19	22	0.64	26.3
	Q9WV18	Gamma-aminobutyric acid type B receptor subunit 1 (Gabbr1)	16	6	0.64	108.1
	Q8C437	PEX5-related protein (Pex5l)	15	5	0.63	63.1
	Q8BPN8	DmX-like protein 2 (Dmxl2)	104	5	0.61	338.0
	Q9JIX8	Apoptotic chromatin condensation inducer in the nucleus (Acin1)	21	6	0.61	150.6
	Q9QXS1	Plectin (Plec)	20	1	0.60	533.9
	Q8VDN2	Sodium/potassium-transporting ATPase subunit alpha-1 (Atp1a1)	150	21	0.60	112.9
	P08553	Neurofilament medium polypeptide(Nefm)	61	18	0.59	95.9
	Q9CYT6	Adenylyl cyclase-associated protein 2 (Cap2)	21	11	0.59	52.8
	Q8C419	Probable G-protein coupled receptor 158(Gpr158)	24	6	0.59	134.3
	A2AN08	E3 ubiquitin-protein ligase UBR4 (Ubr4)	13	1	0.58	571.9
	P14873	Microtubule-associated protein 1B (Map1b)	376	22	0.58	270.1
	Q9WV92	Band 4.1-like protein 3 (Epb41l3)	89	19	0.57	103.3
	Q80TE7	Leucine-rich repeat-containing protein 7 (Lrrc7)	44	5	0.57	166.8
	Q811P8	Rho GTPase-activating protein 32 (Arhgap32)	32	6	0.55	229.6
	Q6I6G8	E3 ubiquitin-protein ligase HECW2(Hecw2)	10	2	0.55	176.1
	Q9WV69	Dematin (Dmtn)	43	21	0.55	45.4
	Q9JIS5	Synaptic vesicle glycoprotein 2A (Sv2a)	8	4	0.54	82.6
	Q9JME5	AP-3 complex subunit beta-2 (Ap3b2)	17	4	0.52	119.1
	P54285	Voltage-dependent L-type calcium channel subunit beta-3 (Cacnb3)	7	6	0.52	54.5
	Q8VD37	SH3-containing GRB2-like protein 3-interacting protein 1 (Sgip1)	93	20	0.46	86.0
	Q91XV3	Brain acid soluble protein 1 (Basp1)	98	63	0.43	22.1
24 h	Q9QYC0	Alpha-adducin (Add1)	114	17	2.83	80.6
	P28652	Calcium/calmodulin-dependent protein kinase type II subunit beta (Camk2b)	71	20	2.28	60.4
	P48962	ADP/ATP translocase 1 (Slc25a4)	11	13	2.06	32.9
	Q04735	Cyclin-dependent kinase 16 (Cdk16)	6	4	1.98	55.9
	P46096	Synaptotagmin-1 (Syt1)	23	11	1.95	47.4
	Q5SNZ0	Girdin (Ccdc88a)	10	2	1.81	215.8
	O70166	Stathmin-3 (Stmn3)	11	18	1.74	20.9
	P54227	Stathmin (Stmn1)	153	42	1.73	17.3
	Q6NS60	F-box only protein 41 (Fbxo41)	8	4	1.72	94.3
	Q3USB7	Inactive phospholipase C-like protein 1 (Plcl1)	17	4	1.71	122.6
	Q8C9H6	Striatin-interacting proteins 2 (Strip2)	11	4	1.64	96.2
	Q91XM9	Disks large homolog 2 (Dlg2)	37	9	1.62	94.8
	O70439	Syntaxin-7 (Stx7)	15	11	1.61	29.8
	G5E829	Plasma membrane calcium-transporting ATPase 1 (Atp2b1)	46	13	1.55	134.7
	P18760	Cofilin-1 (Cfl1)	9	23	1.53	18.5
	P97427	Dihydropyrimidinase-related protein 1 (Crmp1)	40	14	1.51	62.1
	Q8BGU5	Cyclin-Y (Ccny)	12	13	1.41	39.4
	B1AZP2	Disks large-associated protein 4 (Dlgap4)	29	9	0.84	108
	P51954	Serine/threonine-protein kinase Nek1 (Nek1)	7	4	0.82	136.6
	Q8K4G5	Actin-binding LIM protein 1 (Ablim1)	22	9	0.78	96.7
	P47708	Rabphilin-3A (Rph3a)	4	4	0.48	75.4

polypeptide (Nefm), were downregulated at both time points. Proteins related to the networks of synaptic contact and neurogenesis, such as Syt1, Stx7, Camk2b, and Dlg2, were upregulated at 24 h (Fig. 6D). Metacore analysis illustrated Cofilin as the top network for upregulated phosphoproteins at 24 h after the blast.

When we analyzed mouse brains harvested at 30 days and 15 weeks after the blast exposure, we obtained similar results. Analysis of cellular components of phosphoproteins illustrated changes in synaptic proteins (Fig. 7A). Those phosphoproteins were enriched in brains harvested at 30 days (Table 5) and 15 weeks (Table 6) after the blast, respectively.

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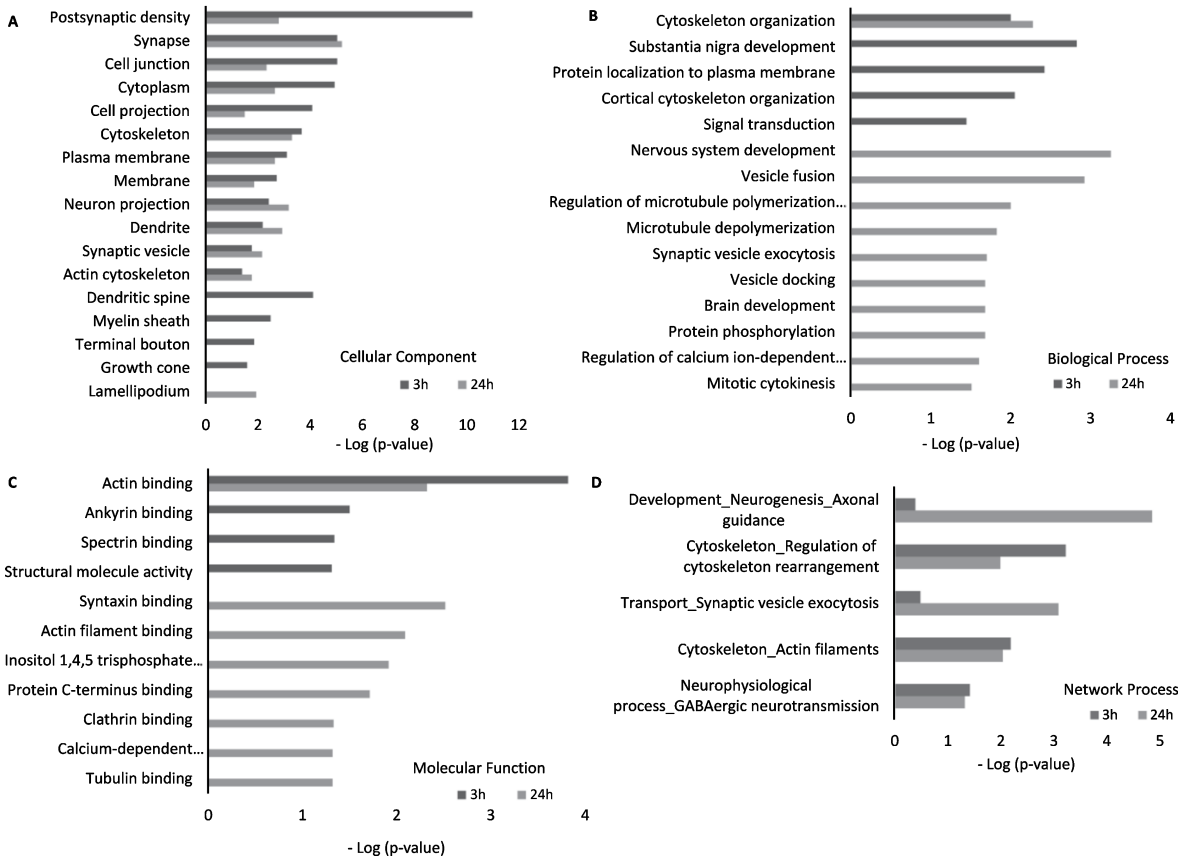

Fig. 6. GO ontology analysis of altered brain phospho-proteomics at 3 h and 24 h post impact. Cellular components (A), biological process (B), molecular functions (C), and network process (D) of phosphoproteins from mouse brains tissue at 3 h and 24 h after the blast were compared to those from control/sham group of animals.

The BP of dysregulated phosphoproteins was examined using GO ontology analysis (Fig. 7B). Similar to what we have observed in tissue collected at earlier time points, one enriched process at all four time points is cytoskeleton organization. Actin binding is the common MF among the phosphoproteins at all time points (Fig. 7C). The networks process of up- and downregulated proteins includes cytoskeleton and actin filament and development of neurogenesis and synaptogenesis (Fig. 7D), similar to those identified at earlier time points (Fig. 6D).

Network analysis revealed up- and downregulated phosphoproteins at 3 h (Fig. 8A), 24 h (Fig. 8B), 30 days (Fig. 8 C), and 15 weeks (Fig. 8D) using STRING. At the early time point (3 h), ATPase, Ca^{2+} transporting, plasma membrane 1 (ATP2b1), calcium/calmodulin-dependent protein kinase II beta (Camk2b) and solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 4 (Slc25a4) are all changed. KEGG pathway analysis shows that calcium signaling pathway is enriched among these altered proteins. At later time points, Cdk16 was a common protein identified by network analysis (Fig. 8B-D).

DISCUSSION

The significance of this study is highlighted by an urgent need to identify critical players involved in neuropathological development in people who suffer mTBI and to investigate how these factors contribute to neuronal damage and neurodegenerative diseases like AD. We have used an animal model exposed to open field blast to model mTBI [25, 26]. This unique platform allows us to compare mice responses to brain injury at the molecular level of in-depth proteomics and biochemistry of brain tissue. Currently we lack mechanistic understanding of neuronal damages caused by mTBI and

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Table 3
The list of altered phosphoproteins (3 h post blast) enriched in DAVID functional analysis

Categories	GO Terms	ACCESSION	GENE NAME
BP	Substantia nigra development	Q9QYG0	N-myc downstream regulated gene 2(Ndrg2)
		Q91XV3	Brain abundant, membrane attached signal protein 1(Basp1)
		P04370	Myelin basic protein(Mbp)
	Cortical cytoskeleton organization	Q9WV92	Erythrocyte membrane protein band 4.1 like 3(Epb41l3)
		O55131	Septin 7(Sept7)
	Signal transduction	Q9CYT6	CAP, adenylate cyclase-associated protein, 2 (yeast)(Cap2)
		Q8C419	G protein-coupled receptor 158(Gpr158)
		Q9QYG0	N-myc downstream regulated gene 2(Ndrg2)
		Q811P8	Rho GTPase activating protein 32(Arhgap32)
		Q9WV18	Gamma-aminobutyric acid (GABA) B receptor, 1(Gabbr1)
		Q61120	Src homology 2 domain-containing transforming protein C3(Shc3)
CC	Postsynaptic density	Q8VDN2	ATPase, Na+/K+ transporting, alpha 1 polypeptide(Atp1a1)
		Q9CYT6	CAP, adenylate cyclase-associated protein, 2 (yeast)(Cap2)
		Q811P8	Rho GTPase activating protein 32(Arhgap32)
		Q9QYC0	Adducin 1 (alpha)(Add1)
		Q9WV69	Dematin actin binding protein(Dmtn)
		Q9WV92	Erythrocyte membrane protein band 4.1 like 3(Epb41l3)
		Q80TE7	Leucine rich repeat containing 7(Lrrc7)
		P14873	Microtubule-associated protein 1B(Map1b)
		P08553	Neurofilament, medium polypeptide(Nefm)
	Dendritic spine	Q811P8	Rho GTPase activating protein 32(Arhgap32)
		Q9QYC0	Adducin 1 (alpha)(Add1)
		Q9WV18	Gamma-aminobutyric acid (GABA) B receptor, 1(Gabbr1)
		Q80TE7	Leucine rich repeat containing 7(Lrrc7)
		P14873	Microtubule-associated protein 1B(Map1b)
	Cytoskeleton	Q9QYC0	Adducin 1 (alpha)(Add1)
		P28652	Calcium/calmodulin-dependent protein kinase II, beta(Camk2b)
		Q9WV69	Dematin actin binding protein(Dmtn)
		Q9WV92	Erythrocyte membrane protein band 4.1 like 3(Epb41l3)
		P14873	Microtubule-associated protein 1B(Map1b)
		P08553	Neurofilament, medium polypeptide(Nefm)
		Q9QXS1	Plectin(Plec)
		O55131	Septin 7(Sept7)
		A2AN08	Ubiquitin protein ligase E3 component n-recognin 4(Ubr4)
	Myelin sheath	Q8VDN2	ATPase, Na+/K+ transporting, alpha 1 polypeptide(Atp1a1)
		P04370	Myelin basic protein(Mbp)
		P08553	Neurofilament, medium polypeptide(Nefm)
		O55131	Septin 7(Sept7)
MF	Actin binding	Q9CYT6	CAP, adenylate cyclase-associated protein, 2 (yeast)(Cap2)
		Q9QYC0	Adducin 1 (alpha)(Add1)
		Q9WV69	Dematin actin binding protein(Dmtn)
		Q9WV92	Erythrocyte membrane protein band 4.1 like 3(Epb41l3)
		P14873	Microtubule-associated protein 1B(Map1b)
		Q9QXS1	Plectin(Plec)
	Ankyrin binding	Q8VDN2	ATPase, Na+/K+ transporting, alpha 1 polypeptide(Atp1a1)
		Q9QXS1	Plectin(Plec)
	Spectrin binding	Q9QYC0	Adducin 1 (alpha)(Add1)
		Q9WV69	Dematin actin binding protein(Dmtn)
	Structural molecule activity	Q9QYC0	Adducin 1 (alpha)(Add1)
		Q9WV92	Erythrocyte membrane protein band 4.1 like 3(Epb41l3)
		P08553	Neurofilament, medium polypeptide(Nefm)

we do not have a target for therapeutic intervention for those suffering mTBI or in people with a history of mTBI. Based on the proteomic profiles of mouse brain exposed to blast, we are exploring novel targets and candidate proteins linked to axonal injury, microtubule cytoskeleton organization, and tau pathology.

Proteomic analysis of the moue brain tissues revealed significant changes in protein expression representing axonal injury and microtubule cytoskeleton organization in response to injury

This study utilized TMT which offers the ability to simultaneously detect ten samples under a single

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Table 4
The list of altered phosphoproteins (24 h post blast) enriched in DAVID functional analysis

Category	GO Terms	ACCESSION	GENE NAME
BP	Nervous system development	P28652	Calcium/calmodulin-dependent protein kinase II, beta(Camk2b)
		Q5SNZ0	Coiled coil domain containing 88A(Ccdc88a)
		P97427	Collapsin response mediator protein 1(Crmp1)
		Q91XM9	Discs, large homolog 2 (Drosophila)(Dlg2)
		P54227	Stathmin 1(Stmn1)
	Cytoskeleton organization	Q8K4G5	Actin-binding LIM protein 1(Ablim1)
		P18760	Cofilin 1, non-muscle(Cfl1)
		Q8C9H6	Striatin interacting protein 2(Strip2)
	Microtubule depolymerization	P54227	Stathmin 1(Stmn1)
		O70166	Stathmin-like 3(Stmn3)
	Synaptic vesicle exocytosis	P47708	Rabphilin 3A(Rph3a)
		P46096	Synaptotagmin I(Syt1)
CC	Synapse	G5E829	ATPase, Ca++ transporting, plasma membrane 1(Atp2b1)
		Q9QYC0	Adducin 1 (alpha)(Add1)
		Q04735	Cyclin-dependent kinase 16(Cdk16)
		Q91XM9	Discs, large homolog 2 (Drosophila)(Dlg2)
		B1AZP2	Discs, large homolog-associated protein 4 (Drosophila)(Dlgap4)
		P47708	Rabphilin 3A(Rph3a)
		P46096	Synaptotagmin I(Syt1)
	Cytoskeleton	P51954	NIMA (never in mitosis gene a)-related expressed kinase 1(Nek1)
		Q8K4G5	Actin-binding LIM protein 1(Ablim1)
		Q9QYC0	Adducin 1 (alpha)(Add1)
		P28652	Calcium/calmodulin-dependent protein kinase II, beta(Camk2b)
		P18760	Cofilin 1, non-muscle(Cfl1)
		P97427	Collapsin response mediator protein 1(Crmp1)
		P54227	Stathmin 1(Stmn1)
	Neuron projection	Q04735	Cyclin-dependent kinase 16(Cdk16)
		P47708	Rabphilin 3A(Rph3a)
		P54227	Stathmin 1(Stmn1)
		O70166	Stathmin-like 3(Stmn3)
		P46096	Synaptotagmin I(Syt1)
	Postsynaptic density	Q8K4G5	Actin-binding LIM protein 1(Ablim1)
		Q9QYC0	Adducin 1 (alpha)(Add1)
		P28652	Calcium/calmodulin-dependent protein kinase II, beta(Camk2b)
		Q91XM9	Discs, large homolog 2 (Drosophila)(Dlg2)
MF	Calmodulin binding	G5E829	ATPase, Ca++ transporting, plasma membrane 1(Atp2b1)
		Q9QYC0	Adducin 1 (alpha)(Add1)
		P28652	Calcium/calmodulin-dependent protein kinase II, beta(Camk2b)
		P46096	Synaptotagmin I(Syt1)
	Syntaxin binding	P47708	Rabphilin 3A(Rph3a)
		P46096	Synaptotagmin I(Syt1)
		O70439	Syntaxin 7(Stx7)
	Actin binding	Q8K4G5	Actin-binding LIM protein 1(Ablim1)
		Q9QYC0	Adducin 1 (alpha)(Add1)
		P18760	Cofilin 1, non-muscle(Cfl1)
		Q5SNZ0	Coiled coil domain containing 88A(Ccdc88a)
	Tubulin binding	P54227	Stathmin 1(Stmn1)
		O70166	Stathmin-like 3(Stmn3)

condition. DAVID analysis of altered proteins show that several functions significantly changed after the blast. Specifically, we found negative effect of blast on a number of cellular processes.

Diffuse axonal injury is a hallmark pathological consequence of non-penetrative mTBI [34]. In neuronal culture model, very mild stretch injury to a localized region of the cortical axon is able to trigger a degenerative response characterized by growth cone collapse and significant abnormal cytoskeleton rearrangement, resulting in the formation of smaller growth cones at the tips of axons and a significantly higher number of collapsed structures at both 24 h and 72 h post injury [34]. Axonal degeneration following stretch injury involves destabilization of the microtubule cytoskeleton. Microtubules are the stiffest structural components of axons and thus may be at risk of damage during mTBI. Loss of

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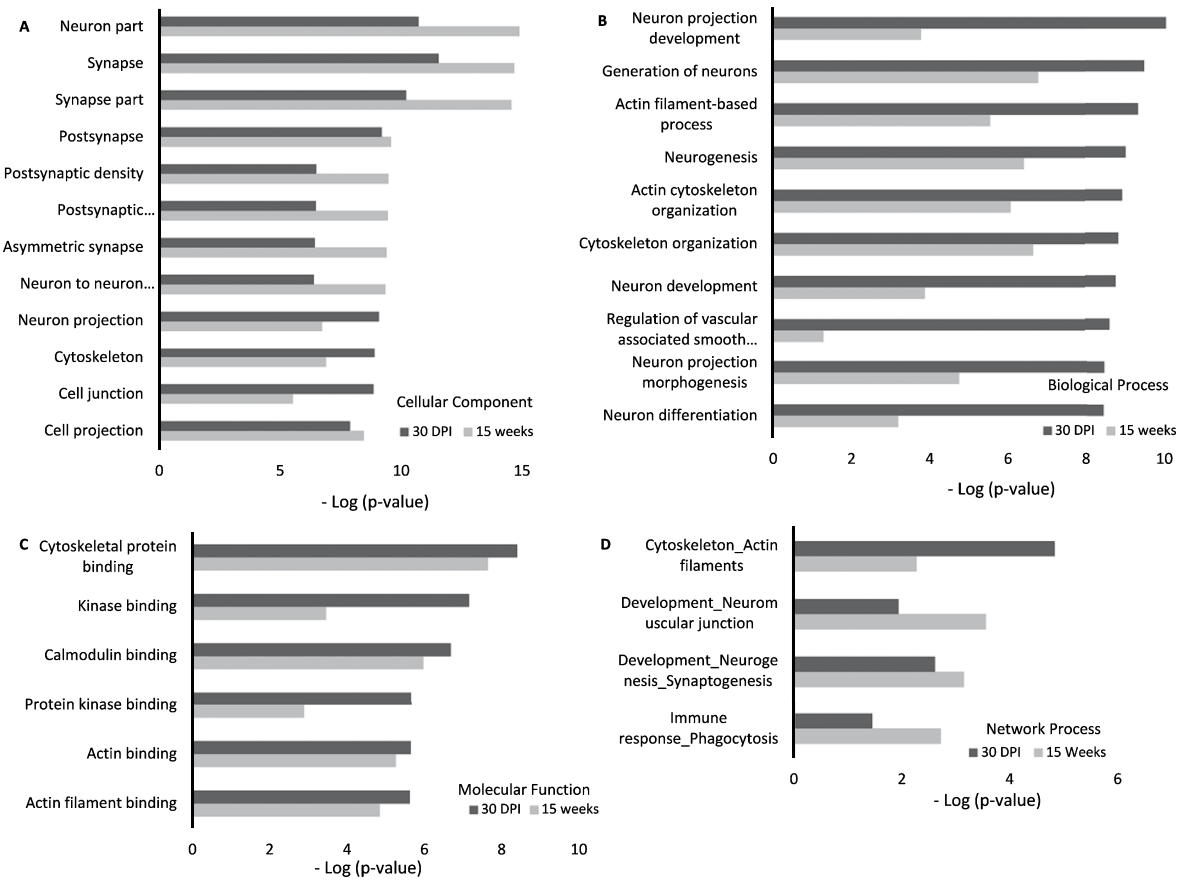

Fig. 7. GO ontology analysis of altered brain phospho-proteomics at 30 days and 15 weeks post impact. Cellular components (A), biological process (B), molecular functions (C), and network process (D) of phosphoproteins from mouse brains tissue at 30 days and 15 weeks after the blast were compared to those from control/sham group of animals.

axonal microtubule was found after stretch injury and progressive disassembly of microtubules along the breakage points [35]. Stabilization of microtubule resulted in a significant reduction in the number of fragmented axons following injury [34]. Consistent with previous reports [36], our studies also revealed depolymerization of microtubules.

We identified several proteins, such as Map1B, Stmn1, Shc3, Sept7, Nefm, Ap3b2, and Mapt. They play important roles in maintaining microtubule stability and axonal integrity. Map1B belongs to a group of microtubule-associated proteins that support microtubule-stabilization, tubulin assembly/disassembly, and physical interactions between microtubules and components of the cytoskeleton. Map1B and its phosphorylated isoform are present in growing axons and concentrated in distal end near the growth cone. Map1B plays a crucial role in the stability of the cytoskeleton, and its function is modulated by the state of phosphorylation. Immuno-histochemical staining showed that phosphorylated Map1B was abundant in developing axons, suggesting its essential role in axonal elongation [37]. Map1B is upregulated after ischemia in aged rats [38] but downregulated in optic nerve after mTBI [36]. In this study, downregulation of phosphorylated Map1B was indicative of axonal injury and cytoskeletal damage following TBI. It is possible that the structural reorganization at the cellular level and shifts in cytoskeletal dynamic associate with injury. This observation is consistent with our previous finding of downregulated phosphorylated Map1B from the inferior prefrontal cortex region of postmortem brain tissues from AD patients [39], clearly linking the TBI-induced cytoskeletal damage to pathological changes in AD brain.

Stathmin (Stmn) plays an essential role in the maintenance of axonal integrity. It is involved in the regulation of the microtubule filament system by destabilizing microtubules, and phosphorylation

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Table 5
The list of altered phosphoproteins (30 days post blast) enriched in DAVID functional analysis

Category	GO Terms	ACCESSION	GENE NAME
BP	Neurotransmitter secretion	Q64332	synapsin II(Syn2)
		Q8JZP2	synapsin III(Syn3)
		P60879	synaptosomal-associated protein 25(Snap25)
	Actin cytoskeleton organization	Q3UHD9	ArfGAP with GTPase domain, ankyrin repeat and PH domain 2(Agap2)
		Q8R0S2	IQ motif and Sec7 domain 1(Iqsec1)
		O88643	p21 protein (Cdc42/Rac)-activated kinase 1(Pak1)
		Q4JIM5	v-abl Abelson murine leukemia viral oncogene 2 (arg, Abelson-related gene)(Abl2)
	Neuron projection morphogenesis	Q62418	drebrin-like(Dbnl)
		P35802	glycoprotein m6a(Gpm6a)
		O88643	p21 protein (Cdc42/Rac)-activated kinase 1(Pak1)
	Synaptic vesicle priming	Q80TJ1	Ca2+-dependent secretion activator(Cadps)
		P60879	synaptosomal-associated protein 25(Snap25)
CC	Cytoskeleton	Q9Z0H8	CAP-GLY domain containing linker protein 2(Clip2)
		A2AJI0	MAP7 domain containing 1(Map7d1)
		P62484	abl-interactor 2(Abi2)
		Q8K4G5	actin-binding LIM protein 1(Ablim1)
		Q80VC9	calmodulin regulated spectrin-associated protein family, member 3(Camsap3)
		Q62418	drebrin-like(Dbnl)
		Q8VDD5	myosin, heavy polypeptide 9, non-muscle(Myh9)
		P26645	myristoylated alanine rich protein kinase C substrate(Marcks)
		Q62417	sorbin and SH3 domain containing 1(Sorbs1)
		Q4JIM5	v-abl Abelson murine leukemia viral oncogene 2 (arg, Abelson-related gene)(Abl2)
		Q8BG89	zinc finger protein 365(Zfp365)
	Lamellipodium	P62484	abl-interactor 2(Abi2)
		Q8K4G5	actin-binding LIM protein 1(Ablim1)
		Q62418	drebrin-like(Dbnl)
		O88643	p21 protein (Cdc42/Rac)-activated kinase 1(Pak1)
		P60879	synaptosomal-associated protein 25(Snap25)
		Q4JIM5	v-abl Abelson murine leukemia viral oncogene 2 (arg, Abelson-related gene)(Abl2)
	Synapse	Q80TJ1	Ca2+-dependent secretion activator(Cadps)
		Q61361	brevican(Bcan)
		Q04735	cyclin-dependent kinase 16(Cdk16)
		Q62418	drebrin-like(Dbnl)
		Q64332	synapsin II(Syn2)
		Q8JZP2	synapsin III(Syn3)
		P60879	synaptosomal-associated protein 25(Snap25)
		P63044	vesicle-associated membrane protein 2(Vamp2)
	Postsynaptic density	UNIPROT_ACCESSION	GENE NAME
		D3YVF0	A kinase (PRKA) anchor protein 5(Akap5)
		Q8K4G5	actin-binding LIM protein 1(Ablim1)
		Q62418	drebrin-like(Dbnl)
		O88643	p21 protein (Cdc42/Rac)-activated kinase 1(Pak1)
		Q64332	synapsin II(Syn2)
		Q8JZP2	synapsin III(Syn3)
MF	Calmodulin binding	D3YVF0	A kinase (PRKA) anchor protein 5(Akap5)
		Q80VC9	calmodulin regulated spectrin-associated protein family, member 3(Camsap3)
		Q8VDD5	myosin, heavy polypeptide 9, non-muscle(Myh9)
		P26645	myristoylated alanine rich protein kinase C substrate(Marcks)
		P63044	vesicle-associated membrane protein 2(Vamp2)
	Protein kinase binding	D3YVF0	A kinase (PRKA) anchor protein 5(Akap5)
		Q3UHD9	ArfGAP with GTPase domain, ankyrin repeat and PH domain 2(Agap2)
		Q80TJ1	Ca2+-dependent secretion activator(Cadps)
		Q9Z2V6	histone deacetylase 5(Hdac5)
		O88643	p21 protein (Cdc42/Rac)-activated kinase 1(Pak1)
		Q62417	sorbin and SH3 domain containing 1(Sorbs1)
	Actin binding	D3YVF0	A kinase (PRKA) anchor protein 5(Akap5)
		Q8K4G5	actin-binding LIM protein 1(Ablim1)
		Q62418	drebrin-like(Dbnl)
		Q8VDD5	myosin, heavy polypeptide 9, non-muscle(Myh9)
		P26645	myristoylated alanine rich protein kinase C substrate(Marcks)
	Calcium-dependent protein binding	Q64332	synapsin II(Syn2)
		P60879	synaptosomal-associated protein 25(Snap25)
		P63044	vesicle-associated membrane protein 2(Vamp2)

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Table 6
The list of altered phosphoproteins (15 weeks post blast) enriched in DAVID functional analysis

Category	GO Terms	ACCESSION	GENE NAME
BP	Negative regulation of phosphatase activity	F8VPU2	FERM, RhoGEF (Arhgef) and pleckstrin domain protein 1 (chondrocyte-derived)(Farp1)
		Q91XM9	discs, large homolog 2 (Drosophila)(Dlg2)
		Q5XJV6	lemur tyrosine kinase 3(Lmtk3)
	Axonogenesis	Q5RJI5	BR serine/threonine kinase 1(Brsk1)
		Q69Z98	BR serine/threonine kinase 2(Brsk2)
		P54227	stathmin 1(Stmn1)
	Regulation of synaptic transmission, GABAergic	Q04690	neurofibromatosis 1(Nf1)
		Q3USB7	phospholipase C-like 1(Plcl1)
	Regulation of cell shape	P05064	aldolase A, fructose-bisphosphate(Aldoa)
		Q9WV69	dematin actin binding protein(Dmtn)
		Q6URW6	myosin, heavy polypeptide 14(Myh14)
CC	Synapse	Q5RJI5	BR serine/threonine kinase 1(Brsk1)
		Q80TJ1	Ca2+-dependent secretion activator(Cadps)
		F8VPU2	FERM, RhoGEF (Arhgef) and pleckstrin domain protein 1 (chondrocyte-derived)(Farp1)
		Q811P8	Rho GTPase activating protein 32(Arhgap32)
		Q9QYC0	adducin 1 (alpha)(Add1)
		O70174	cholinergic receptor, nicotinic, alpha polypeptide 4(Chrna4)
		Q91XM9	discs, large homolog 2 (Drosophila)(Dlg2)
		Q80TE7	leucine rich repeat containing 7(Lrrc7)
		Q8BSS9	protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 2(Ppfia2)
		Q9EQZ7	regulating synaptic membrane exocytosis 2(Rims2)
		Q8BJI1	solute carrier family 6 (neurotransmitter transporter), member 17(Slc6a17)
		Q71LX4	talin 2(Tln2)
	Postsynaptic density	Q9QYE3	B cell CLL/lymphoma 11A (zinc finger protein)(Bcl11a)
		Q811P8	Rho GTPase activating protein 32(Arhgap32)
		Q8K4G5	actin-binding LIM protein 1(Ablim1)
		Q9QYC0	adducin 1 (alpha)(Add1)
		Q80YN3	breast carcinoma amplified sequence 1(Bcas1)
		Q9WV69	dematin actin binding protein(Dmtn)
		Q91XM9	discs, large homolog 2 (Drosophila)(Dlg2)
		Q80TE7	leucine rich repeat containing 7(Lrrc7)
		Q04690	neurofibromatosis 1(Nf1)
	Myelin sheath	P05064	aldolase A, fructose-bisphosphate(Aldoa)
		P07901	heat shock protein 90, alpha (cytosolic), class A member 1(Hsp90aa1)
		P04370	myelin basic protein(Mbp)
		Q6URW6	myosin, heavy polypeptide 14(Myh14)
		Q9Z1B3	phospholipase C, beta 1(Plcb1)
		Q60932	voltage-dependent anion channel 1(Vdac1)
MF	Calmodulin binding	P28667	MARCKS-like 1(Marcksl1)
		Q9QYC0	adducin 1 (alpha)(Add1)
		Q8C078	calcium/calmodulin-dependent protein kinase kinase 2, beta(Camkk2)
		Q6URW6	myosin, heavy polypeptide 14(Myh14)
		Q9Z1B3	phospholipase C, beta 1(Plcb1)
	Actin binding	P28667	MARCKS-like 1(Marcksl1)
		Q8K4G5	actin-binding LIM protein 1(Ablim1)
		Q9QYC0	adducin 1 (alpha)(Add1)
		Q9WV69	dematin actin binding protein(Dmtn)
		Q6URW6	myosin, heavy polypeptide 14(Myh14)
		Q71LX4	talin 2(Tln2)
	Tau-protein kinase activity	Q5RJI5	BR serine/threonine kinase 1(Brsk1)
		Q69Z98	BR serine/threonine kinase 2(Brsk2)

of Stmn regulates microtubule disassembly. In our study, phosphorylated Stmn1and Stmn3 were found upregulated at 24 h after the blast, and the level of Stmn1 was found 2-fold of that of the control group at 15 weeks after the blast (data not shown). A recent study demonstrated that Stmn-dependent changes in microtubule stability are crucial for synaptic function and memory formation [40]. Stmn acts on microtubule disruption and/or tubulin sequestration, and elevation of Stmn1 after blasts was found in rat model of mTBI [41]. High Stmn expression induced microtubule structures disruption and enhanced tau hyperphosphorylation [42]. Similarly, brain injury causes reduced polymerization of Gephyrin and

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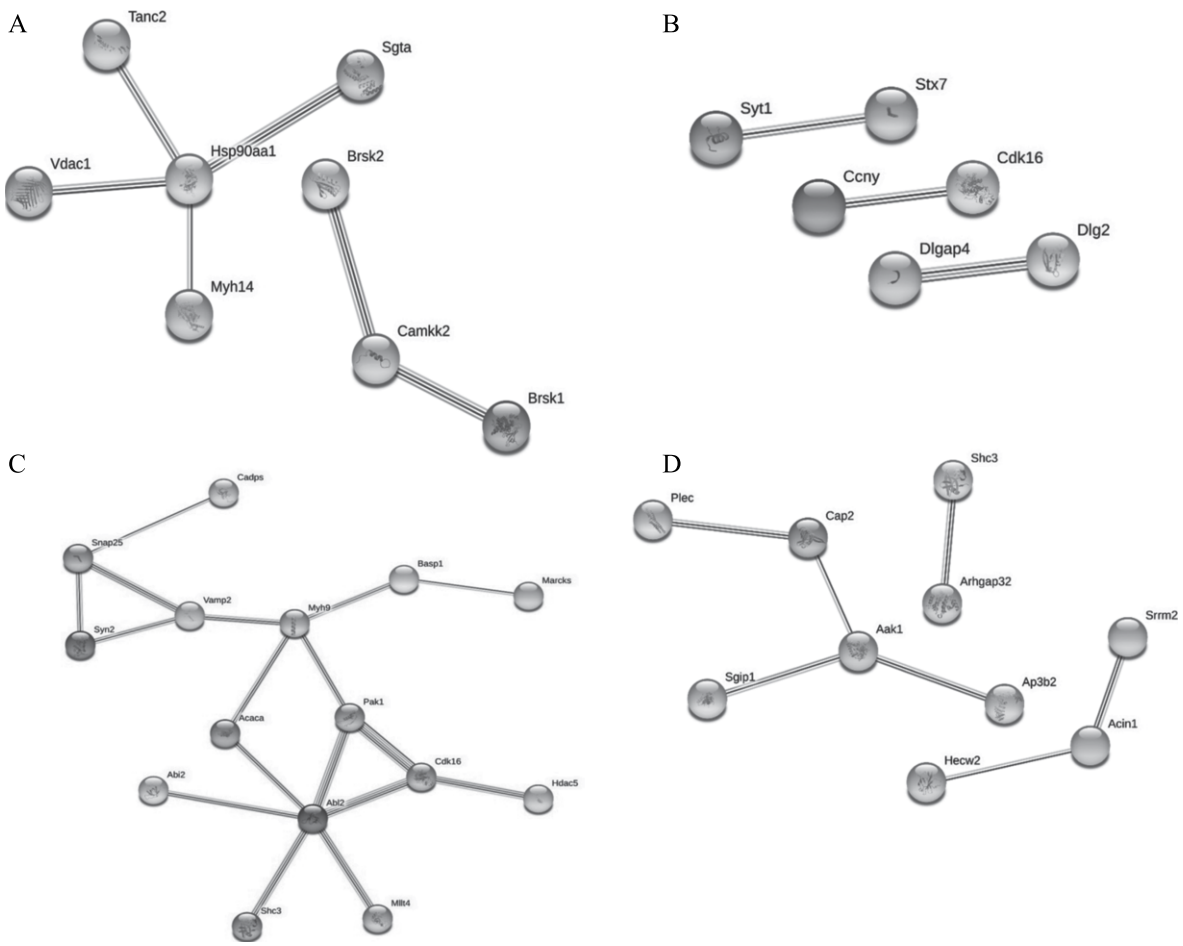

Fig. 8. Network analysis of all captured phosphoproteins was illustrated using the web-based tool STRING. Proteins from mouse brains at 3 h (A), 24 h (B), 30 days (C), and 15 weeks (D) after the blast were compared to those from the control group using LC-MS/MS, and the enriched cellular components and altered proteins were analyzed by DAVID.

subsequent synapse destabilization [43], and progressive disassembly and loss of axonal microtubule along the breakage points [35].

A number of proteins altered in our mice exposed to open field blast were also reported from previous proteomic analysis of optic nerves from mice suffering repetitive mTBI (rmTBI) [36]. Various negative effects of rmTBI on cellular processes were reported, including depolymerization of microtubules, disassembly of filaments and loss of neurons, evidenced by a reduction of several proteins, including neurofilaments, tubulin (Tubb2A, Tuba4A), and microtubule-associated proteins (Map1A, Map1B) [36]. In our study, phosphorylated neurofilament medium polypeptide (Nefm) was downregulated. Nefm belongs to the group of neurofilament triplet proteins, the major cytoskeletal components of neurons in the CNS. They interact with microtubules and microfilaments to form and maintain the axonal structural integrity and neuronal shape, and also participate to the axonal mechanism of transport. Significant decrease in neurofilament levels in optic nerve of rat retina after 21 days mTBI was reported [36]. Furthermore, increased level of Nefm was observed in CSF and serum from patients with a history of mTBI, but the effect could come from peripheral nerve injury [44].

We also observed several altered dynein proteins in addition to those proteins related to microtubule and tubulin binding. Axonal transport is related to microtubule stability and essential for transducing extracellular signals and recycling misfolded proteins from the nerve terminals to cell soma, thus avoiding the build-up of toxic aggregates. Axonal

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transport requires the cytoskeletal microtubule scaffold to serve as tracks, and the motor proteins kinesin and dynein that exert mechanical force to move cargoes.

In this study, we found changes in a large proportion of proteins located in dendritic spines, those highly dynamic membranous protrusions on postsynaptic dendrites. Structural changes in dendritic spines form the basis for learning and memory formation, making them central hubs for the processing and storage of information in the brain [45]. Study in Map1B-deficient mice showed that Map1B was required for dendritic spine development and synaptic maturation [46]. Study of mouse hippocampal neuron cultures showed inhibition of microtubule dynamics alters dendritic spine morphology through the actin cytoskeleton, suggesting that the microtubule and actin cytoskeleton act together in dendritic spines to regulate synaptic plasticity [47]. We have observed changes in several proteins with actin binding activities. Three phosphorylated actin binding proteins, Cofilin, Add1, and Ccdc88a, were found upregulated 24 h after the blast in our study. Cofilin is a major component of intranuclear and cytoplasmic actin rods, and Ccdc88a is required for formation of actin stress fibers and lamellipodia.

Consistent with previous study [36], phosphomyelin basic protein (Mbp) was found reduced in mice exposed to blast in our study. Mbp is a major constituent of CNS myelin and mature oligodendrocytes, and the decrease in Mbp levels could be a direct reflection of an ongoing demyelination. We observed continuous decrease of phospho-Mbp at 15 weeks (data not shown), suggesting that demyelination was a long-lasting outcome of TBI. In Schwann cells responding to 2 h of sustained shear stress, Mbp appeared to be downregulated, suggesting that shear stress alone is sufficient to induce pathological changes [48]. In rats, extensive degradation of Mbp isoforms was observed after TBI [49], suggesting that TBI-mediated axonal injury causes secondary structural damage to the adjacent myelin membrane and instigate Mbp degradation. This could initiate myelin sheath instability and demyelination, which might further promote axonal vulnerability [49].

Upregulated calcium signaling after TBI

In our study, several phosphorylated proteins involved in calcium signaling pathway were upregulated. There is strong evidence in support of the concept that abnormal neurotransmission and Ca^{2+}-mediated signal transduction pathways could be responsible for neuronal dysfunction following trauma. Calcium is crucial for several aspects of plasticity at glutamatergic synapses. Changes in the levels of intracellular Ca^{2+} or alterations of Ca^{2+} signaling could contribute to a variety of pathologies. One of the major causes of secondary injury following TBI is related to an excess influx of Ca^{2+} into cells in the brain [50].

Calcium/calmodulin-dependent protein kinase type II subunit beta (Camk2b) is essential for the integrity of the actin cytoskeleton and for cell migration. Camk2b is expressed in motor neurons during axon outgrowth and is part of slow axonal transport [51]. Consistent with previous findings [52, 53], we found increased phosphorylation of CamkIIb at 24 h after the TBI. Atp2b1, which mediates calcium efflux, was upregulated in our blast-induced mouse brains. Slc25a4, which catalyzes the exchange of ADP and ATP across the mitochondrial inner membrane, was also upregulated. Since the response to CNS injury includes an apparent increase in energy mobilization capacity, elevated intracellular Ca^{2+} after TBI may be related to reduced ATP levels, as previously reported [50]. Another key protein, phosphorylated Synaptotagmin I (Syt1), was upregulated at 24 h after the blast. Synaptotagmins are integral membrane proteins of synaptic vesicles thought to serve as Ca^{2+} sensors in the process of vesicular trafficking and exocytosis. Calcium binding Syt-1 participates in triggering neurotransmitter release at the synapse [53].

Increases of p-tau, behavioral and pathological changes in mice exposed to blast link mTBI to AD

Our study revealed pathways related to p-tau (Fig. 1), which is the component of neurofibrillary tangles found in brains of AD patients. In addition, our exploratory SNAP tests revealed a significant difference in behavioral outcomes in the blast group compared to sham controls (Fig. 2). Degenerating axon-terminals and dendrites were visualized on silver-stained tissue sections from mouse brains exposed to blast (Fig. 3), supporting a contributing role of mTBI toward neurodegenerative process commonly found in brains of AD patients.

Tau as axonal injury marker has been reported in previous studies following mTBI [54, 55]. Traumatic brain injury can lead to diffuse traumatic axonal injury and blood–brain barrier disruption. Shearing of axons results in the disruption of tau binding

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to tubulin [56]. Subsequent hyperphosphorylation of tau leads to formation of tau oligomers in the neuronal soma. When tau becomes hyperphosphorylated, it binds to other normal tau proteins, which leads to aggregation. Accumulation of insoluble tau within neurons contributes to the development of tau oligomers [56]. Tau oligomers are granular intracellular buildups of mutated tau, which precede the development of NFTs. Tau hyperphosphorylation is common after mTBI and other brain injuries [57]. Previous study in patients with mTBI showed the level of phosphorylated tau was significantly higher and correlated with the Glasgow Coma Scale (GCS) score [58]. We found a similar increase of p-tau in brains of mice collected at 3 and 24 h after open field blast exposure, which is consistent with previous findings in humans and animals [58–60]. While increase in tau immunostaining in brain tissue was not observed in mini-pig from 2 weeks to 8 months after blast-induced TBI [61], our ELISA-based quantitation of tau and phospho-tau in mice exposed to blast illustrate a significant difference at 3 and 24 h. Levels of tau and p-tau were significantly changed within one day after the blast and returned to pre-blast stage at 15 weeks after blast (Fig. 1). It seems that blast-exposed animals were capable of recovering from impact, and the amount of ptau gradually reduced to the pre-blast levels. Several protein kinases (e.g., GSK3β) phosphorylate tau at multiple sites, and activities of these kinases might be enhanced upon low impact blast, leading to an increase in phosphorylated tau levels [7]. Once the animals were recovered from the blast, kinase activities might return to pre-blast levels, and diminish transient increases of ptau. This is consistent with previous findings in mini-pigs [61] and mice [62]. In transgenic mice expressing human tau (hTau), only transient increases in p-tau was observed after single or repetitive mild TBI, and no tau pathology was found at one year after TBI [62].

Tau-related pathological change is a common feature linking mTBI to AD. Whether mTBI-induced phospho-tau increase triggers AD pathogenesis needs to be investigated. Elucidation of tau-related molecular changes in people with a history of mTBI will open a new window to determine whether personnel who have suffered mTBI have asymptomatic AD. This is essential for designing future preventive measures for AD. Future effort to suppress p-tau-related neuropathological changes will potentially reduce the burden on affected individuals, especially those vulnerable to developing AD.

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Paclitaxel Reduces Brain Injury from Repeated Head Trauma in Mice

Donna J. Cross[a,*], James S. Meabon[b,d], Marcella M. Cline[c,e], Todd L. Richards[f], Amanda J. Stump[a], Chloe G. Cross[a], Satoshi Minoshima[a], William A. Banks[c,g] and David G. Cook[c,h]
[a]*Department of Radiology and Imaging Sciences, University of Utah, Salt Lake City, UT, USA*
[b]*The Mental Illness Research Education and Clinical Center (MIRECC), and VA Puget Sound Health Care System, Seattle, WA, USA*
[c]*Geriatric Research Education and Clinical Center (GRECC) and VA Puget Sound Health Care System, Seattle, WA, USA*
[d]*Department of Psychiatry University of Washington, Seattle, WA, USA*
[e]*Department of Molecular and Cellular Biology, University of Washington, Seattle, WA, USA*
[f]*Department of Radiology, University of Washington, Seattle, WA, USA*
[g]*Department of Medicine, University of Washington, Seattle, WA, USA*
[h]*Department of Pharmacology, University of Washington, Seattle, WA, USA*

Abstract. Repetitive mild traumatic brain injury (rmTBI) is known to disturb axonal integrity and may play an important role in the pathogenic cascades leading to neurodegeneration. One critical approach to reduce the future onset of neurodegeneration is to intervene in this process at an early stage following a brain injury. Previously we showed that direct application of the microtubule-stabilizing drug, *paclitaxel,* on the brain following controlled cortical impact improved motor function and reduced lesion size. Herein, we extended these findings to a model of mild brain injury induced by repeated closed-skull impacts. *Paclitaxel* was administered intranasally to circumvent its poor transport across the blood-brain barrier. Mice received five mild closed-skull impacts (one per day for five days). Intranasal *paclitaxel* was administered once only, immediately after the first impact. We found that *paclitaxel* prevented injury-induced deficits in a spatial memory task in a water tread maze. *In vivo* magnetic resonance imaging (MRI) and positron emission tomography with 18F-flurodeoxyglucose (FDG-PET) revealed that *paclitaxel* prevented structural injury and hypometabolism. On MRI, apparent, injury-induced microbleeds were observed in 100% of vehicle-treated rmTBI mice, but not in *paclitaxel*-treated subjects. FDG-PET revealed a 42% increase in whole brain glucose metabolism in paclitaxel-treated mice as compared to vehicle-treated rmTBI. Immunohistochemistry found reduced evidence of axonal injury and synaptic loss. Our results indicate that intranasal *paclitaxel* administration imparts neuroprotection against brain injury and cognitive impairment in mice. The results from this study support the idea that microtubule-stabilization strategies hold therapeutic promise in mitigating traumatic brain injury.

Keywords: Axonal injury, imaging, microtubule-stabilizing drug, repeat mild traumatic brain injury, synaptic preservation

INTRODUCTION

There is increasing evidence that repetitive mild traumatic brain injury (rmTBI) is associated with neurodegenerative disease [1–4]. Although the process by which rmTBIs lead to neurodegeneration is still under investigation, there is emerging evidence that loss of axonal integrity and cytoskeletal derangement may play a prominent role in the evolving pathogenic cascades that in later life can cause significant neurocognitive impairment [5–7]. One critical approach to reduce the future onset of neurodegeneration is to intervene in this process at an early stage following a brain injury. However, much work remains in the search for clinically effective pharmacological interventions to treat traumatic brain injury

*Correspondence to: Donna J. Cross, PhD, Department of Radiology and Imaging Sciences, University of Utah, 30 N. 1900 E. #1A71, Salt Lake City, UT 84132-2140, USA. Tel.: +1 801 585 1346; E-mail: d.cross@utah.edu.

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(TBI). Microtubules play a role in many homeostatic brain processes including cytoskeletal integrity and axonal transport, as well as in regulating cell morphology, proliferation, motility, and intracellular protein trafficking [8–11]. All of these processes may be disturbed by the primary mechanical insult in TBI [12]. In a previous study, we administered a microtubule-stabilizing (MT) drug, typically used to treat cancer, to mitigate acute head trauma [13].

Long used for chemotherapy to prevent mitosis in tumor cells, the prototypical, microtubule-stabilizing compound, *paclitaxel* has been well-studied in terms of binding and target characterization [14]. *Paclitaxel* prevents depolymerization by binding to β tubulin on the inner surface of microtubules [15, 16]. Studies have indicated that low doses of *paclitaxel* may be beneficial for acute CNS injuries, as well as neurological diseases [8, 17–21]. However, the neurotherapeutic potential of *paclitaxel* has been investigated only sparingly in models of focal neuronal injury [19, 20, 22, 23]. An *in vivo* rat study by Adlard et al. used intracortical delivery of *paclitaxel* after a penetrating needle stick injury and found reduced axonopathy and a preservation of MAP2 labeling in the injury area [22]. Another study showed that intrathecal administration of *paclitaxel* facilitated axonal regeneration in acute spinal cord injury in rats [19]. Sengottuvel et al. found *paclitaxel* promoted axonal growth *in vitro* and optic nerve regeneration after crush injury *in vivo* [20]. In addition, Ertürk et al. delivered *paclitaxel* to a dorsal root ganglion injury and found that the drug limited axonal retraction bulbs. This same study found that *paclitaxel* promoted axonal growth when applied *in vitro* to cultured neurons [23]. Collectively, these findings suggest that *paclitaxel* may be neurotherapeutic for focal neuronal injuries.

Previously we applied *paclitaxel* topically to the injury site in the brains of mice following a single, unilateral controlled cortical impact with craniotomy and found significant improvement in neurological function using "Catwalk" gait analysis [13]. This improvement corresponded to imaging biomarkers indicating reduced injury volume and edema, as well as myelin preservation with *paclitaxel.* These encouraging results support the idea that acute TBI might benefit from treatment with *paclitaxel*. The aim of this present study was to determine if *paclitaxel c*ould mitigate repeated mild brain injury.

One significant challenge for the application of *paclitaxel* to brain injury is its low blood-brain barrier penetration when delivered systemically [24]. To address this issue and, more importantly, to limit systemic distribution and target specifically to the brain, we delivered *paclitaxel* via the intranasal (IN) route. IN administration for CNS drug delivery was proposed by Frey and colleagues more than two decades ago [25]. Our group has used IN administration as an alternative delivery approach in studies that included insulin for treatment of Alzheimer's disease (AD) [26], imaging agents such as manganese [27], and administration of hormones such as progesterone and testosterone [28, 29]. Brain uptake after IN administration is not dependent on classic distribution mechanisms. Research suggests that transport via IN administration is within the perivascular spaces of the brain, resulting in rapid distribution, which reaches steady state quickly [30, 31]. In this study, we assessed the pharmacokinetics of IN administration in mice using [3H]-*paclitaxel.*

To test the hypothesis that *paclitaxel* will prevent diffuse axonal injury from TBI, we utilized a mouse model with repeat mild TBI, which produced axonal injury in brain white matter and synaptic loss in the CA1 region of the hippocampus. We administered IN *paclitaxel* only one time after the first impact of a rmTBI protocol consisting of five impacts over five days without craniotomy. We tested the therapeutic efficacy of *paclitaxel* to mitigate injury-induced deficits with outcomes that included cognitive function on the radial water tread maze, imaging of structure and function using magnetic resonance imaging (MRI) and ^{18}F-flurodeoxyglucose positron emission tomography (FDG-PET) and immunohistochemistry.

MATERIALS AND METHODS

Subjects

All procedures and animal handling were conducted in accordance with the animal care guidelines issued by the National Institutes of Health and by the University of Washington Animal Care and Use Committee. For the rmTBI treatment and sham groups, adult male C57BL/6J mice were purchased from the Jackson Laboratory, Bar Harbor, ME, USA. Animals were kept on a 12-h light/12-h dark cycle with *ad libitum* access to food and water, and were randomly assigned to four groups: sham surgery with IN saline ($n = 5$), or IN *paclitaxel* ($n = 3$), repeat impact with IN saline ($n = 9$), or IN *paclitaxel* ($n = 7$). Statistical analyses of imaging and behavioral outcomes revealed no statistical differences or trends of

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differences between sham controls receiving saline or *paclitaxel* ($F[1, 6] = 0.010$, $p <= 0.922$, n.s.), so these groups were combined as a single control group (sham). For paclitaxel pharmacokinetic assessment, male CD-1 mice were purchased from Charles River, Wilmington, MA, USA and were kept on a 12-h light/12-h dark cycle with ad libitum access to food and water.

Intranasal paclitaxel pharmacokinetics

With a protocol similar to a previously published study [28], male CD-1 mice (8 weeks, $n = 3$–5 per time point) were anesthetized with urethane and administered 1 μl with 1,000,000 DPM tritium labeled paclitaxel (American Radiolabeled Chemicals, St. Louis, MO, USA) with unlabeled paclitaxel in PBS to equal 0.6 mg/kg (dose used in *in vivo* studies below). The radiolabeled paclitaxel was administered IN using a micropipette tip inserted 4 mm into the left nostril of each mouse. A subset of animals ($n = 3$, 20 min only) received intravenous (IV) administration (left jugular vein) of radiolabeled paclitaxel for brain/serum ratio quantification. At 10, 20, 40, or 60 min after administration, the carotid artery was exposed and blood taken. The mouse was then decapitated and the brain removed and dissected [32] on ice into 11 regions: olfactory bulb, occipital cortex, striatum, frontal cortex, hypothalamus, thalamus, hippocampus, parietal cortex, cerebellum, midbrain, and pons-medulla. Each region was weighed. Collected whole blood was allowed to clot at room temperature, centrifuged at 5400 g for 10 min at 4°C, and 50 μl of the resulting serum removed. Radioactivity in each of brain region and the matching serum sample was measured in a gamma counter (Perkin Elmer; Waltham, MA, USA) for 30 min. Percent injected dose in 1 ml of serum (%Inj/ml) was calculated by: %Inj/ml = [100*(CPM/ml)]/Inj (Inj = counts per minute (CPM) administered, CPM/ml = radioactivity in 1 ml of serum). Percent injected dose taken up per gram of brain region (%Inj/g) was calculated at each time point by: %Inj/g = [100*(CPM/R)](/Inj(W)) (W = weight of brain region in grams, CPM/R = radioactivity in brain region).

Repeat impact TBI and drug administration

Mice were anesthetized and maintained with isoflurane (1.5–2.5% in flowing O_2 at 2 L/min) during surgery and core temperature was maintained at 37°C via paraffin heating pad. The mice were secured in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA), and the scalp depilated with topical cream diluted with saline (Nair; Church & Dwight Co. Inc., Ewing, NJ, USA). An impactor device (Impact One; Leica Biosystems, Buffalo Grove, IL, USA) was positioned directly above the midline of the skull between bregma and lambda and a mark was made with a permanent marker to ensure the same area was impacted daily. Mice received a mild closed-skull TBI or sham impact (anesthesia and treatments, no impact) once daily for five consecutive days (5 mm impact tip, 1 mm depth, 5 m/s velocity, 200 ms dwell time). Low-dose IN paclitaxel (0.0175 μmol of paclitaxel in 5 μl volume) or 5 μl saline (vehicle) was administered (2.5 μl in each naris) immediately after the initial impact, but not on subsequent days. Immediately following surgery, mice were removed from anesthesia, placed in a warmed recovery box, and given a subcutaneous injection of buprenorphine (0.5 mg/kg).

Outcome study design

The study protocol was designed to accommodate multiple endpoints, thus necessitating accommodations for logistical work flow and timing constraints of the experimenters. The injury paradigm of 5 impacts in 5 days was performed Monday-Friday. MRI imaging was conducted early (4 days) after the injury to assess if the injury protocol resulted in observable lesion on structural imaging. Maze testing requires a full week (M-F) for acquisition plus short-term memory test as well as one more day of Friday testing the following week for the long-term memory. Since imaging requires anesthesia we did not plan any imaging experiments during the maze testing time frame, nor within 3 days prior to maze testing. Depending on the cohort size, FDG-PET requires 1–2 days and "hot" animals needed to be housed overnight in shielded housing then moved back to regular housing, therefore imaging could not be performed on a Friday. Euthanasia was performed within 1 week of the FDG-PET imaging.

Radial water tread maze (RWT)

Water maze testing began at 10 days after final impact. The full procedure and application to a mouse model of TBI has been published previously by our group [33]. This test requires no swimming, capitalizes on the natural tendency of mice to avoid open areas in favor of hugging the edges of an apparatus (thigmotaxis), and has been used successfully to

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distinguish TBI from sham mice previously [33, 34]. The RWT maze consists of a 32-inch galvanized steel tub with 9 holes placed at equal intervals around the device, roughly $1\frac{1}{2}$ inch above the apparatus floor. Of these, 8 terminate after roughly 1 inch (decoy exits), and 1 leads to a safety box hidden behind a 90-degree angle bend to prevent visual confirmation of escape route. The safety box consists of a dark plastic container (30"L × 15"W × 15"H) heated externally via electronic heating pad. Five large unique visual cues line the sides of the apparatus for spatial orientation.

Testing was conducted in a brightly lit room. Before each mouse was tested, the maze was sanitized with 70% ethanol and filled with 1 inch of 12–14°C water. The temperature was monitored during filling to ensure it was within the desired range. After maze preparation, each mouse was placed in the center of the maze and given 180 s to find the escape hole. If the mouse was unable to find the escape within the time period, the mouse was gently guided by hand to the exit and the trial was recorded as 180 s. If a mouse attempted to enter a decoy hole and did not voluntarily re-enter the maze after 5 s, they were returned to the center of the maze by hand. Once inside the safety box, the mouse was presented with a high-reward food treat and allowed to remain in the box for 60 s. The safety box and maze were sprayed with 70% ethanol between trials to prevent olfactory cues. Each mouse was tested for three sequential trials, with an inter-trial interval of one minute. Water was then changed between mice, so each mouse was tested in fresh water. Mice were given 3 trials a day for 4 days (acquisition period), followed by a short-term memory test consisting of 3 trials on day five. Mice were then unexposed to the maze until day 12 (day 22 after final impact), at which they underwent 3 final trials (long-term memory test).

Imaging and image analysis

Magnetic resonance imaging (MRI), including diffusion tensor imaging (DTI), has been proposed as a biomarker to assess rmTBI clinically. We acquired MR imaging including T1-weighted, T2-mapping, and DTI at 4 days after the final impact. Mice were anesthetized with isoflurane and scanned on an ultra-high resolution 14T MRI (Avance III, Bruker BioSpin Corp, Billerica, MA). T1-weighted imaging to assess gross brain structure and obvious lesions, was acquired using a 3D Modified Driven Equilibrium Fourier Transform (MDEFT) sequence: voxel size: $0.14 \times 0.14 \times 0.25$ mm^3, 64 slices, flip angle/repetition time/echo time (FA/TR/TE): 121/5000 ms/1.9 ms. The presence of edema under the impact region was assessed by T2 quantitative mapping (T2) with a voxel size of $0.12 \times 0.12 \times 1.0$ mm^3, 15 slices, TR 1/4 2000 ms,16 echoes, spacing: 6.7 ms, TE Effective 1:6.7 ms, TE Effective 2:13.4 ms. DTI was acquired as a 4 shot echo planar image (EPI), TR/TE = 500/16.482 ms, 30 diffusion directions, Max bval = 6477.26849710899 s/mm^2, multislice 3d, FOV $22.4 \times 22.4 \times 22.4$ mm, Matrix size = $112 \times 80 \times 82$, time was 1 h 35 min and 40 s. MR image sets including T1-weighted and T2 maps were manually inspected (DC) for the presence of anatomical abnormalities, edema and/or lesions resulting from the impact procedure. Manual inspection consisted of visual review of synchronized image sets through approximately 20 axial slices covering the area under impact region. During this manual inspection, it was noted that there were small lesions under the impact area in all of the saline-treated rmTBI subjects that were not present in the paclitaxel-treated mice.

DTI processing included the application of a custom algorithm running within the Neuroimaging Informatics Tools and Resources Clearinghouse (NITRC) DTIPrep software package (http://www.nitrc.org/projects/dtiprep/), which allows elimination of image frames contaminated by motion-derived artifacts, followed by eddy current and B0 corrections and confers several advantages. The resultant diffusivity maps had little spatial distortion, artifacts of subject motion or inhomogeneities of magnetic (B0) or RF fields; and showed a high degree of white/gray matter contrast. Fractional anisotropy (FA) maps are constructed from DTI by FSL software (Analysis Group, FMRIB, Oxford, UK) and compared between groups using FSL tract-based spatial statistics (TBSS). Manual regions of interest (ROI) analysis as well as left and right regions were hand-drawn by a blinded investigator over 3 consecutive slices as indicated and averaged.

Cerebral glucose activity was assessed at 31 days after last impact using ^{18}F-flurodeoxyglucose positron emission tomography (FDG-PET) (Inveon micro-PET; Siemens, Munich, Germany). PET imaging was performed under isoflurane anesthesia with 30 min uptake after 250 μCi intraperitoneal injection of FDG. High-resolution images were acquired over the whole brain for 30 min and processed using 3D OSEM/MAP reconstruction with a spatial resolution of approximately 1 mm. Given the limited spatial resolution of FDG-PET of the mouse brain, whole brain

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analysis (rather than region-based) was performed on standardized uptake values (SUV) calculated images using ImageJ software.

Immunostaining

Mice were euthanized using intraperitoneal ketamine/xylazine until respiratory arrest and then transcardially perfused with PBS followed by 4% formaldehyde. Brains were removed and postfixed overnight in 4% paraformaldehyde in PBS at 4°C, followed by 24 h in 20% sucrose in PBS, and then 24 h in 30% sucrose. Tissue was then paraffin embedded, cut coronally to 5 μm sections, and slide mounted. Sections were deparaffinized in xylenes (2 times at 10 min each), followed by 100% ethanol (2 times for 5 min each), 95% ethanol (once for 5 min), 70% ethanol (once for 5 min) and finally distilled H_2O (2 times for 5 min each). Tissues were permeabilized (1 h, room temperature, 0.2% Triton X-100 and 0.01% sodium azide in PBS) followed by three 5 min washes in PBS. Antigen retrieval was performed by heating at 80°C for 30 min in 50 mM sodium citrate (pH 8.5–9.0). Sections were then cooled to room temperature, washed in PBS (3 times for 5 min each) and blocked in PBS with 10% normal goat serum (NGS) for 2 h at room temperature with gentle agitation on an orbital shaker (10 rpm). After circling individual tissue sections using a hydrophobic barrier pen, sections were covered with primary antibody solution (PBS, 5% NGS; 200 μl per section) and incubated at 4°C overnight at 1:1000 with rabbit mAb PSD-95 (Cell Signaling, Danvers, MA). Following a brief rinse and 3 PBS washes (each 10 min in duration) secondary antibodies were applied. Anti-rabbit/AF555 secondary antibody (Molecular Probes, Grand Island, NY) were applied to tissue sections for 3 h at room temperature, followed by three 10 min washes in PBS. Slides were lastly rinsed into water to remove buffer salts, mounted and cover slipped. Slides were mounted in Prolong gold anti-fade solution with DAPI (Life Technologies, Grand Island, NY, USA). Laser scanning confocal imaging was conducted using a Leica TCS SP2 confocal/multiphoton hybrid microscope with tunable emission gating and using sequential, between stack, single photon excitation at 488, 543, and 633. Z-plane images acquired the full volume thickness of each slice imaged (i.e., 50 μm for floating sections and 5 μm for paraffin-embedded sections) using system optimized stepping. Within an experiment, all directly compared sections/slides were identically and simultaneously prepared.

Gallyas silver stain was used to detect axonal retraction bulbs. Gallyas method was performed using a commercial kit (FD Neurosilver Kit, FD Neurotechnologies, Inc. Columbia, MD, USA) and is characterized by its stability, reproducibility, and ability to visualize abundant deposits. This commonly used method allows reproducible examination for pathological deposits such as neurofibrillary tangles or glial cytoplasmic inclusions from TBI. Mouse brains were coronally sectioned between [Bregma: –3.4 and –2]. Samples were cut serially with each successive tissue slice being placed onto the next microscope slide. Anatomically-matched tissue sections from 2–4 members of each group were represented on each slide to ensure that each group was well-matched in terms of anatomy and received equal downstream processing treatment. Random planes relative to Bregma were examined and all subjects were matched to the selected plane. Slides were stained together as a full group using the FD Neurosilver Kit according to the manufacturer's instructions. Slides from all study conditions were simultaneously processed, darkened for ~3 min at room temperature and stopped in parallel. After development, slides were coverslipped. Using brightfield microscopy, the entire surface of each tissue slice was briefly examined for argyrophilic degeneration. Due to a lack of notable staining elsewhere, quantitation of retraction bulbs was determined in the dorsal hippocampal commissure / corpus collossum / alveus inferior to the site of TBI and proximal (superior) to where the PSD-95 micrographs in Fig. 6 were taken. A total of two slices per animal were examined from two separate silver-stained preparations.

PSD-95 expression was indirectly quantified using the histogram function of Adobe Photoshop to determine the PSD-95-positive immunofluorescence (i.e., green channel only; mean ± standard deviation) within matched ROIs. The ROIs used for statistical analyses are indicated by a white box for each subject in Fig. 6.

Statistical analysis

Where appropriate, one-factor between-group ANOVAs or Student *T* tests were used to compare paclitaxel-rmTBI to Sham or saline-rmTBI groups. All reported *p* values were based upon two-tailed critical values ($p \leq 0.05$). Radial water tread maze data were analyzed by two-way analysis of variance (ANOVA) with training or test days as a repeated measures within-subjects factor and

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treatment (drug/no drug) as a between-subjects factor. Following ANOVA the a priori hypothesis that injured/untreated groups would be statistically significantly different from both vehicle/sham-treated and injured/paclitaxel-treated groups was performed using a Helmert individual comparisons test [35, 36]. PK data for time versus %Inj/g brain tissue were analyzed by two-way ANOVA. Statistical analyses were carried out using SPSS software (IBM, Armonk, NY, USA).

RESULTS

Pharmacokinetics of [3H] Paclitaxel in the brain after intranasal administration

We first assessed the pharmacokinetics of [3H]-paclitaxel after IN administration at a dose of 0.0175 μmol (0.6 mg/kg for 25 g mice). Inspection of the time activity curves in several brain regions (Fig. 1A) indicated that overall, [3H]-paclitaxel concentration in the brain remained fairly stable over the interval of 10–60 min. However, there were different levels of [3H]-paclitaxel uptake among the 11 brain regions analyzed. This was confirmed by a two-factor ANOVA where the time factor was not statistically significant ($F[3, 30] = 2.583$, n.s.) and the brain region factor was statistically significant ($F[1, 30] = 59.36$, $p < 0.00001$). Brain region differences in [3H]-paclitaxel uptake at 20 min post injection are further illustrated in Fig. 1B. As expected olfactory bulb uptake was higher than other brain regions [28]. Interestingly, uptake was also comparatively high in the striatum and hypothalamus, which is unique for paclitaxel. We also assessed [3H]-paclitaxel distribution in peripheral organs. Only 0.3% Inj/ml was found in serum and 2% injected dose/g-tissue in the kidney (2.06 ± 1.8, 0.66 ± 0.7, 0.30 ± 0.2, and 0.02 ± 0.0, mn ± sd of %Inj/ml of [3H]-paclitaxel, in kidney, liver, serum and urine, respectively). Taken together these results demonstrate that paclitaxel delivered IN is taken by multiple brain regions at levels that remain stable for at least 60 min.

Paclitaxel prevents white matter injury from repeat mild impacts

Diffusion tensor images were acquired in all subjects, however due to a coil malfunction, we were unable to acquire usable artifact-free FA maps in 5 of 7 paclitaxel-treated subjects. In the remaining subjects of the saline-treated rmTBI and sham

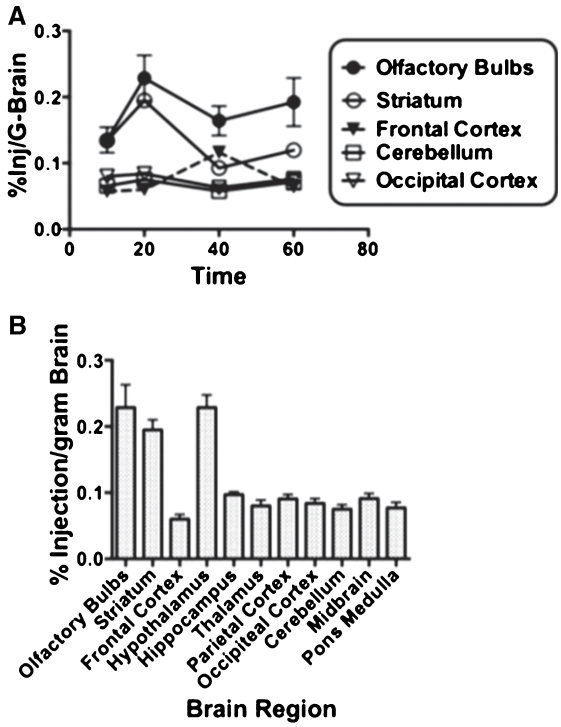

Fig. 1. Brain distribution of *paclitaxel* after intranasal administration. A) Time-activity curves compare the % Inj/g-brain in five brain regions. Although there are regional differences, tissue concentrations are stable over the 1 hour of sampled time. B) All brain region concentrations of 3H-*paclitaxel* at the 20 min time point from after IN administration show a classic pattern of high olfactory bulb uptake; however, the high striatum and hypothalamus uptake is unique. Results are expressed as the percent of administered dose taken up by a gram of brain region. Error bars indicate standard deviation across the group ($n = 5$).

groups, the FA maps were assessed on a voxel-wise basis using tract-based spatial statistics (TBSS). The rmTBI group had extensive regions of decreased FA throughout the white matter tracts of the brain, in particular the external capsule, fimbria of the hippocampus, the ventral hippocampal commissure, and the corpus callosum (Fig. 2A). This voxel-based analysis was confirmed by manual region of interest analysis. Manual ROI indicated that rmTBI resulted in decreased FA in the external capsule (green arrows) at 4 days after final impact (17% (0.21 ± 0.01 versus 0.25 ± 0.01, unpaired t-test $p \leq 0.01$) as compared to sham mice (Fig. 2B). These imaging results were used to define the location of the post-mortem immunohistochemistry. At 45 days after rmTBI, a corresponding post-mortem examination of the external capsule using Gallyas silver stain confirmed *in vivo* imaging findings of diffuse axonal injury. More importantly, we were able to assess the white

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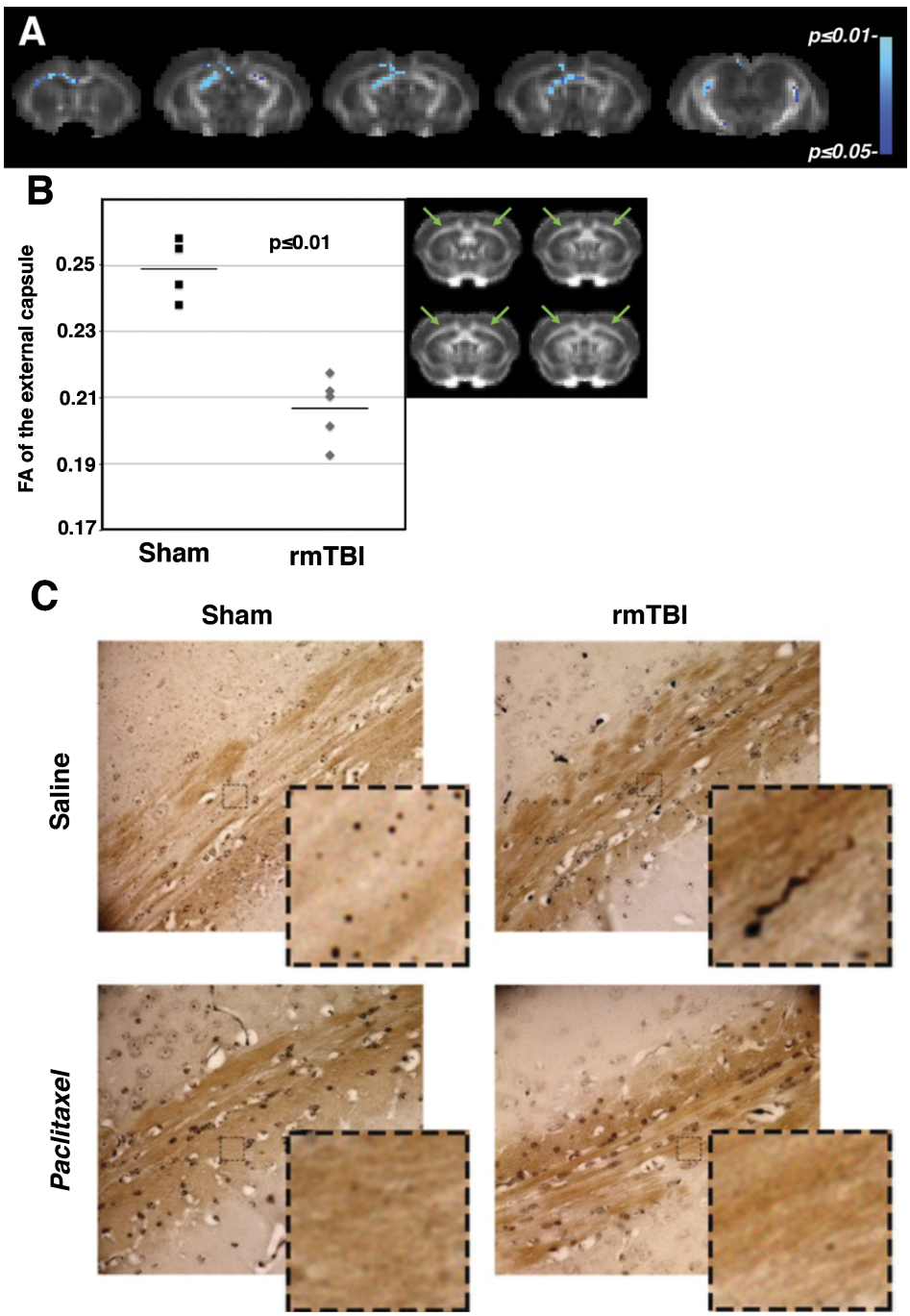

Fig. 2. White matter injury in the external capsule indicated on DTI and confirmed by silver stain is ameliorated by *paclitaxel.* A) TBSS statistical maps (blue) are superimposed on coronal slices from an FA template brain aligned to the mouse brain atlas for anatomical reference [65]. TBSS indicated that repeat mild brain injury resulted in decreased fractional anisotropy (FA) in diffuse white matter regions throughout the brain, including the external capsule at 4 days after final impact. Color scale bar to the right indicates *p*-values. B) Scatterplot of the manual ROI analysis of the external capsule (location indicated by green arrows), shows that FA was decreased significantly by 17% (0.21 ± 0.01 versus 0.25 ± 0.01, mn $\pm$ sd, $p \leq 0.01$) compared to sham mice. Horizontal line indicates mean. C) Representative samples indicate axonal injury in cortical white matter of the external capsule/alveus by rmTBI as demonstrated by Gallyas silver staining. Mice were perfused for immunohistochemistry at 45 days after rmTBI. Argyrophilic axonal degeneration was found in 5/6 saline-treated but not *paclitaxel*-treated mice ($n = 0/5$) or sham-*paclitaxel*-treated controls ($n = 0/3$). A single, unilateral dystrophic axonal bulb was observed in 1/5 sham mice. Insets represent magnifications of boxed areas.

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matter histologically in the paclitaxel-treated subjects. Argyrophilic axonal degeneration is seen in cortical white matter of the alveus/external capsule in 5 of 6 saline-treated rmTBI mice with varicosities (bilateral, and at least 1 argyrophilic bulb per side observed). One mouse had very prominent degeneration (15 argyrophilic bulbs in a single field in the dorsal hippocampal commissure / corpus collossum). 1 of 5 sham mice had a single unilateral, translucent bulb which is likely artifactual. However, to favor a conservative assessment it was counted as a bona fide argyrophilic retraction bulb. 0 of 3 paclitaxel-treated sham mice had evidence of argyrophilic degeneration in any region. 0 of 5 paclitaxel-treated rmTBI mice had at least 1 argyrophilic retraction bulb in the white matter regions within, or contiguous with, the alveus. However, in 2 of 5 paclitaxel-treated rmTBI treated mice argyrophilic degeneration was observed in the area consisting of the external capsule, brachium of the superior colliculus, and the optic tract (Fig. 2C).

Paclitaxel administration prevents apparent microbleed

In the same imaging session at 4 days after final impact, T1-weighted (T1-w) images and T2 maps were acquired to reveal any gross anatomical injury and/or edema. Manual inspection consisted of visual review of synchronized image sets through approximately 20 axial slices covering the area under impact region. During this manual inspection, it was noted that there were small lesions under the impact area in all of the saline-treated rmTBI subjects that were not present in any slices of either MR acquisition of the paclitaxel-treated mice. A likely tissue strain microhemorrhage was observed in the cortical region directly below the impact area (Fig. 3A). This lesion was found in 100% (9/9) of rmTBI mice receiving saline-only treatments and no paclitaxel-treated mice (0%, 0/6, one PTX subject MRI had a slight artifact in the region of the impact therefore it was not possible

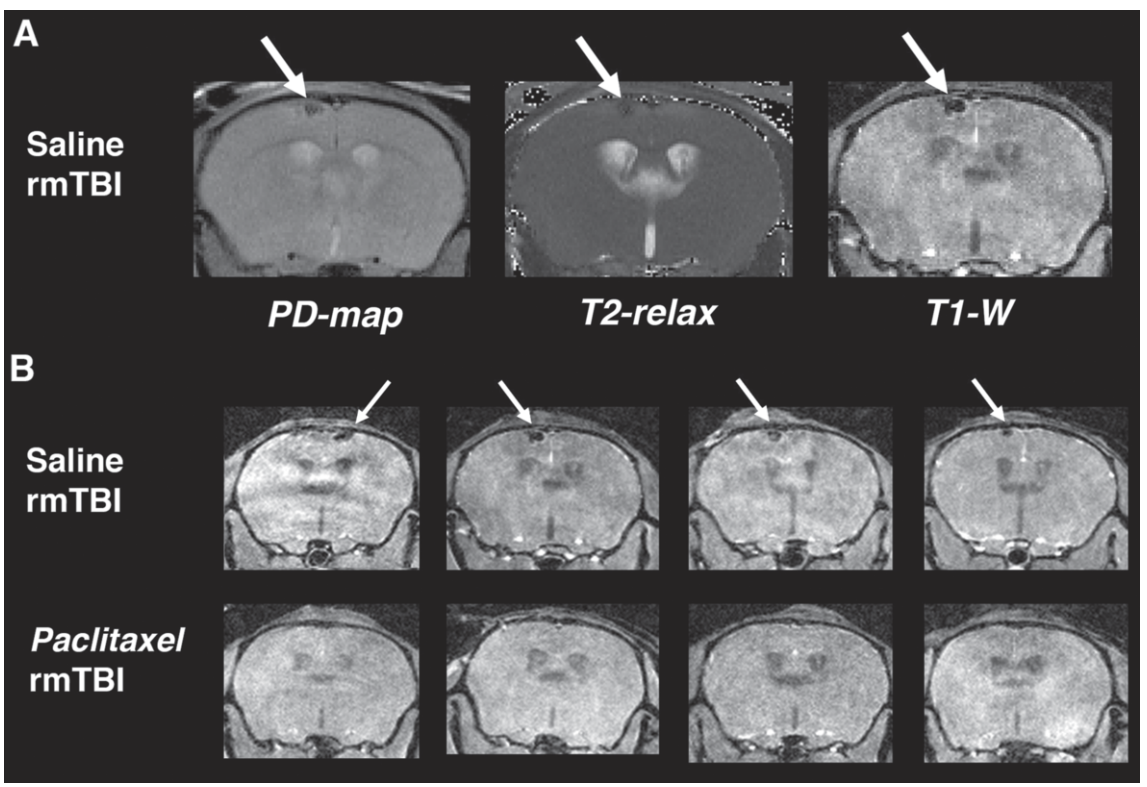

Fig. 3. *Paclitaxel* prevents brain lesions associated with rmTBI. MR imaging was acquired at 4 days post-rmTBI to assess injury and response to *paclitaxel* treatment *in vivo*. A) Left to right: Proton Density mapping (PD-map), T2-relaxation quantitative image (T2-relax) and T1-weighted image (T1-W) from the same saline-treated mouse (coronal view). Green arrows indicate a dark spot in the cortex just below the impacted region. Spot is dark in all 3 images indicating a likely tissue strain microbleed. B) T1-W imaging of saline- (top row) and *paclitaxel*-treated (bottom row) from 4 subjects of each group. 100% (8/8) saline-treated subjects had microbleed lesion at 4 days post injury and 0% (0/6) of the *paclitaxel*-treated showed evidence of the lesion.

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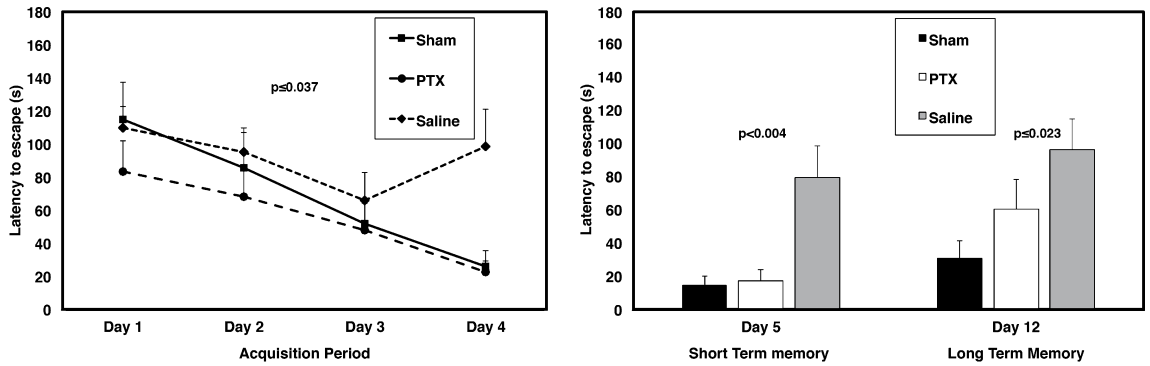

Fig. 4. *Paclitaxel* preserves short- and long-term memory. The radial water tread (RWT) maze tests spatial learning and memory. Maze test was administered from Day 10 to 22 post-injury. A) Graph indicates learning/acquisition phase over Days 1–4, where saline-treated rmTBI mice did not learn the maze as compared to sham and PTX-treated rmTBI. *Paclitaxel* (PTX) rmTBI mice learned the maze similar to Sham. B) *Paclitaxel* subjects were significantly improved from saline in both short-term memory (Day 5) as well as long term memory (tested on Day 12).

to definitively conclude no lesion) showed evidence of the injury (Fig. 3B). This defect resolved by time of euthanasia/immunohistology, so pathologic confirmation of the microbleed was not performed.

Spatial memory impairment from repeat mild impacts is prevented by paclitaxel

Mice were tested at ten days after final impact for spatial memory impairment using a radial water tread (RWT) maze, which was developed specifically to test spatial memory in mice [33, 37]. A two-way ANOVA (Days 1–12, between and within subjects factors, respectively) indicated that there was not a statistically significant difference between sham-saline versus sham-paclitaxel mice for either testing day, or the acquisition phase ($F[1, 6] = 0.010$, $p \leq 0.922$, n.s). Therefore, in further analyses these two sham groups were pooled. As shown in Fig. 4A, a two-way ANOVA (Days1–4) comparing Shams, rmTBI paclitaxel-treated, and rmTBI saline-treated animals revealed an overall statistically significant difference between groups ($F[2, 20] = 3.903$, $p \leq 0.037$), indicating that paclitaxel treatment significantly improved learning of the water tread maze by mice with repetitive mTBI. In keeping with this, a Helmert a priori individual comparisons test [36] further confirmed that vehicle-treated rmTBI mice performed significantly worse than the paclitaxel-treated rmTBI and sham groups ($p \leq 0.014$) and that the paclitaxel-treated rmTBI group performance was not significantly different from non-injured sham controls ($p \leq 0.324$, n.s.). In addition, Fig. 4B shows that RWT performance on post-exposure (sham or mTBI) mice at Days 5 and 12 also showed that paclitaxel significantly reduced escape latencies, indicative of improved performance ($F[2, 20] = 7.254$, $p \leq 0.004$ and $F[2, 20] = 4.595$, $p \leq 0.023$; Day 5 and Day 12, respectively). Finally, an overall two-way ANOVA (treatment X Days; between and within subjects factors, respectively) encompassing Days 1–12 further confirmed that paclitaxel significantly reduced injury-induced deficits on the RTW maze (Day $5{:}24.2 \pm 15.7$ versus 69.78 ± 70.5 and Day $12{:}26.58 \pm 21.4$ versus 68.33 ± 50.16, mn $\pm$ sd, paclitaxel-rmTBI versus saline-rmTBI, respectively, $F[1, 20] = 12.423$, $p \leq 0.001$).

Paclitaxel preserves cerebral glucose metabolic activity and prevents synaptic loss from rmTBI

An alternative imaging modality used to investigate TBI in humans is FDG-PET, which assesses the metabolic activity associated with glucose uptake in the brain. Our group has previously reported regional metabolic deficits in Veterans years after mild repetitive blast TBIs using FDG-PET [38–40]. In our current study, mice underwent FDG-PET imaging at 31 days post rmTBI. Brain-injured mice showed significantly decreased whole brain FDG uptake, which was not observed in brain-injured mice receiving IN paclitaxel (Fig. 5). Whole brain standardized uptake values (SUV, pseudo-quantitative assessment of FDG-PET, dimensionless) were 120.5 ± 30.1, 90.3 ± 18.7, and 129.2 ± 23.0, mn $\pm$ sd for sham, saline, and paclitaxel (PTX), respectively, which is 43% increased in mice treated with paclitaxel as compared to saline (One-way ANOVA, $p = 0.0154$,

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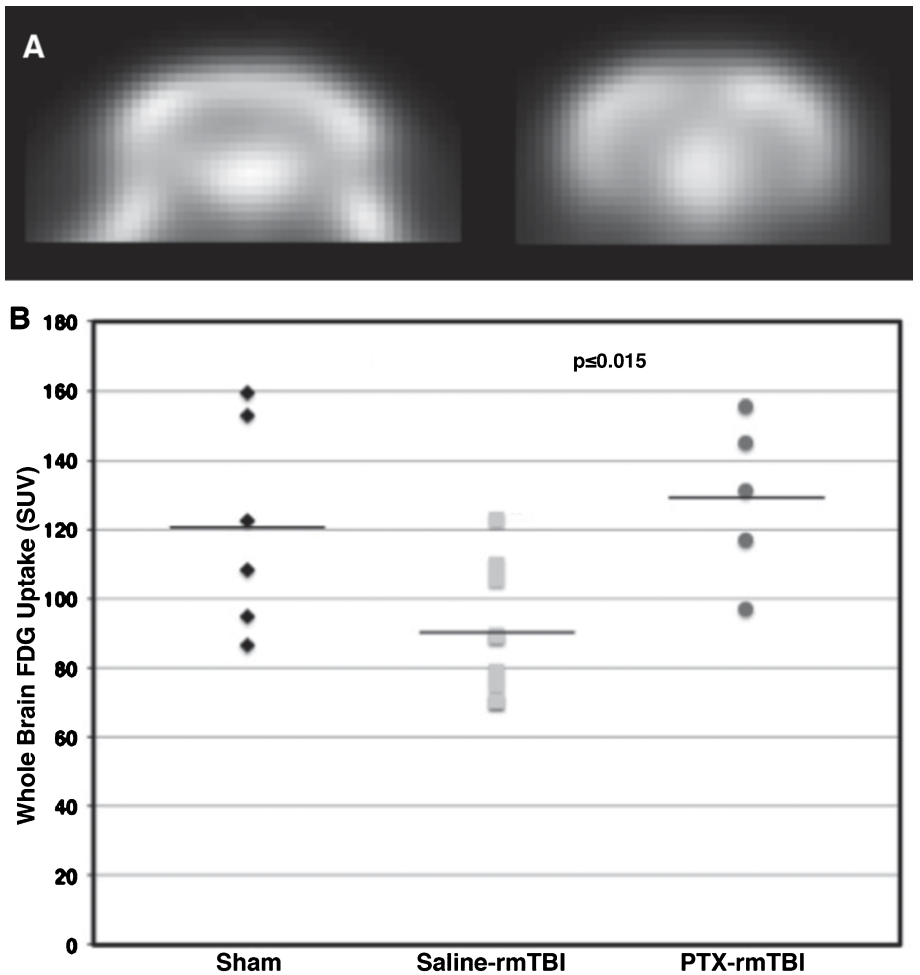

Fig. 5. *Paclitaxel* preserves FDG brain uptake. A) Representative mouse brain FDG-PET image (coronal view) from a single sham subject to illustrate image quality. B) Scatterplot of standardized uptake values (SUV) from saline-treated rmTBI mice shows significantly decreased brain FDG uptake at 31 days post injury, which was preserved in mice administered IN *paclitaxel*. Horizontal lines indicate mean.

$F(2,17) = 5.386$). Glucose consumption in the brain is associated with synaptic activity [41]. Since synapses are also supported by microtubule structural components, we further reasoned that paclitaxel could maintain post-synaptic regions in the hippocampus. Therefore, we examined synaptic density in the CA1 hippocampi after rmTBI (45 days) by immunostaining postsynaptic density-95 (PSD-95) protein, which is a well-established synaptic marker [42]. Figure 6 shows that overall PSD-95 immunostaining intensity was reduced in saline-treated mice exposed to rmTBI, while paclitaxel administration blocked the TBI-induced loss in PSD-95 immunostaining intensity. We also quantified PSD-95 expression in the demarked regions shown in Fig. 6 (mean PSD-95 immunostaining intensity (relative units) = 23.5 ± 3.7 s.e.m., 46.5 ± 7.7 s.e.m., and 47.6 ± 6.9 s.e.m.; corresponding to the saline/rmTBI, saline/Sham, and PTX/rmTBI, respectively; overall $F[2, 6] = 4.62$, $p \leq 0.06$; $N = 3$ for each group). Confirming the specific hypothesis that paclitaxel would restore injury-induced PSD-95 expression to saline-treated sham control levels, a Helmert a priori planned comparison test revealed that PSD-95 levels were significantly lower ($p \leq 0.023$) in the saline/rmTBI group compared to $\pm$ saline/Shams and PTX/rmTBI animals; and that there was no significant difference ($p > 0.90$, n.s.) in PSD-95 levels among the PTX-treated rmTBI animals compared to the saline/Sham group. This conclusion was further confirmed with separate hypothesis-independent *post-hoc* paired comparisons (saline/

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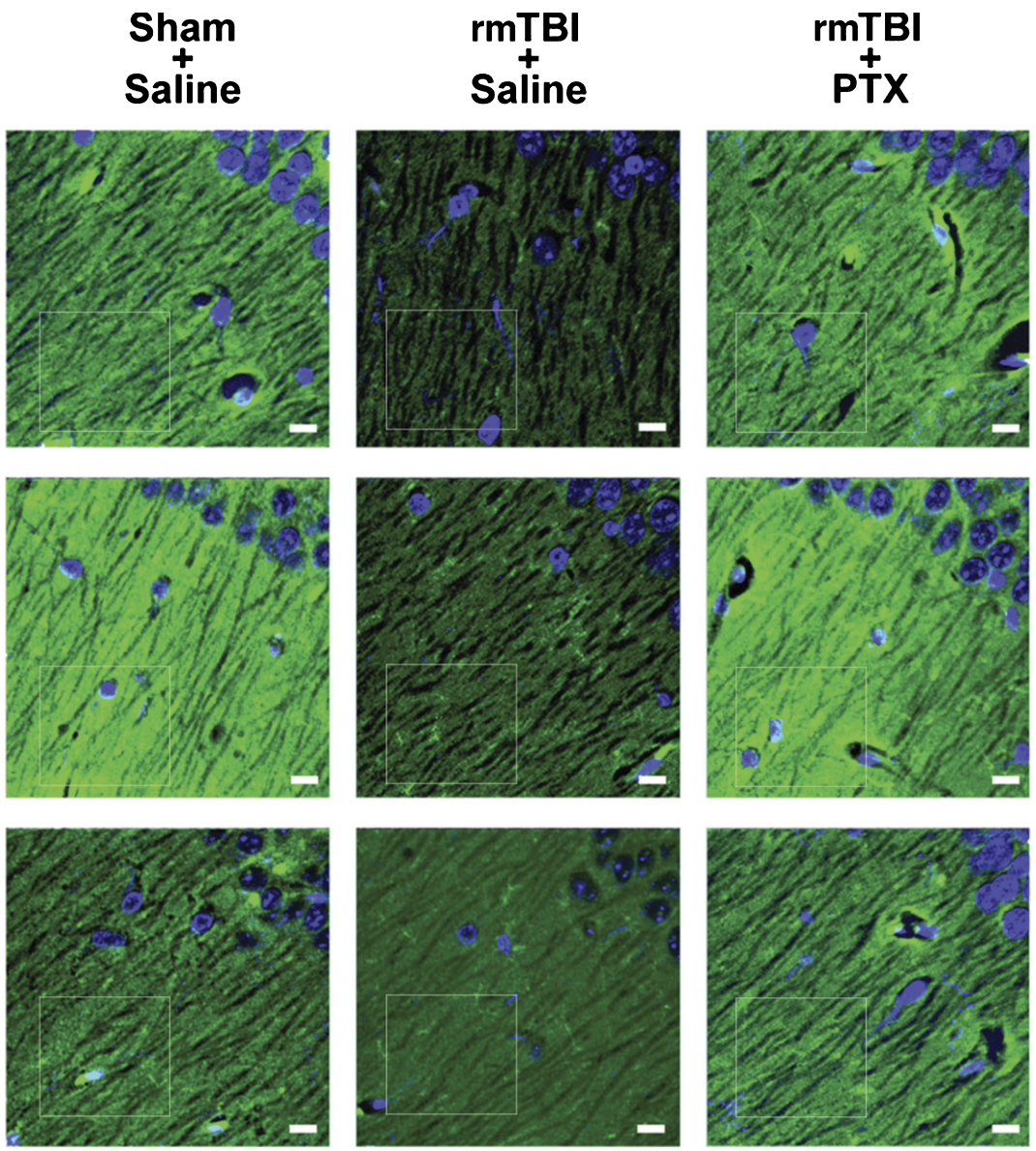

Fig. 6. *Paclitaxel* treatment blocks rmTBI-induced loss of postsynaptic density-95 immunoreactivity in hippocampus. Representative PSD-95 (green) expression in CA1 stratum oriens hippocampi. Tissue was counterstained with DAPI (blue) to delineate prominent nuclei of the stratum pyramidale. In saline-treated mice exposed to rmTBI, hippocampal PSD-95 immunofluorescence was reduced as compared to sham or rmTBI *paclitaxel* mice. CA1 (just superior to the dentate lateral horn) is near the location of the Gallyas silver stain from Fig. 2C. Confocal micrographs are from 3 mice in each group. White boxes indicate region of fluorescence analysis. Bars = 10 μm.

rmTBI versus saline/Sham, $p \leq 0.04$; saline/rmTBI versus PTX/rmTBI, $p \leq 0.04$; PTX/rmTBI versus saline/Sham, $p \leq 0.91$, n.s.).

These data support that paclitaxel reduces overall PSD-95 loss induced by rmTBI and also lends support to our FDG-PET findings (Fig. 5), in that synapse activity is associated with glucose consumption [41]. Taken collectively, the findings in this report argue that microtubule stabilization can provided protection against mild repetitive TBI that is reflected at the anatomical structural and functional level, as well as by improved cognitive performance.

DISCUSSION

In this report, we demonstrate that IN *paclitaxel* treatment delivered after the first impact of a multi-impact model of repeat mild brain injury prevented

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axonal injury in mice. This finding was supported by improved spatial memory, preserved post-synaptic density in the CA1 hippocampus, and *in vivo* imaging that indicated structural and metabolic outcomes similar to sham subjects. To our knowledge, this is the first evidence that this therapeutic strategy can mitigate structural and functional impairments from repeated mild TBI. Although the use of microtubule-stabilizers has been proposed for neurotherapeutic uses, including AD disease and other tauopathies, this approach has been limited to models of focal axonal injury. Previous studies have focused specifically on the potential of microtubule-stabilizers to maintain axonal function and structure [19–23]. Synaptic loss is a well-described feature of many animal models of brain injury [43], as well as in clinical TBI [21, 44–47]. A previous study by Erez et al found *paclitaxel* rescued tau-induced decreases in synaptic transmission *in vitro* [48]. However, the focus was on presynaptic vesicle release and alterations in excitatory postsynaptic potential amplitude and did not look specifically at post-synaptic density. Preservation of post-synaptic density may simply be explained by prevention of deafferentation from axonal loss or may be a direct effect of *paclitaxel*-microtubule stabilization on dendrites, which could not be distinguished in the current study.

We found significant improvement in spatial memory and increased FDG brain uptake measured by PET imaging in mice treated with a single dose of *paclitaxel*. These results are consistent with findings that FDG uptake in the brain is generally associated with brain activity [49, 50]. Numerous clinical studies, including those from our group examining Veterans with repetitive blast-related rmTBI, and others of mild impact TBI have indicated decreased FDG uptake on PET imaging [38–40, 51, 52]. Therefore, the finding that our mouse model of rmTBI model resulted in significantly decreased brain FDG uptake, at one month after the injury, is translationally consistent with human studies. Although infection and inflammation are known to increase FDG uptake on PET images, [53] research suggests that the neuroinflammation specifically may contribute less to overall brain FDG-PET uptake than brain activity [54]. The spatial memory preservation seen in mice treated with *paclitaxel* is a novel finding. Although it has been proposed that microtubules may be involved in learning and memory processes, the mechanism of action is not yet fully understood [55]. In 2006, insulin, delivered via the IN route, was shown to improve memory in patients with memory impairment [56]. A subsequent study by the same group indicated increased brain uptake of FDG in amnestic cognitive impairment after IN insulin [26]. In a more recent study, IN insulin improved memory and decreased neuroinflammation in a model of traumatic brain injury [57]. Although it is beyond the scope to this study to determine the exact mechanism by which IN *paclitaxel* preserved brain FDG uptake as well as spatial memory, we may also speculate that these effects could be attributed to modulation of brain energetics. Further studies on effect of *paclitaxel* to preserve memory function in TBI and other neurological conditions are warranted.

The prevention of the injury-induced cortical lesion, possibly a microbleed, after administration of *paclitaxel* was not anticipated. However, our previous research applying *paclitaxel* directly to the site of controlled cortical impact found a significant reduction in injury volume and edema [13]. Hemorrhage is a common feature in moderate to severe TBI [58]. Microbleeds, observed in acute TBI, are a result of vascular damage. The detection of microbleeds after mild TBI is an area of considerable clinical research effort [59–61]. In our study we only acquired MR imaging at 4 days after the final impact, therefore we do not know if *paclitaxel* administration after the first impact completely prevented the lesion, or facilitated tissue recovery. However, this finding raises intriguing possibilities for the application of microtubule-stabilizers to more severe trauma where hemorrhage is a serious complication [62]. Also, because the imaging took place at 4 days after impact, and the mice were not euthanized until 45 days it was not possible to confirm histologically that the post-injury lesion was actually a microbleed. This finding will be further investigated in future research.

The pharmacokinetics of IN *paclitaxel* delivery indicated a brain uptake of 0.08–0.23 %injection/g brain tissue. These values convert to a tissue concentration of 14–40 pmol/g of brain tissue. For kidney, 2% uptake of the administered dose would give a tissue concentration of about 350 pmol/g of tissue. These biologic effects, despite low brain uptake are consistent with previous research of other small molecules [28, 29]. Although MT-stabilizers including *paclitaxel* may be neurotoxic at doses required to treat cancer [63], other research indicates an opposite (neurotherapeutic) effect of *paclitaxel* in lower concentrations and doses. An *in vivo* study with intracortical delivery of 853.9 ng *paclitaxel* after penetrating needle injury found reduced axonopathy [22]. Another showed that 256 ng/day via

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intrathecal administration facilitated axon regeneration in acute spinal cord injury in rats [19]. In another study, 8.54 ng *paclitaxel* delivered to a dorsal root ganglion injury limited axonal retraction bulbs, while in the same study, only 10 nM applied *in vitro* to cultured neurons promoted axonal growth [23]. Sengottuvel et al. found 3 nM *paclitaxel* promoted axon growth *in vitro* and 4.27 ng promoted optic nerve regeneration *in vivo* [20]. Collectively, these findings along with those from our study suggest that low dose *paclitaxel* is neurotherapeutic. In fact, the finding that *paclitaxel* can be neurotherapeutic at low doses and neurotoxic at higher ones, should not be surprising. Medical practice has many examples of therapeutics, from botulimun toxin to acetaminophen, that have a range of beneficial effects despite high dose toxicity.

One advantage to IN delivery is that using a commercially available drug delivery device [64], a drug can be quickly administered to injured patients by medical personnel (from the sidelines of football and hockey games, or in the military field) under up-tempo conditions (i.e., light weight and without requiring refrigeration, electricity, or sophisticated administration equipment). Some skepticism for IN drug delivery remains within the field of central nervous system drug development, particularly with respect to translation of results from mouse studies to human. Main objections include: the mouse brain is diminutive and diffusion distances are shorter than in humans, and the olfactory bulb of the mouse is large compared to that of the human so it is not surprising that mice have drug uptake via the olfactory nerve tract. The success of IN insulin as a therapeutic for AD, argues against these concerns [26]. Also, and more directly, distribution of substances into the brain after IN administration is not dependent on classic brain distribution mechanisms. Instead, distribution is very rapid, reaches steady state quickly, and is fairly stable over one hour [64]. The exact mechanism of action is still not entirely clear, however. It has been suggested that transport after IN administration is within the perivascular space of the brain [30, 31].

This study has some limitations. First, a coil malfunction that was not apparent at the time of imaging and did not compromise the other imaging acquisitions (T1 w and T2 mapping), was discovered after the study was completed. The malfunction affected the quality of the DTI, particularly in the middle of the brain and we were unable to analyze the FA maps from the *paclitaxel*-treated group that were acquired at 4 days post-injury. However, we applied silver stain of the external capsule at 45 days for all subjects, which showed no evidence of axonal injury in *paclitaxel*-treated mice. We also examined the *in vivo* structural T1-weighted and T2 mapping and confirmed that *paclitaxel*-treated subjects did not have overt brain lesions at the 4 days time point. Second, the neuropathological evidence of synaptic preservation based on PSD-95 stain is considered preliminary and support for this finding will be investigated in future studies using electron microscopy. Third, we do not know the temporal dynamics for repeat mild impacts on cytoskeletal derangements and the maximum interval during which the administration of microtubule-stabilizers will mitigate impairments. In this study we administered the drug immediately following the first impact. In future studies, we will determine future translational potential of microtubule-stabilizing therapeutics by providing insight into the temporal dynamics related to delivery. Lastly, only male mice were used in this study. Research is underway to investigate sex differences in the rmTBI model as well as response to *paclitaxel* treatment.

Conclusions

We found that *paclitaxel* treated mice did not suffer from the spatial memory impairment seen in the vehicle-only rmTBI mice. *In vivo* imaging of treated mice revealed preservation of glucose uptake on FDG-PET and no significant differences as compared to sham mice in white matter integrity on DTI. Apparent micro-hemorrhage seen on imaging in vehicle treated mice was not observed in *paclitaxel*-treated mice. Postmortem examination of *paclitaxel*-treated brains confirmed *in vivo* imaging results. In particular, we found no indication of axonal injury after treatment, and, more importantly, *paclitaxel* protected against synaptic loss in the CA1 region of the hippocampus. These results indicate that microtubule-stabilizers, more specifically *paclitaxel*, may be neuroprotective against repeated mild brain injury.

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Long-Term Effects of Traumatic Brain Injury in a Mouse Model of Alzheimer's Disease

Marlena Zyśk[a], Fredrik Clausen[b], Ximena Aguilar[a], Dag Sehlin[a], Stina Syvänen[a] and Anna Erlandsson[a,*]
[a]*Department of Public Health and Caring Sciences, Uppsala University, Rudbeck Laboratory, Uppsala, Sweden*
[b]*Department of Neuroscience, Uppsala University, Rudbeck Laboratory, Uppsala, Sweden*

Abstract. Alzheimer's disease (AD) is the leading cause of dementia worldwide, affecting over 10% of the elderly population. Epidemiological evidence indicates that traumatic brain injury (TBI) is an important risk factor for developing AD later in life. However, which injury-induced processes that contribute to the disease onset remains unclear. The aim with the present study was to identify cellular processes that could link TBI to AD development, by investigating the chronic impact of two different injury models, controlled cortical impact (CCI) and midline fluid percussion injury (mFPI). The trauma was induced in 3-month-old tg-ArcSwe mice, carrying the Arctic mutation along with the Swedish mutation, and the influence of TBI on AD progression was analyzed at 12- and 24-weeks post-injury. The long-term effect of the TBI on memory deficiency, amyloid-β (Aβ) pathology, neurodegeneration and inflammation was investigated by Morris water maze, PET imaging, immunohistochemistry, and biochemical analyses. Morris water maze analysis demonstrated that mice subjected to CCI or mFPI performed significantly worse than uninjured tg-ArcSwe mice, especially at the later time point. Moreover, the injured mice showed a late upregulation of reactive gliosis, which concurred with a more pronounced Aβ pathology, compared to uninjured AD mice. Our results suggest that the delayed glial activation following TBI may be an important link between the two diseases. However, further studies in both experimental models and human TBI patients will be required to fully elucidate the reasons why TBI increases the risk of neurodegeneration.

Keywords: Alzheimer's disease, amyloid-β, inflammation, Morris water maze, neurodegeneration astrocytes, PET, traumatic brain injury

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia, estimated to affect around 50 million people worldwide [1]. Due to the fact that AD is a progressive neurodegenerative disorder, developing over several years, the pathophysiology of the disease is very complex. The key neuropathological hallmarks of AD are the extracellular plaques, mainly consisting of aggregated amyloid-β (Aβ), and the intracellular neurofibrillary tangles, composed of hyperphosphorylated tau (*p*-tau). In addition, oxidative stress, apoptosis, and neuroinflammation are elevated during the course of the disease [2].

It has been demonstrated that there is a clear epidemiological association between traumatic brain injury (TBI) and AD [3], but the mechanism behind this link is still unclear. Interestingly, Aβ plaques may be found in patients within hours following TBI [4]. If these injury-induced plaques are temporary or result in permanent Aβ pathology is not clear.

*Correspondence to: Anna Erlandsson, Department of Public Health and Caring Sciences/Molecular Geriatrics, Rudbeck Laboratory, Uppsala University, SE-751 85 Uppsala, Sweden. E-mail: anna.erlandsson@pubcare.uu.se.

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Since the onset of AD often occurs many years after TBI, long-term cellular mechanisms, such as chronic inflammation, most likely play a central role in the process. In addition to TBI, some of the known risk factors for AD include hypertension and diabetes, which display vascular morbidities [5], indicating that the vascular system of the brain is important for the development of the disease. Several studies suggest that brain tissue damage following TBI is a chronic process with ongoing brain tissue atrophy for several years after the primary injury [6, 7]. Despite that, most TBI studies concentrate on the first weeks following injury and very little is known about the inflammation at later time points. However, it has been reported that both reactive astrocytes and macrophages/microglia are still present in the injured brain one year following trauma in rats [8].

A number of TBI studies have reported effects on cognition and changes in Aβ or tau pathology in mouse models of AD [9–19]. Especially, the short-term effect of TBI has been in focus and only a few investigations have included later time points [9, 10]. None of these studies followed the animals longer than 16 weeks after the inflicted brain trauma or addressed the effect on glial activation at later time points.

Here, we aimed to study the impact of two different TBI models in tg-ArcSwe mice up to 24 weeks following the injury. In order to examine both focal and diffuse aspects of TBI, we use one mostly focal model, controlled cortical impact (CCI), and one mostly diffuse model, midline fluid percussion injury (mFPI). As this is the first long-term study on TBI in tg-ArcSwe mice, we designed the experiments in this way, to not miss an effect elicited by either type of TBI. The tg-ArcSwe mice show elevated levels of soluble Aβ aggregates already at a very young age and develop plaque pathology from around 6-7 months of age [20, 21]. The trauma was induced when the animals were 3 months old, which is prior to any visible Aβ pathology. The first investigated time point, when animals were 6 months old (12 weeks following TBI), coincides with the first appearance of plaques in uninjured tg-ArcSwe mice and the second time point, when the animals were 9 months old (24 weeks following TBI), coincides with pronounced Aβ pathology in uninjured tg-ArcSwe mice.

The two trauma models cause different damage to the brain; while CCI results in a local ipsilateral cortical contusion with scattered neuronal loss in the underlying hippocampus, mFPI results in widespread axonal injury throughout the brain [22]. Using immunohistochemistry, biochemical analyses, Morris water maze (MWM), and PET imaging, we could demonstrate that the Aβ burden and astrocyte gliosis were clearly increased in injured tg-ArcSwe mice compared to uninjured tg-ArcSwe control mice, 24-weeks post-TBI. Notably, there was no elevated pathology at 12 weeks after the injury. These results were in line with our findings that the injured animals showed increased neurodegeneration and severe memory deficiency at the latest time point.

MATERIALS AND METHODS

Animals

Transgenic mice carrying the Arctic (E693G) and Swedish (KM670/6701NL) amyloid-β precursor protein (AβPP) (tg-ArcSwe mice) were obtained by in-house breeding on a C57bl/6J background. The tg-ArcSwe mice develop Aβ deposits in the brain that resemble AD neuropathology. More specifically, the tg-ArcSwe mice express elevated levels of soluble Aβ aggregates, such as Aβ protofibrils, accumulate Aβ inside neurons, and develop robust amyloid plaques. In total 51 gender balanced tg-ArcSwe mice were included in the study, 27 males and 24 females. However, 11 mice died before the experiment was finalized (during the 6 months). Hence, the total number of animals that were included in the MWM study and the immunohistochemistry analysis were 40 animals. The following groups were included: 14 naïve mice (7 of these were sacrificed at 3 months and 7 were sacrificed at 6 months), 12 CCI mice (7 of these were sacrificed at 3 months and 5 were sacrificed at 6 months), and 14 mFPI mice (6 of these were sacrificed at 3 months and 8 were sacrificed at 6 months). The mice analyzed with PET were the same individuals that were included in the MWM test and then euthanized at 6 months. Hence, PET scans were obtained for 7 naïve, 5 CCI, and 8 mFPI, but two PET scans of naïve mice were omitted due to technical problems. The animals that were euthanized at 3 months did not undergo PET scanning. The mice were housed at the National Veterinary Institute in Uppsala at 20–22°C, with access to food and water *ad libitum* on a 12 h light/dark cycle. All experiments were approved by the Uppsala County Animal Ethics board, and followed the rules and regulations of the Swedish Animal Welfare Agency (approval number C17/13). An experimental outline is shown in Supplementary Figure 1A.

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Anesthesia

Anesthesia was induced with inhalation of 4% isoflurane in air. During surgery, general anesthesia was maintained with a mix of isoflurane (1.2–1.4%) and N_2O/O_2 (70/30%), delivered through a nose cone. Lubricant eye ointment (Viscotears; Novartis, Basel, Switzerland) was used for corneal protection during the procedure. After being shaved and cleaned with ethanol on the scalp, the mice were placed in a stereotaxic frame and core temperature was maintained at 37°C, using a heating pad controlled by a rectal thermometer. Local anesthesia (Marcain, AstraZeneca, Sweden) was applied to the scalp and the skull was exposed by an incision along the midline. Uninjured controls did not undergo any surgical intervention or anesthesia.

Controlled cortical impact (CCI)

A craniotomy (4 mm diameter) was made over the right parietal cortex between the sutures of bregma and lambda using a dental drill. The cortical contusion was delivered by a 2.5 mm diameter piston set to an impact depth of 0.5 mm from a pneumatically driven CCI device (VCU Biomedical Engineering Facility, Richmond, VA, USA). The velocity of the piston was set to 2.8 m/s. The bone fragment was put back in place, secured with tissue adhesive (Histoacryl, Braun, Germany), and the scalp was sutured.

Midline fluid percussion injury (mFPI)

A 3 mm-diameter craniotomy was performed, centered at the midline halfway between bregma and lambda, leaving the underlying dura intact. A plastic cap was secured over the craniotomy with dental cement (Heraeus Kulzer, Hanau, Germany). Injury was produced by attaching the saline filled cap to the Luer-Lok fitting on the fluid percussion device (VCU Biomedical Engineering Facility, Richmond, VA, USA) and releasing a pendulum hitting a saline-filled reservoir, producing moderate injury, into the closed cranial cavity. The peak pressure pulse was 1.40 ± 0.06 atm, measured by a transducer displayed on an oscilloscope and recorded on a computer. Immediately after the injury, each mouse was visually monitored for apnea duration and seizures. Anesthesia was then resumed, the cement and the cap were removed, the bone flap was replaced, and the skin was closed with sutures. Mice were moved to a cage with a heating pad until they had recovered from anesthesia and were fully ambulatory.

Morris water maze

To evaluate spatial learning and memory, we used the MWM test [23], in which the mice are placed in white 1.4 m-diameter circular tank, filled 20 cm with 22°C water. The test is performed by putting the mice into different starting positions from where they have to find a fixed 10 cm-diameter platform placed in the southwest quadrant of the tank and submerged 1 cm below the surface. Simple visual cues to aid navigation are placed on roller curtains surrounding the tank. 16 training trials over a 4-day interval (4 trials per day) were performed in the MWM at week 12 or week 24 post-injury. Each swim trial was performed by placing the mouse in the tank at one of four designated entry points facing the wall. The trial was recorded using a digital tracking system (HVS Image, Buckingham, UK). The trial was terminated when the mouse located and stayed on the platform. The mouse was allowed to remain undisturbed on the platform for 15 s or placed there if it had not located the platform in order to acquire the visual cues surrounding the pool. For each MWM learning trial, the latency to find the platform, swim speed and path length were analyzed.

PET scanning

To investigate the *in vivo* brain accumulation of Aβ aggregates, PET scanning was performed with the Aβ protofibril selective antibody-based radioligand [^{124}I]RmAb158-scFv8D3 [24] in mice ($n = 7$ uninjured, $n = 5$ CCI, $n = 7$ mFPI) 24 weeks after TBI. Animals were i.v. administered with 8.7 ± 1.2 MBq [^{124}I]RmAb158-Scfv8D3 3 days prior to PET/CT imaging. PET scanning, 60 min, was conducted under 1.5–2.0% isoflurane anesthesia (Baxter Medical AB, Kista, Sweden) in a Triumph Trimodality System (TriFoil Imaging, Inc., Northridge, CA, USA) followed by a 2 min CT scan obtained in the same scanner. Image reconstruction and analysis of radioligand accumulation in the brain was performed as previously described [25]. All animals were euthanized directly after scanning.

Mouse brain homogenization and fixation

At the time of euthanization, mice were anesthetized with 2.7–3.2% isoflurane (Baxter Medical

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AB, Kista, Sweden) followed by intracardiac perfusion with 50 ml physiological saline during 2 min. The brain was removed from the cranium and divided coronally in two parts, just anterior to the brain injury site. The anterior part was snap frozen on dry ice to be used for biochemical quantification of Aβ. The posterior part was placed in paraformaldehyde for 24 h. After 24 h, it was cryoprotected in sucrose (10%, 20%, and finally 30%) and stored in 30% sucrose at 4°C until cryostate-sectioned coronally at a thickness of 14 μm for immunohistochemical analysis.

The anterior tissue was extracted by adding 5 μl cold TBS/mg tissue weight (20 mmol/L Tris and 137 mmol/L NaCl, pH 7.6, complete protease inhibitor cocktail (Roche)). A tissue grinder with Teflon pestle were used with 2 × 10 strokes on ice. The TBS homogenate was mixed with concentrated formic acid to a concentration of 70%, followed by homogenization and centrifugation at 16 000×g at 4°C for 60 min, and the supernatants were stored at –70°C prior to analysis. The reason for mixing TBS with concentrated formic acid to a concentration of 70% is to completely dissolve the tissue and obtain a brain preparation of total Aβ, i.e., including plaque Aβ.

ELISA

Levels of $A\beta_{40}$ and $A\beta_{42}$, corresponding to total Aβ load, was measured in the formic acid (FA) treated brain homogenates, as previously described [25]. In brief, 96-well plates were coated overnight with 100 ng/well of rabbit polyclonal anti-$A\beta_{40}$ or anti-$A\beta_{42}$ (custom made by Agrisera) and blocked with 1% BSA in PBS. FA extracts from frontal cortex were neutralized with 2 M tris and diluted 2000× for $A\beta_{40}$ and 200× for $A\beta_{42}$ analysis, then incubated overnight, followed by detection with biotinylated mAb1C3 (0.5 μg/ml) [26] and streptavidin-HRP (Mabtech AB). Signals were developed with K Blue Aqueous TMB substrate (Neogen Corp.) and read with a spectrophotometer at 450 nm. All dilutions were made in ELISA incubation buffer (PBS with 0.1% BSA, 0.05% Tween20 and 0.15% Kathon).

Immunohistochemistry

Immunohistochemistry was performed on sections localized right before the injury area; Bregma: 1.32–0.38, to evaluate the expression of Aβ, GFAP, Vimentin, Iba-1, Mac-2, MAP-2, synaptophysin and double cortin (DCX) in brain tissue sections from mice at 12- and 24-weeks post injury. All steps were performed at room temperature unless otherwise mentioned. The slides were air dried for 3 min before rehydration in PBS for 5 min. Non-specific binding was blocked with 5% normal goat serum (NGS, Bionordica) in phosphate buffered saline (PBS) + 0.3% Triton X-100 for permeabilization for one hour in a humidity chamber. The blocking buffer was poured off from the slides before incubating with primary antibody, diluted with 0.5% NGS in PBS + 0.03% Triton X-100, in humidity chamber overnight at 4°C. For specific antibodies and dilutions, see Table 1. The slides were washed 3 × 10 min in PBS before incubation with secondary antibody (Table 1), diluted with 0.5% NGS in PBS 0.03% Triton X-100, in humidity chamber at 37°C for 1 h. After washing 3 × 10 min in PBS, slides were mounted with Vectashield Hard Set™ Mounting medium with DAPI (Vector), staining the nuclei.

Quantification and statistics

Morris water maze

Two-way ANOVA was performed for injury group and trial day. Bonferroni's *post-hoc* test was used to describe the differences between the groups (Statistica, Statsoft, Sweden).

PET

Radioligand accumulation was quantified as concentration of [^{124}I]RmAb158-scFv8D3 in frontal cortex relative to that in cerebellum, i.e., a region largely spared of pathology at this age in tg-ArcSwe mice [25, 27]. Thus, the concentration ratio indicates radioligand accumulation above what is expected in normal pathology-free tissue. The concentration ratio, often denoted SUVR, is a standard read-out in preclinical as well as in clinical PET studies. The PET data was analyzed with one-way ANOVA followed by Bonferroni's *post hoc* test and presented as mean ± standard error of the mean (SEM). Significance levels indicated in the figures correspond to: $^{*}p$-value <0.05, $^{**}p$-value <0.01.

ELISA

Aβ concentrations were calculated from a standard curve of serially diluted synthetic $A\beta_{40}$ and $A\beta_{42}$, using a four-parameter equation. The ELISA data was analyzed with one-way ANOVA followed by Bonferroni's *post hoc* test and presented as mean ± standard error of the mean (SEM). Significance levels

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Table 1
Overview of antibodies used for immunohistochemistry

Antibody	Host/Dilution	Target	Supplier/product number
Anti-$A\beta_{1-16}$ (6E10)	Mouse, 1:500	Aβ/AβPP	Covance, SIG-39320
Anti-$A\beta_{1-16}$ (82E1)	Mouse, 1:100	Aβ N-terminus	IBL International, JP10323
Anti-DCX	Guinea pig, 1:800	Newborn neurons	Millipore, AB2253
Anti-GFAP	Mouse, 1:400	Reactive astrocytes	DAKO, Z0334
Anti-Iba1	Rabbit, 1:200	Microglia	Wako, 019-19741
Anti-Mac2	Rat, 1:200	Microglia/macrophages	Cedarlane, CL8942AP
Anti-Map2	Rabbit, 1:200	Neurons	Millipore, AB5622
Anti-Synaptophysin	Rabbit, 1:500	Presynaptic marker	Abcam, ab32127
Anti-Vimentin	Chicken, 1:500	Reactive astrocytes	Millipore, AB5733
Anti-chicken 488	Goat, 1:200	Secondary antibody	Thermo Fisher, A11039
Anti-guinea pig 488	Goat, 1:200	Secondary antibody	Thermo Fisher, A11073
Anti-mouse 488	Goat, 1:200	Secondary antibody	Thermo Fisher, A11001
Anti-mouse 555	Goat, 1:200	Secondary antibody	Thermo Fisher, A21424
Anti-rat 488	Goat, 1:200	Secondary antibody	Thermo Fisher, A11006
Anti-rabbit 488	Goat, 1:200	Secondary antibody	Thermo Fisher, A11008
Anti-rabbit 555	Goat, 1:200	Secondary antibody	Thermo Fisher, A21428

indicated in the figures correspond to: $^{*}p$-value <0.05, $^{**}p$-value <0.01.

Immunohistochemistry

The stained brain sections were visualized in a Zeiss Observer Z.1 microscope (Carl Zeiss Microimaging GmbH, Jena, Germany) with objectives 20×/0.4 W, 40×/0.75 W, and 63×/1.4 oil immersion. Images were captured of the regions of interest (ROI) (Supplementary Figure 1B) and the intensity was measured with Zeiss Zen software. For each animal, 3 sections were stained and 10 images/section for cortex and 2 images/section for hippocampus were captured. The data was analyzed by nonparametric Mann Whitney test, using GraphPad Prism (version 5.03) and shown as mean ± standard error of the mean (SEM). Significance levels indicated in the figures correspond to: $^{*}p$-value <0.05, $^{**}p$-value <0.01, and $^{***}p$-value <0.001.

RESULTS

Memory deficits in tg-ArcSwe subjected to TBI

To investigate if CCI or mFPI results in long-term negative effects on spatial learning in tg-ArcSwe mice, we performed MWM analysis at 12 and 24 weeks following the injury and compared their performance to age-matched uninjured tg-ArcSwe mice. At both time points, we found a significant increase in the latency to find the platform ($p < 0.05$) in mice that received TBI, as compared to the uninjured tg-ArcSwe mice (Fig. 1A, B). At the later time point, the mFPI mice performed worse than the CCI mice (Fig. 1B). We did not detect any difference in swim speed between the groups, but the swim distance was significantly increased for the injured mice at 24-weeks post-TBI, compared to uninjured mice, corresponding to the increased latency to find the platform (Supplementary Figure 2).

PET analysis shows earlier Aβ pathology following TBI

PET imaging revealed significantly higher accumulation of [^{124}I]RmAb158-scFv8D3 in the frontal cortex of mFPI and CCI tg-ArcSwe mice, compared to uninjured tg-ArcSwe mice, indicating an increased accumulation of Aβ in this region (Fig. 2A, B). Quantification of total Aβ levels in brain tissue homogenates showed no significant increase in the TBI groups, compared to tg-ArcSwe uninjured mice (Fig. 2C). While this difference was not significant due to high variability, mainly caused by two individuals with very high Aβ load in the mFPI group, there was an overall good correlation with the PET quantification (Fig. 2D).

Increased Aβ deposits and reactive astrocytes 24 weeks following TBI

In order to study the long-term glial response and Aβ pathology in tg-ArcSwe mice following CCI or mFPI, we performed immunohistochemistry with brain sections from animals sacrificed 12 weeks and 24 weeks after the injury. Immunostainings with antibodies specific to the astrocyte marker GFAP, revealed that there was no significant increase in

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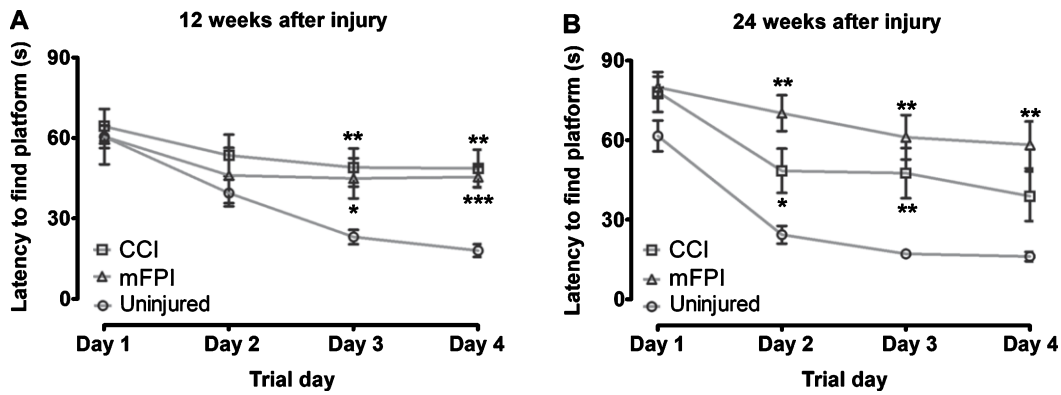

Fig. 1. Severe long-term defects in spatial learning in tg-ArcSwe mice following TBI. The tg-ArcSwe mice performed significantly worse in the Morris water maze test, compared to age-matched uninjured tg-ArcSwe mice at both 12-weeks (A) and 24-weeks (B) post-injury. At the later time point, the mice that had received mFPI mice performed worse than the mice that had received CCI. Two-way ANOVA was performed for injury group and trial day. Bonferroni's *post-hoc* test was used to describe the differences between the groups.

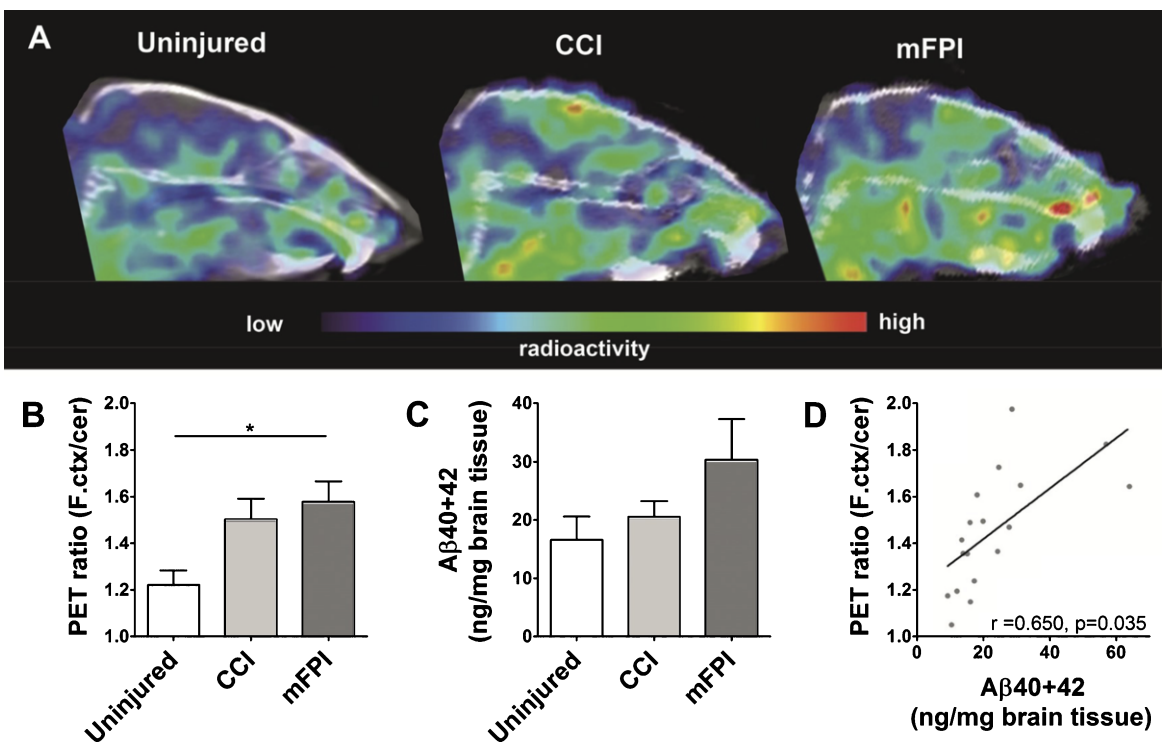

Fig. 2. Earlier Aβ pathology following TBI. Sagittal view of PET images obtained with [^{124}I]RmAb158-scFv8D3 administered to mice 3 days before scanning (A). Quantification of PET ratio (frontal cortex/cerebellum) from PET images revealed that frontal cortex signal was significantly higher in mice subjected to both forms of TBI compared to uninjured mice (B). ELISA Quantification of total total Aβ40 + 42 in frontal cortex showed a similar trend as PET (C), with a significant correlation to the PET ratio (D). The PET data and the ELISA data were analyzed with one-way ANOVA followed by Bonferroni's *post hoc* test. Graphs display group mean with error bars for SEM. $*p < 0.05$; $**p < 0.01$.

GFAP expression 12 weeks after injury (Fig. 3A, B). Instead, there was lower GFAP expression in mice that received mFPI, compared to uninjured animals, while there was no difference between animals that revived CCI and uninjured animals. Interestingly, there was a two-fold increase in GFAP-expression in both groups of injured mice at 24 weeks, compared to uninjured controls. These results indicate a prominent long-term effect of TBI, resulting in a pronounced chronic astroglial response half a year

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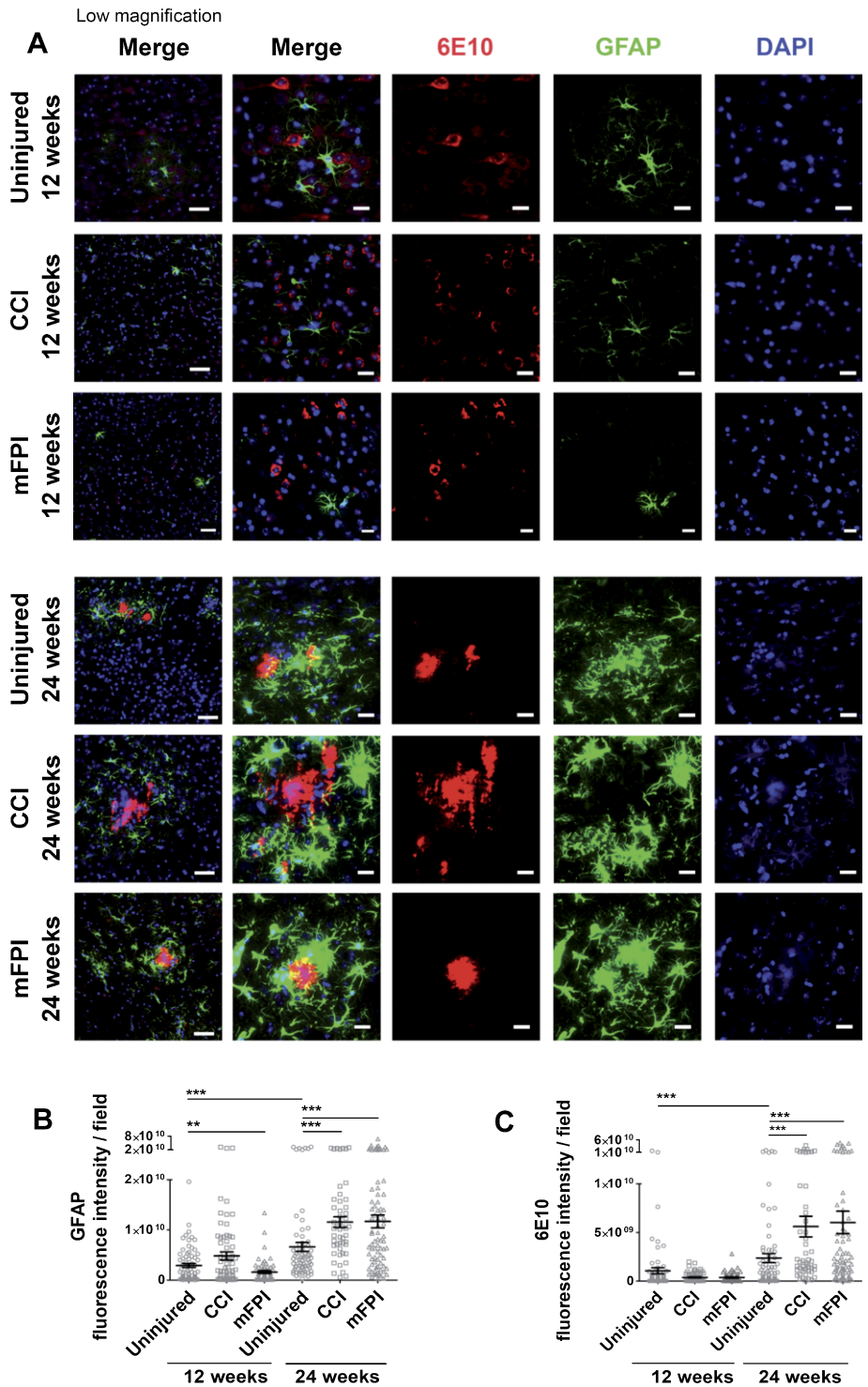

Fig. 3. Elevated GFAP expression and Aβ deposition at 24 weeks following CCI or mFPI. Immunostainings, revealed that there was no significant increase in reactive astrocytes, GFAP (green) and Aβ deposits (6E10, red) in tg-ArcSwe mice at 12 weeks after CCI or mFPI. At24 weeks, there was a clear increase in both GFAP-expression and Aβ deposition in both injury groups at, compared to uninjured controls (A). Analysis of the fluorescence signal confirmed that there was a significant increase in GFAP-expression and Aβ deposition 24 weeks after CCI or mFPI (B-C). The data was analyzed by nonparametric Mann Whitney test and the graphs display mean ± SEM. Scale bars: Low magnification = 20 μm, high magnification = 50 μm. $^{**}p < 0.01$, $^{***}p < 0.001$.

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after the trauma. The analysis of Aβ burden in mice, following CCI or mFPI, using the Aβ specific antibody 6E10, confirmed PET and ELISA analyses and demonstrated that both injuries resulted in an increased Aβ pathology as compared to uninjured mice at 24-weeks, but not at 12-weeks post-injury, which is in line with the astrocyte response. Moreover, the immunostainings illustrate that the reactive astrocytes were mostly situated around Aβ plaques (Fig. 3A, C).

Next, we performed additional stainings with specific antibodies to vimentin, a protein that is highly expressed in reactive astrocytes. As predicted, there was a very low expression of vimentin in the uninjured tg-ArcSwe brain. Following CCI, the vimentin expression was markedly induced and could easily be detected (Fig. 4). At 12-weeks post-injury, vimentin was prominently expressed in astrocytes that were in close proximity to the injury-site. However, vimentin expression could not be detected in regions distant from the CCI injury-site or in the brains of mice that received mFPI at this time point. At week 24 after injury, the pattern of vimentin expression was different. Vimentin positive, reactive astrocytes were then frequently found in both injured and uninjured animals, but exclusively around the plaques where it overlapped with GFAP expression (Fig. 4). However, at this time point vimentin expression was very sparse in the region close the CCI lesion site.

Increased number of activated microglia 24 weeks after CCI

In order to analyze the microglia activity in the tg-ArcSwe mouse brain following CCI or mFPI, we performed stainings with antibodies specific for Iba-1 (Fig. 5A, B). At the earlier time point, 12-weeks post-TBI, there was a very sparse expression of Iba-1 in both uninjured and injured mice. Interestingly, there was significantly less Iba-1 expressing microglia in mice that received CCI or mFPI at this time point, compared to uninjured controls (Fig. 5B). At 24 weeks after TBI, there was a two-fold increase of Iba-1 reactivity in mice that received CCI, compared to uninjured tg-ArcSwe mice, indicating that similar to astrocytes, microglia are activated at late time points following injury (Fig. 5B). Notably, CCI resulted in a more prominent late microglial activation, compared to mFPI. Double stainings with the Aβ antibody 82E1 clearly demonstrated that microglia were predominately found in close proximity to the plaques (Fig. 5A).

Next, we examined the number of activated, Mac-2 positive microglia/macrophages in the in tg-ArcSwe mouse brain 12 and 24 weeks following CCI or mFPI (Fig. 6). There was a very low number of Mac-2 positive cells in both uninjured and injured mice. At the later time point, Mac-2 positive cells were found around the plaques of both uninjured and injured mice. However, the Mac-2 staining was strongest in CCI-injured animals at both time points (Fig. 6). Quantification of the Mac-2 positive cells revealed 0.4 ± 0.7 positive cells/field in uninjured animals, 10.9 ± 5.0 positive cells/field in CCI-mice and 1.9 ± 2.6 positive cells/field in mFPI-mice at 12 weeks. At 24 weeks there was 2.1 ± 1.0 positive cells/field in uninjured animals, 11.5 ± 6.1 positive cells/field in CCI-mice, and 2.7 ± 1.9 positive cells/field in mFPI-mice.

Elevated expression of synaptophysin 24 weeks after TBI

In our investigation of cellular processes that could be altered by CCI or mFPI, we next studied the neuronal dendrite network in tg-ArcSwe mouse brain following CCI or mFPI, by using specific antibodies to MAP2 (Fig. 7). Our immunostainings showed that there was a clear disruption of the MAP2 positive processes in the areas of Aβ pathology at the 24-week time point that was not seen at week 12. Where the plaques had developed, there were clear gaps in the MAP2-staining. However, no obvious differences between uninjured mice and mice that received CCI or mFPI was observed and around the plaques, the dendrite network appeared intact (Fig. 7).

Synapse alterations are widespread in AD and we therefore sought to investigate the effect of CCI and mFPI on the synapse specific marker synaptophysin in the region of hippocampus (Fig. 8A, B). Immunocytochemistry demonstrated that there was no significant difference in synaptophysin expression at 12 weeks following TBI, as compared to uninjured controls. However, at the later time point, 24 weeks post-injury, there was a clear increase in synaptophysin expression in both the CCI and mFPI animals, indicating a possible compensatory effect due to synaptic dysfunction (Fig. 8B).

Increased cell loss in hippocampus at later time points following TBI

It is well-known that TBI, as well as early AD pathology, could stimulate neurogenesis [28, 29].

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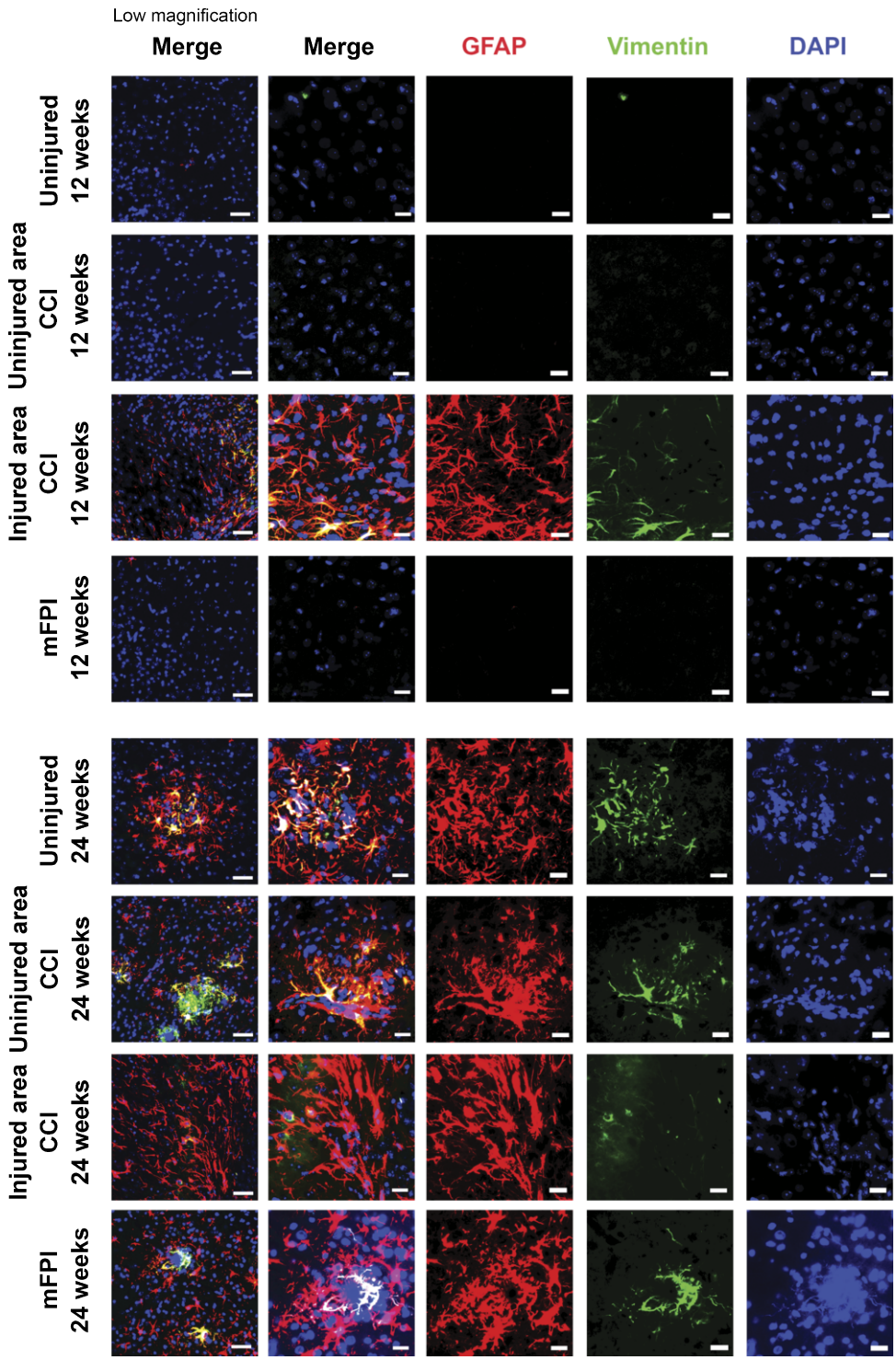

Fig. 4. Increased expression of Vimentin at 12 weeks following CCI. Immunostainings demonstrated that Vimentin (red) and GFAP (green) had different expression patterns. There was a clear increase of Vimentin positive astrocytes in close proximity to the lesion site at 12 weeks following CCI (injured area CCI), while the expression in distant regions was very low. At week 24, Vimentin positive astrocytes were frequently found in both injured and uninjured animals, but exclusively around the plaques where the Vimentin expression overlapped with the GFAP expression. The Vimentin expression close to the CCI lesion site was very sparse at this time point. Scale bars: Low magnification = 20 μm, high magnification = 50 μm.

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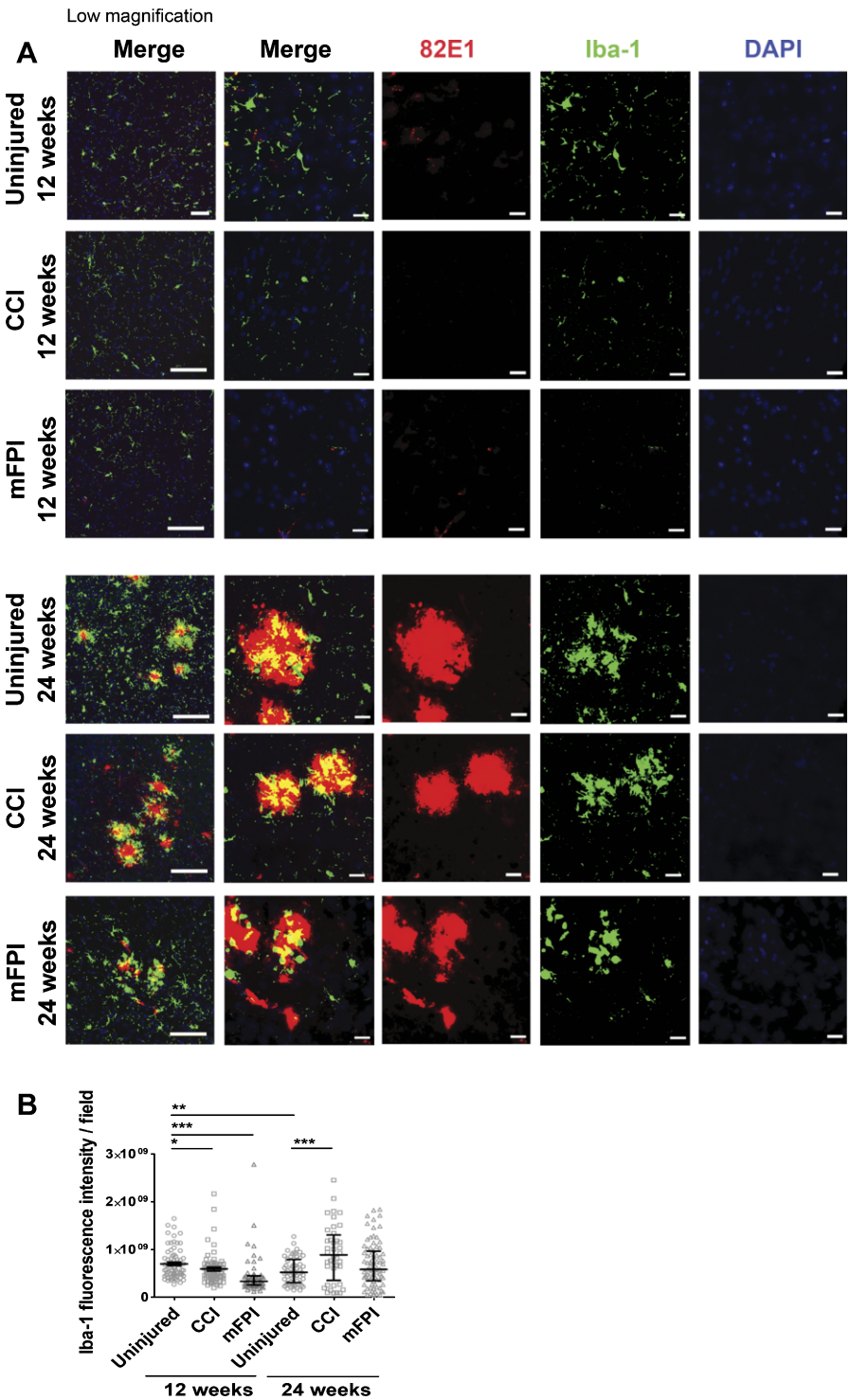

Fig. 5. Induced number of activated microglia 24 weeks after CCI. Immunostainings with antibodies specific for Iba-1 (green) and the Aβ antibody 82E1 (red) demonstrated that microglia were found in close proximity to the plaques (A). At 12-weeks post-TBI there was a sparse expression of Iba-1 in both uninjured and injured mice. There was significantly less Iba-1 expression in mice that received CCI or mFPI at this time point, compared to uninjured controls. 24 weeks after TBI there was a two-fold increase of Iba-1 reactivity in mice that received CCI, compared to uninjured tg-ArcSwe mice (B). The data was analyzed by nonparametric Mann Whitney test and the graph displays mean ± SEM. Scale bars: Low magnification = 20 μm, high magnification = 50 μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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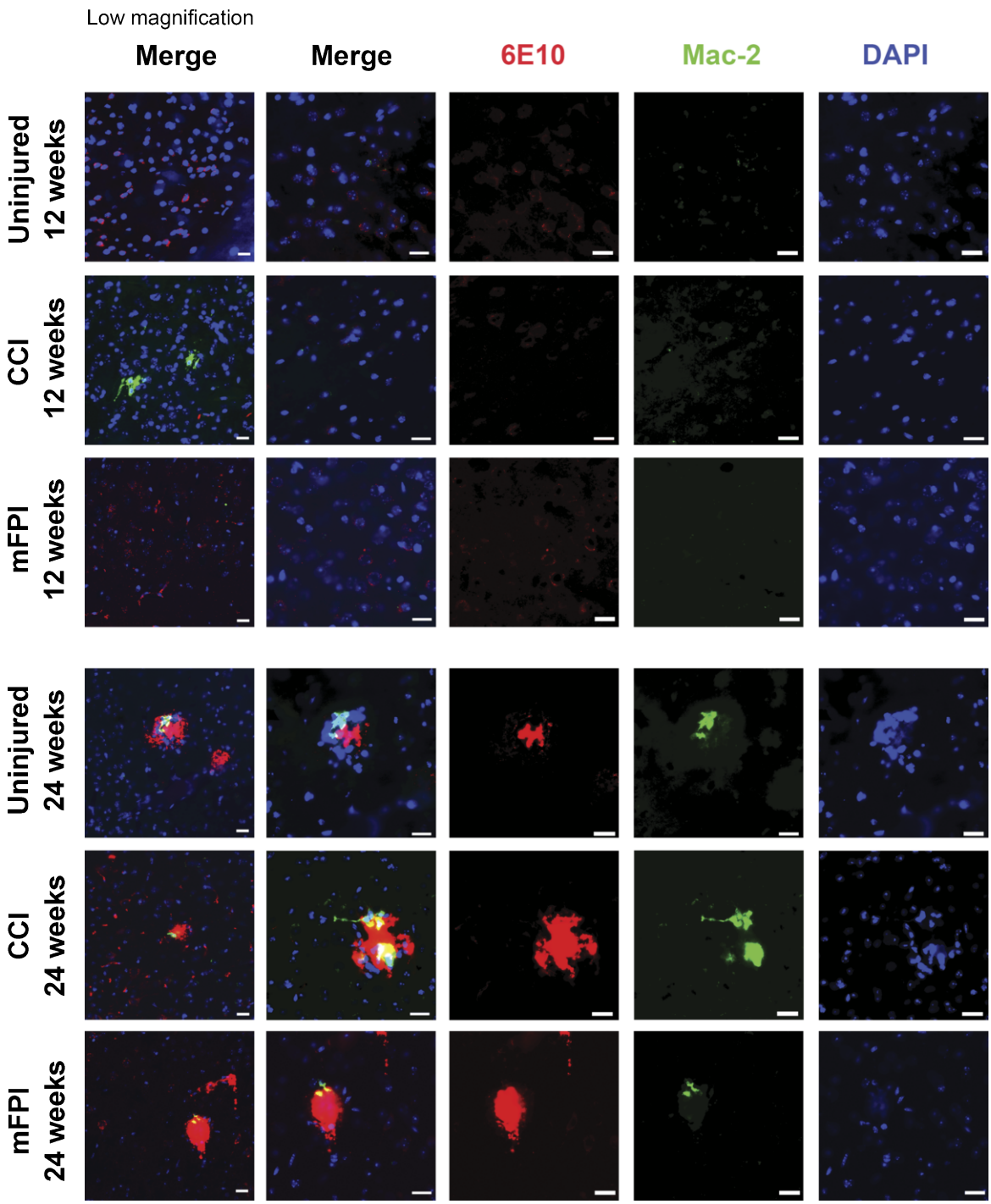

Fig. 6. Mac-2 positive cells around plaques in both uninjured and injured mice. Immunostainings using specific antibodies to amyloid-β (6E10, red) and activated microglia/macrophages (Mac-2, green) showed that there was a very low number of Mac-2 positive cells in the brains of both uninjured and injured mice at 12 weeks post-injury. At 24 weeks post-injury, there was an increased number of Mac-2 positive cells found around the plaques of both uninjured and injured mice, but the Mac-2 staining was strongest in CCI-injured animals. Scale bars: 20 μm.

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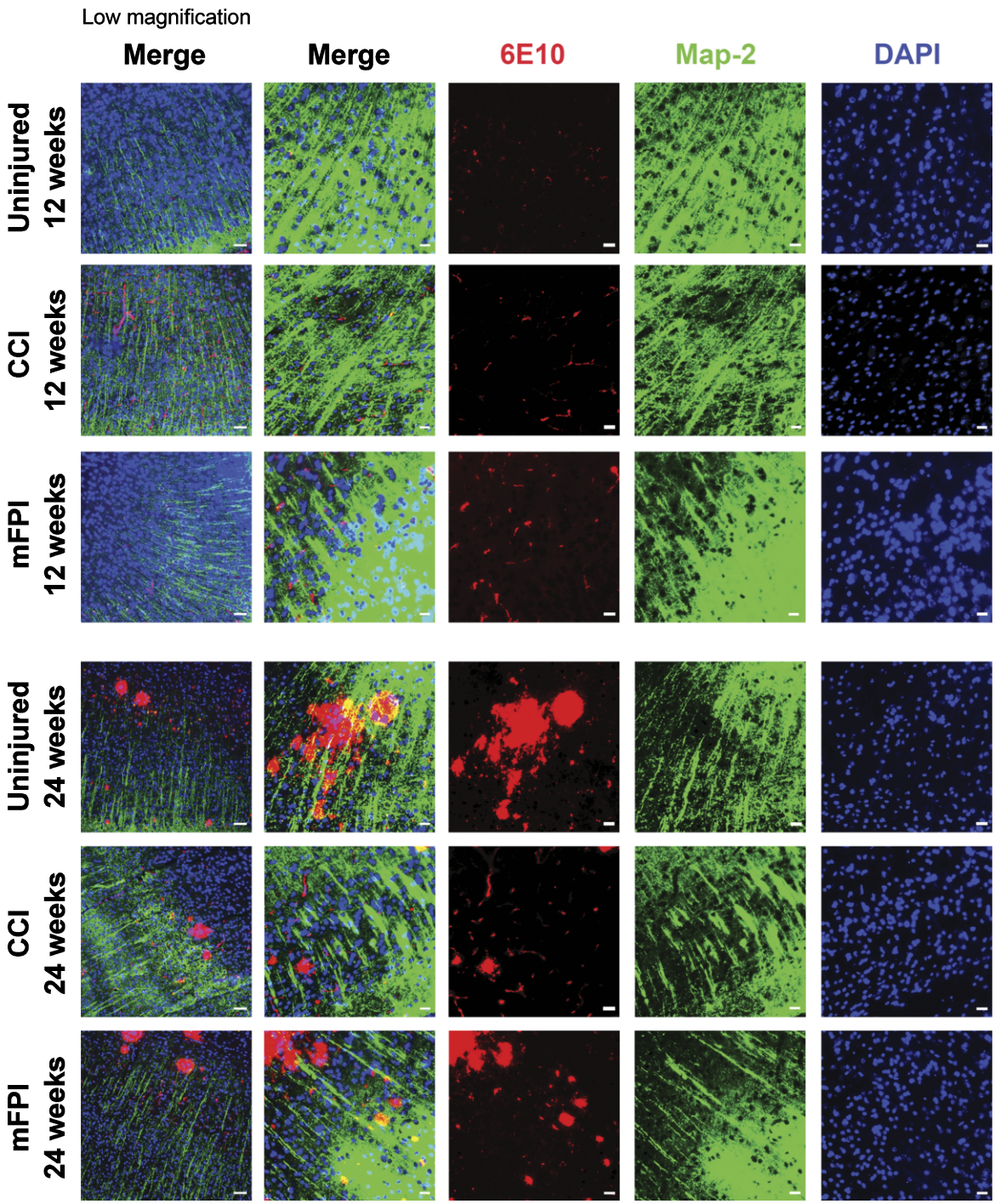

Fig. 7. Aβ pathology interrupts neuronal processes in older animals. Immunocytochemistry showed that there is a clear disruption of the MAP2 positive (green) neuronal processes in the areas of Aβ pathology (6E10, red), especially at the 24-week time point. Scale bars: Low magnification = 20 μm, high magnification = 50 μm.

Therefore, we analyzed the number of DCX positive neuroblasts in the tg-ArcSwe mouse brain following CCI or mFPI (Fig. 9A, B). We found newly formed neuroblasts in all mice at both time points, but there were fewer DCX positive cells at the later time point in both uninjured and injured animals. However, the

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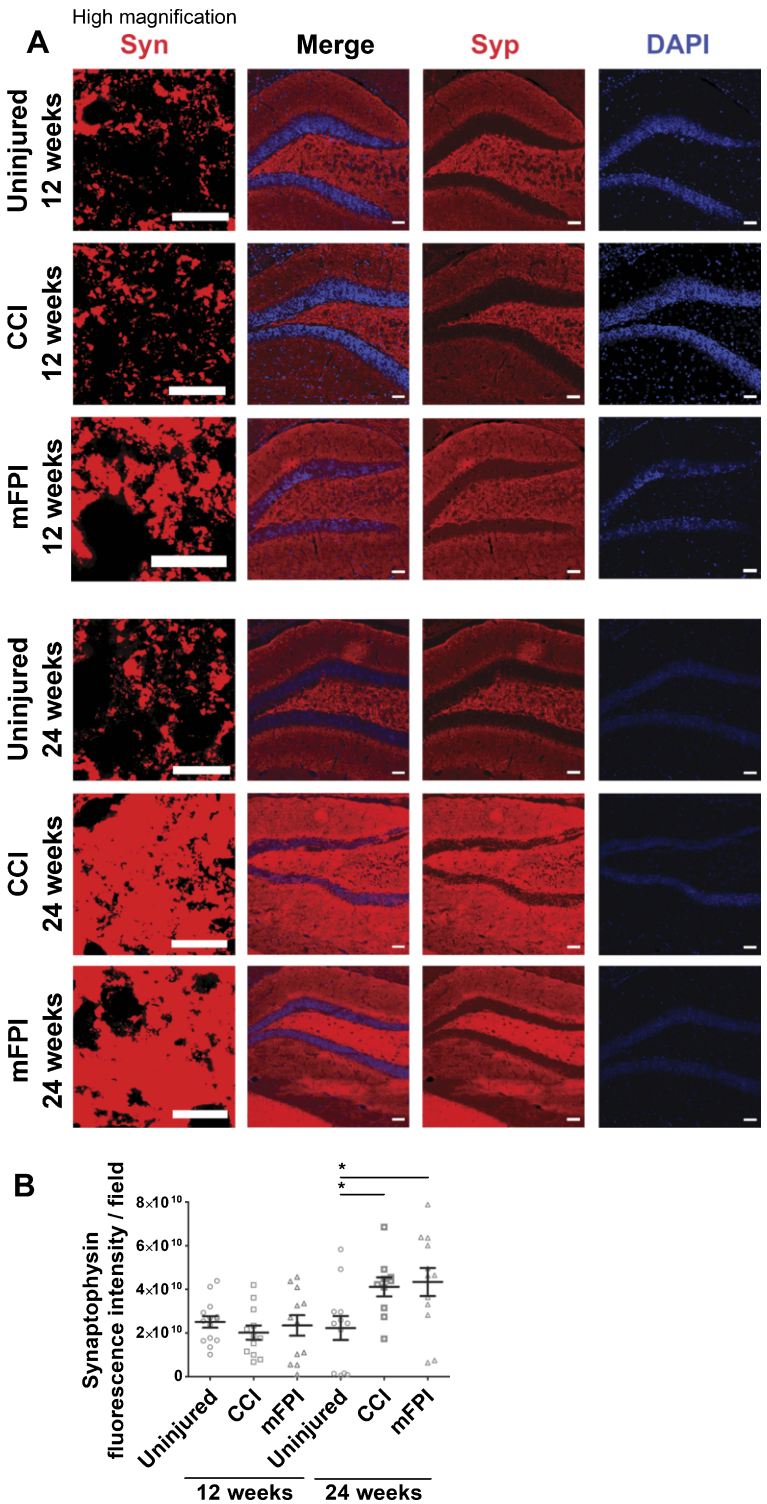

Fig. 8. Elevated expression of synaptophysin 24 weeks after TBI. Immunocytochemistry (A) followed by analysis of the fluorescence signal (B) demonstrated that there was no significant difference in synaptophysin (Syn, red) expression at 12 weeks following TBI, as compared to uninjured controls. At 24-weeks post-injury, it was a clear increase in synaptophysin expression in both the CCI and mFPI animals. The data was analyzed by nonparametric Mann Whitney test and the graph displays mean ± SEM. Scale bars: Low magnification = 10 μm, high magnification = 50 μm. *$p < 0.05$.

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TBI had no significant impact on the number of newly formed neuroblasts at these time points (Fig. 9B).

In order to investigate the effect of CCI and mFPI on hippocampal cell loss, we analyzed the cell number by labelling the cell nuclei with DAPI (Fig. 10A–C). Measurements of the hippocampus thickness revealed a significant reduction at 12-weeks post-TBI in mice that received CCI or mFPI, compared to uninjured controls (Fig. 10B). At the later time point, 24 weeks, the hippocampus were reduced in all animal groups and there was no longer any difference between uninjured and injured mice (Fig. 10B). A significant decline in the number of DAPI stained nuclei per area was also found in the CCI and mFPI animals at 12-weeks post-TBI (Fig. 10C). For the mFPI animals, the DAPI stained nuclei per area was still significantly lower than the uninjured animals at 24-weeks post-injury (Fig. 10C).

DISCUSSION

TBI is one of the leading causes of death and disability worldwide. In addition, it is known from epidemiological studies that moderate to severe TBI constitutes a risk factor for AD. Brain trauma induces multiple acute and chronic changes in the brain, for example oxidative stress, axonal degeneration, vascular damage, and widespread neuroinflammation [30]. However, the exact mechanism behind the increased risk to develop AD remains elusive.

The aim with the present study was to identify cellular processes that could link TBI to AD development, by investigating the chronic impact of two different injury models, controlled cortical impact (CCI) and midline fluid percussion injury (mFPI). It is important to remember that cellular events in animal models of AD and TBI may or may not reflect the disease processes in the human brain. In the human situation, the trauma often occurs years before AD onset. However, to mimic the disease process in a mouse model, the time line had to be adjusted. To study the effect of TBI on AD initiation and progression, it was important to induce the trauma before the mice show any visible Aβ pathology. Hence, 3 months was a good time point. The first investigated time point, when animals were 6 months old (12 weeks following TBI), coincides with the very first appearance of plaques in uninjured tg-ArcSwe mice and the second time point, when the animals were 9 months old (24 weeks following TBI), coincides with pronounced Aβ pathology in uninjured tg-ArcSwe mice. The long-term effect of the TBI on memory deficiency, Aβ pathology, neurodegeneration, and inflammation was investigated by MWM, PET imaging, immunohistochemistry, and biochemical analyses.

Our results from the MWM analysis demonstrate that mice that received CCI or mFPI performed significantly worse than uninjured tg-ArcSwe mice, especially at the later time point, which corresponds to an increased Aβ accumulation. Interestingly, we found that at the later time point, the mFPI mice performed worse than the CCI mice. A possible explanation for this could be that the diffuse injury induced by mFPI progresses more severely over time, compared to the more focal injury induced by CCI. The diffuse injury affects the white matter and axons to a much larger extent than the focal injury, and it is possible that the amyloid protein accumulation causes more cognitive deficits over time in this scenario compared to the more encapsulated focal injury caused by CCI. In a recent study, using a novel technique of tissue preparation, it was found that many axonal swellings after CCI probably were not as severe as reported earlier in the literature. This could suggest that the progression after CCI is rather slow [31]. In another strain of AD-mice (APP-PS1), it has been reported that young mice develop more post-TBI problems than older mice [32].

AD is characterized by key neuropathological features, including extracellular accumulation of Aβ in plaques, intracellular aggregation of microtubule-associated protein tau in neurofibrillary tangles and neuroinflammation. According to the amyloid cascade hypothesis, Aβ is the causative agent of AD, consequently driving the formation of neurofibrillary tangles, vascular damage, inflammation, and neuronal cell loss [2, 33]. Accumulation of Aβ in the AD brain is caused by an imbalance between Aβ production and Aβ clearance and mutations in amyloid-β protein precursor (AβPP) are known to result in early-onset AD by increasing the Aβ levels [34]. However, since the majority of patients with late-onset AD do not have an increased Aβ production, it has been suggested that the main cause of this form of the disease is instead insufficient lysosomal degradation [35, 36] or other factors resulting in increased cellular stress, such as oxidative stress, inflammation, or bacterial infections, which in turn result in the accumulation of aggregated proteins [5, 33].

Importantly, both Aβ and neurofibrillary changes begin during preclinical AD when cognitive deficits are not apparent [37]. It was shown in the present

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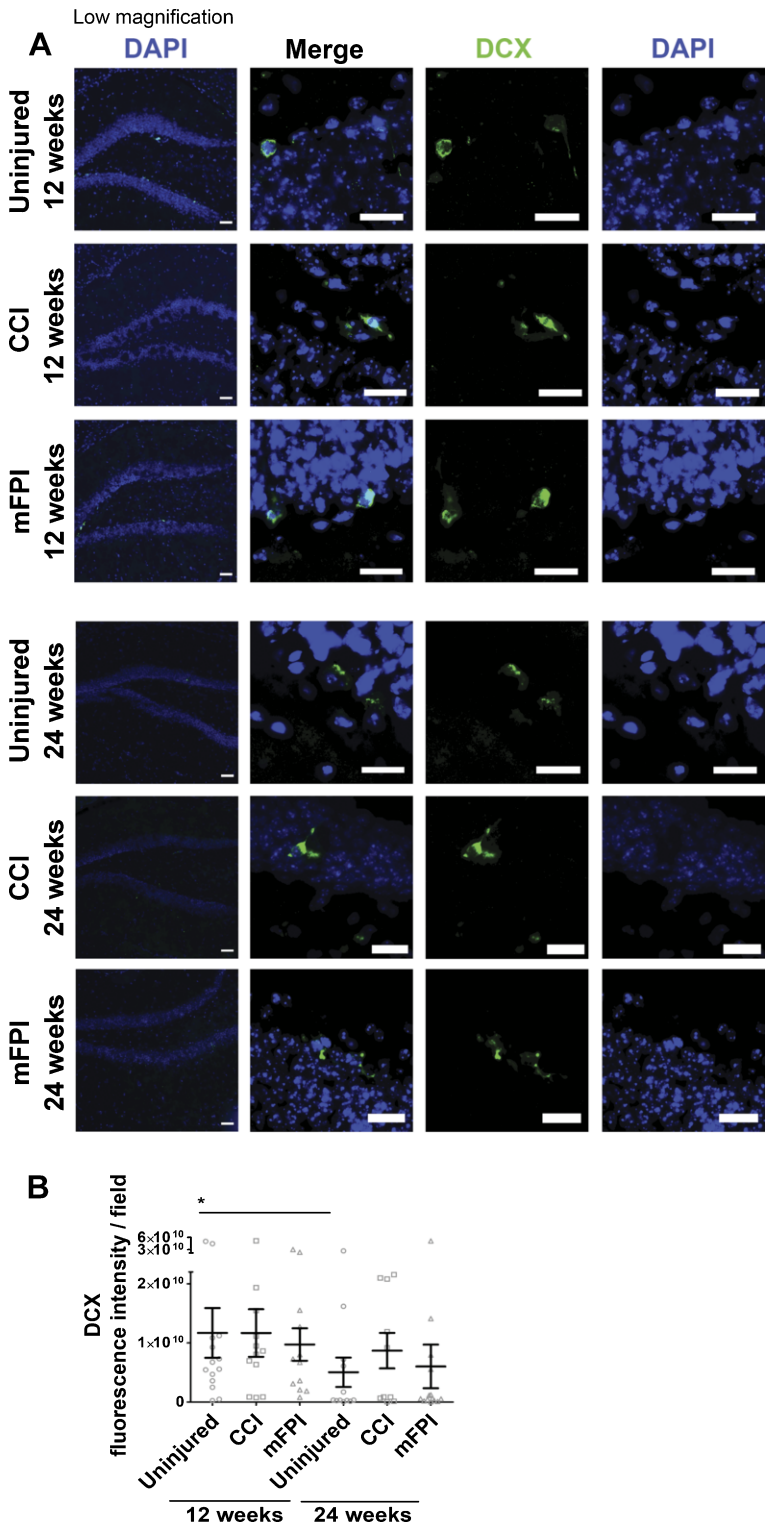

Fig. 9. No effect on neurogenesis 12 and 24 weeks after TBI. Immunohistochemistry demonstrated that double cortin (DCX, green) positive neuroblasts were present in the tg-ArcSwe mouse brain following CCI or mFPI and in uninjured controls (A). There was fewer DCX positive cells at the later time point in both uninjured and injured animals (B). The data was analyzed by nonparametric Mann Whitney test and the graph displays mean ± SEM. Scale bars: Low magnification = 20 μm, high magnification = 50 μm. *$p < 0.05$.

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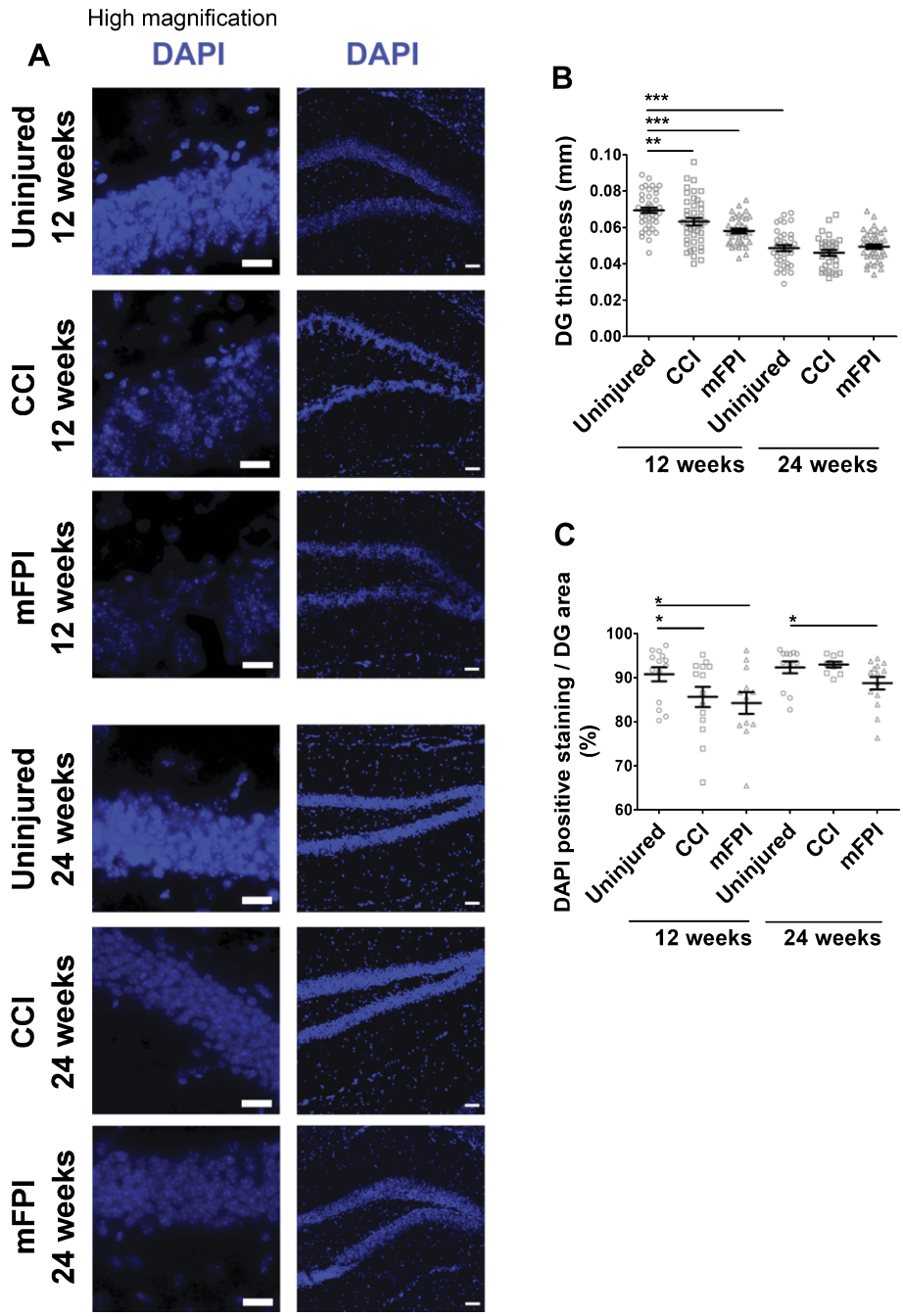

Fig. 10. Increased cell loss in hippocampus at 24 weeks following TBI. The nuclei of the cells were labelled with DAPI (A). There was a significant reduction in hippocampal thickness in both injury group at 12 weeks, as compared to uninjured tg-ArcSwe mice. At 24 weeks, the hippocampal thickness was reduced in all animal groups (B). The DAPI stained nuclei per area was also reduced in the CCI animals at 12 weeks and the mFPI animals at 12 and 24 weeks (C). The data was analyzed by nonparametric Mann Whitney test and the graphs display mean ± SEM. Scale bars: Low magnification = 20 μm, high magnification = 50 μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

study that TBI caused an earlier formation of Aβ pathology, compared to what is observed in the uninjured tg-ArcSwe brain, and further that Aβ levels could be monitored by PET. Likewise, the emergence of tau PET radioligands [38] might be an aid in the future to monitor *in vivo* changes leading to dementia in the brain after TBI. Interestingly, studies in patients using PET ligands for activated microglia

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found abnormal chronic inflammatory response up to 17 years after the TBI event [39], pointing to neuroinflammation as a chronic process which may affect brain function for years after TBI.

Immunohistochemistry analysis of Aβ burden in mice, following CCI or mFPI confirmed PET and ELISA results and demonstrated that both injuries resulted in increased pathology at 24-weeks, but not 12-weeks post-injury. Hence the injury induces a delayed accumulation of Aβ in the brain, several months following TBI. Studies of brain autopsies from young TBI patients who died during the acute phase after injury have demonstrated that Aβ plaques, similar to those found in AD patients, are present at the lesion site shortly after the trauma [40]. It is possible that this acute accumulation of Aβ promotes cellular dysfunction in the brain, i.e., degradation impairment that eventually results in the chronic Aβ pathology. Effective waste removal is crucial for maintaining a healthy brain and any alteration in the clearance capacity may contribute to the development of AD and dementia [41]. We have previously shown that astrocytes ingest large amounts of aggregated Aβ, but then store, rather than degrade the ingested material. The incomplete digestion results in a high intracellular load of neurotoxic Aβ species and severe lysosomal dysfunction [42]. Hence, the Aβ-induced degradation impairments will easily turn into a negative spiral.

Interestingly, the severe memory deficits in tg-ArcSwe mice following TBI could not be explained by synaptic loss. Instead, the synapse marker synaptophysin was increased in TBI mice compared to uninjured controls, indicating a compensatory induction of synaptogenesis. However, it was obvious that the increased synapse formation could not rescue the memory function, indicating that the regenerating synapses might be none-functional. Neuroregenerative processes are known to be induced by trauma or diseases in the brain, but apparently the plasticity cannot effectively compensate for the lost behavioral functions. It is possible that the increased regeneration can even result in adverse effects. For example, sprouting of mossy fibers following TBI has been suggested to result in epilepsy [43–45]. In contrast to the increased synaptogenesis, our quantification of DCX positive cells, showed that there is no long-term effect on neurogenesis following TBI in the tg-ArcSwe mice.

At 24-weeks post-injury, the mFPI mice demonstrated more severe memory deficits than the CCI mice. This result was in line with the long-term decline in the number of DAPI stained nuclei per area in hippocampus following mFPI, compared to uninjured control mice. Since mFPI results in widespread axonal injury throughout the brain, while CCI results in a local ipsilateral cortical contusion [22], there is much evidence that white matter injury is linked to much later cognitive impairment in patients (exhaustively reviewed by Filley and Kelly) [46]. One of the proposed mechanisms is widespread and chronic neuroinflammation [47] and another is an accelerated aging of the brain [48]. It is important to remember that wild type mice recover surprisingly well following TBI. For example, it was recently shown that mice that receive a single TBI perform as well as the uninjured controls in the MWM test at 6-months post-injury. However, repetitive injuries result in long-term memory deficits [49]. It is obvious from our analysis of cell density in the hippocampus that the mice recover rather well from the acute injury.

Carrying *APOE* ε4 is one of the strongest genetic risk factors in developing late-onset AD [50]. Interestingly, recovery from TBI also seem to be influenced by *APOE*. Inducing CCI in PDAPP mice (that develop AD symptoms), expressing human *APOE3*, human *APOE4*, or no *APOE*, indicated that *APOE4* may indeed influence the long-term neurodegenerative cascade after TBI via an effect on Aβ [51].

In this study we demonstrate that TBI resulted in a pronounced chronicastroglial response half a year after the trauma, based on the GFAP expression. Interestingly, there was a two-fold increase in GFAP-expression in both groups of injured mice at 24 weeks, compared to uninjured tg-ArcSwe controls, but there was no significant increase in GFAP expression, at 12 weeks. On the contrary, mice subjected to mFPI had lower GFAP expression 12 weeks after injury, compared to uninjured animals. This is a novel and interesting finding, since at 24 weeks the mFPI-subjected mice showed increased amount of GFAP, suggesting a transient decrease in reactive astrocytes after mFPI. In a more acute study of mild (not midline) FPI in wild type mice, Powell et al. found a peak of GFAP expression 3 days after injury and that the levels had returned to uninjured control levels 21 days after injury [52]. In our own laboratory, we have previously noticed an increase of GFAP-immunoreactivity up to 7 days after injury in wild type mice. However, the signs of a compromised blood-brain barrier peaked at 3 days after injury and decreased until day 7 [53]. This suggests that the mice recover after mild FPI initially. Our

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finding in the present study suggest that the diffuse injury in tg-ArcSwe mice may cause a decrease in reactive astrocytes compared to uninjured animals. To fully elucidate this phenomenon will require more research, but hypothetically, an initial stimulation of the astrocytes could result in a downregulation of GFAP-expression temporarily. As the diffuse injury, progresses between 12 and 24 weeks the astrocytes turn more reactive again, as seen in the increased GFAP-immunoreactivity at 24 weeks.

In a previous a study, we have demonstrated that the expression pattern of vimentin and GFAP differ in old tg-ArcSwe mice [54]. In 16-month-old tg-ArcSwe mice, vimentin-positive cells are found distinctly around the plaques, while GFAP-positive cells are found throughout the brain. Many of the reactive astrocytes co-express GFAP and vimentin. Among the cells only express one of the markers, there are more single positive cells for GFAP than for vimentin. Moreover, the expression of vimentin in the wild type mice is much lower than the GFAP expression and vimentin positive cells are only found in the hippocampus and surrounding the ventricles. In the present study we found that the expression of vimentin was markedly induced at 12-weeks post-injury, in astrocytes that were in close proximity to the injury-site. At the later time point vimentin positive, reactive astrocytes were frequently found in both injured and uninjured animals, but exclusively around and not at the injury site. Hence, the timing of the increase of the two markers are different. While the vimentin expression peaks at the earlier time point the expression of GFAP comes later. The explanation for this could be that the markers are expressed at different stages of the chronic inflammation or by different subclasses of astrocytes. For example, a subclass of the reactive astrocytes is known to acquire neural stem cell potential after injury [55]. Our immunostainings show that the vimentin + GFAP + cells were situated tightly around the plaques at the later time point, while vimentin– GFAP + cells were found also in the surrounding tissue. The role of reactive astrocytes in AD development has recently received much attention [56]. Their complex role in the AD brain is thought to depend largely on their release and uptake of substances from the microenvironment that they share with neurons. Being the most abundant glial cell, astrocytes play an important role in maintaining brain homeostasis [57]. Neurodegenerative diseases, including AD, are defined by loss of brain homeostasis, which at least partly could be due to the severely stressed astrocytes that are unable to fulfil their normal tasks [58]. Our previous studies indicate that astrocytes play a central role in the progression of AD, by accumulating and spreading toxic Aβ species [42, 59]. Interestingly, it was recently shown that wild type rats subjected to CCI, displayed several-fold increased MHCII expression as well as accumulation of phosphorylated tau and AβPP 6 months later [60]. Whether the MHCII expressing cells were astrocytes or microglia (or both) were however not studied.

Analysis of the Iba-1 reactivity following TBI in the present study showed that the activation of microglia followed the same pattern as the GFAP+astrocytes, with a two-fold increase of Iba-1 at 24 weeks after TBI. Similar to the GFAP expression there was no significant increase in Iba-1 expression at the earlier time point. Interestingly, CCI resulted in a more prominent late microglial activation, compared to mFPI. Moreover, Mac-2 positive microglia/macrophages were most prominent in the CCI-injured animals at 24 weeks post-injury. Taken together, the immunohistochemistry to glial markers indicate that the chronic inflammation is peaking rather late following injury and that CCI results in a more prominent long-term macrophage/microglial activation, compared to mFPI.

Conclusions

The relationship between TBI and AD is not understood. In the present study, we demonstrate that TBI induces severe memory deficits in a mouse model of AD, several months after the injury. The injured mice showed a late upregulation of reactive gliosis, which concurred with a more pronounced Aβ pathology, compared to uninjured AD mice. Our results suggest that delayed glial activation may occur following TBI. However, further studies in both experimental models and human TBI patients will be required to explore the relationship between this phenomenon and the onset of AD. By answering the question, whether and how TBI contributes to the development of AD later in life, we may also understand the crucial mechanisms of AD development in general.

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Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/19-0572r2).

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-190572.

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VEGF-C Induces Alternative Activation of Microglia to Promote Recovery from Traumatic Brain Injury

Shiming Ju, Chen Xu, Gan Wang and Lin Zhang*
Department of Neurosurgery, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

Abstract. Traumatic brain injury (TBI), a brain disorder that causes death and long-term disability in humans, is increasing in prevalence, though there is a lack of protective or therapeutic strategies for mitigating the damage after TBI and for preserving neurological functionality. Microglia cells play a key role in neuroinflammation following TBI, but their regulation and polarization by a member of the vascular endothelial growth factor (VEGF) family, VEGF-C, is unknown. Here, we show that VEGF-C induced M2 polarization in a murine microglia cell line, BV-2, *in vitro*, by a mechanism that required signaling from its unique receptor, VEGF receptor 3 (VEGFR3). Moreover, in a TBI model in rats, VEGF-C administration induced M2 polarization of microglia cells, significantly improved motor deficits after experimental TBI, and significantly improved neurological function following TBI, likely through a reduction in cell apoptosis. Together, our data reveal a previously unknown role of VEGF-C/VEGFR3 signaling in the regulation of post-TBI microglia cell polarization, which appears to be crucial for recovery from TBI.

Keywords: Microglia, polarization, traumatic brain injury, VEGF-C, VEGFR3

INTRODUCTION

Traumatic brain injury (TBI) causes death and long-term disability in humans, and its prevalence increases each year [1]. Although the majority of TBI cases only lead to mild to moderate injury, about 10% of severe cases require long-term health care and support services for substantial and lifelong cognitive, physical, and behavioral impairments [2–4]. The elderly appears to be much more vulnerable to TBI, and suffer more severe outcomes in terms of disability and the development of neurodegenerative and neuropsychiatric issues [5]. Recent studies have shown that TBI can predispose patients to develop neural disorders like Parkinson's disease (PD) and Alzheimer's disease (AD) [6]. Since there is a significant lack of therapeutic options for TBI, it is imperative to seek any protective or therapeutic strategies for alleviating the damage after TBI and for preserving neurological functionality.

TBI-triggered pathological processes involve a broad spectrum of cellular and molecular pathways, and can be classified into two phases [7]. The first phase is a primary damage phase from the initial insult. The pathological changes in this phase include

*Correspondence to: Lin Zhang, Department of Neurosurgery, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, School of Medicine, Shanghai Jiao Tong University, 600 Yishan Road, Xuhui District, Shanghai 200233, China. Tel.: +86 18930170167; E-mail: 18930170167@163.com.

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contusion, laceration, injury to axons, swelling of brain tissue, intracranial hemorrhage, and neural cell death [7]. The first phase is followed by the second phase, which lasts weeks. The pathological changes in the second phase include breakdown of the blood-brain barrier (BBB), increases in oxidative stress and glutamate excitotoxicity, progressive neurodegeneration, and delayed cell death [7]. Importantly, the inflammatory response during the first and second phase after TBI is often disorganized, which sometimes lead to further tissue injury and impairment of tissue repair [8].

Neuroinflammation is mediated by a number of cell types from both the CNS and the circulation. Astrocytes and microglia are cells from the CNS that play a pivotal role in the post-TBI inflammatory response [8]. Microglia, derived from erythromyeloid precursor cells, are specialized immune cells in the brain with phagocytic and antigen-presenting capabilities [7]. Extreme heterogeneity has been found in microglia and is largely a function of their activation state [9].

In the healthy brain, microglia are described as 'ramified' in a resting or quiescent state. Upon activation, microglia transform to a hypertrophic morphology for antigen-presentation, phagocytosis or the release of cytokines or hormones for tissue repair [9]. Previous studies have shown that microglia may have various roles after injury, through their complex responses in the context of brain injury [9]. The plasticity of microglia likely results from their phenotypic changes, much like macrophages outside the brain. However, the polarization of microglia has been minimally studied, especially during TBI [9].

Vascular endothelial growth factor (VEGF) receptor signaling plays a central role in vascularization and angiogenesis-related events [10]. The VEGF family is composed of 6 members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF) [11]. VEGFR3 mediates lymphangiogenesis in response to VEGF-C and VEGF-D [12]. Very recently, VEGF-C/VEGFR3 signaling has been shown to be involved in macrophage differentiation in non-brain tissue [13–17]. Moreover, VEGF-C/VEGFR3 signaling has been shown to regulate glial cell responses in mesenchymal stem cell transplantation in the rat brain [18]. Based on these previous reports, we hypothesized that VEGF-C/VEGFR3 signaling may play a role in the microglia cell response in TBI, and this hypothesis was examined in the current study.

MATERIALS AND METHODS

Protocol approval

The study protocol and experimental design were approved by the institutional Ethics Committee at Shanghai Jiao Tong University. All the animal experiments were approved by the Institutional Animal Care and Use Committee at Shanghai Jiao Tong University, and were performed strictly according to the guideline.

Cell culture and treatment with VEGFR3 inhibitor

BV-2 is a murine cell line generated by infecting primary microglia cells with a v-raf/v-myc oncogene-carrying retrovirus [19]. We obtained BV-2 cells from The Chinese Academy of Sciences (Beijing, China), and cultured them in Dulbecco's Modified Eagle's Medium supplied with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) in a 37°C incubator with 5% CO_2. Recombinant VEGF-C was purchased from R&D Systems (West Grove, PA, USA), and was used at a dose of 100 ng/ml. For suppression of VEGFR3 signaling, SAR131675 (Selleckchem, Houston, TX, USA) was applied to BV-2 cells at a concentration of 50 nM.

Cell transfection

The BV-2 cells were transfected with either short hairpin small interfering RNA for VEGFR3 (shVEGFR3) or a scrambled sequence under the control of a cytomegalovirus (CMV) promoter. The plasmids were prepared from a backbone pcDNA3.1-CMV-GFP plasmid (Clontech, Mountain View, CA, USA). The sequence for shVEGFR3 is 5′-CCCAGTATTGTGTGGTACAAA-3′. The sequence for the scrambled is 5′- GTGCCAAGAC GGGTAGTCA -3′, as described [20]. Transfection was performed using the Lipofectamine-3000 assay (Invitrogen).

Flow cytometry

F4/80 + Iba-1 + microglia cells were isolated by flow cytometry, after the fixed cells were incubated with APC-conjugated anti-F4/80 and Pacific blue-conjugated anti-Iba-1 antibodies. CD206 + M2 cell analysis and sorting were performed after the living cells were labeled with PEcy7-conjugated

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anti-CD206 antibodies (Becton-Dickinson Biosciences, San Jose, CA, USA). For analysis of cell apoptosis, dissociated living cells were double-stained with FITC-Annexin V and propidium iodide (PI) with an FITC Annexin V Apoptosis Detection Kit I (Becton-Dickinson Biosciences). Annexin V + PI- cells were determined to be (early) apoptotic cells. Flow cytometry was performed using a FACSAria flow cytometer (Becton-Dickinson Biosciences). Negative controls were applied to remove background noise and to confirm positive cells. Data were analyzed and quantified using Flowjo software (Flowjo LLC, Ashland, OR, USA).

Arginase activity measurement

Arginase activity was assessed in cell lysates indirectly by measuring urea concentration generated by the arginase-dependent hydrolysis of l-arginine, using an arginase activity assay kit (Sigma-Aldrich). The samples and standards (100 μL/well) were transferred in triplicate to a 96-well plate for checking the optical density at 540 nm with a 690 nm correction. Sample concentrations were determined from the standard curve and converted to Arginase units.

RT-qPCR

RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany), and then was used as a template for cDNA synthesis. Quantitative PCR (RT-qPCR) was performed in duplicates using the QuantiTect SYBR Green PCR Kit (Qiagen). Data were assessed using 2-ΔΔCt method. The primers used for quantitative PCR sequences were as follows: VEGFR3: GCCCGAGGACGAGGGTGACT, CCTGGCTGCGCCTATCCTGC; Arg-1: TGAGAGACCACGGGGACCTG, GCACCACACTGACTCTTCCATTC; Fizz1 (Retnla: resistin-lke-α): CCATAGAGAGATTATCGTGGA, TGGTCGAGTCAACGAGTAAG; Ym1 (Chi3l3: chitinase 3-like 3): TGGAATTGGTGCCCCTACAA, AACTTGCACTGTGTATATTG; β-actin: AAATCTGGCA CCACACCTTC, GGGGTGTTGAAGGTCTCAAA. The values of genes were determined by sequential normalization to β-actin and experimental controls.

Western blotting and immunocytochemistry

Cell or tissue extracts were lysed in radioimmunoprecipitation assay (RIPA) buffer (Invitrogen) supplied with the Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA). Protein concentrations were determined with a BCA protein assay kit (Bio-rad, Bejing, China). Primary antibodies were rabbit anti-caspase 3, anti-brain-derived neurotrophic factor (BDNF), and anti-β-actin (Cell Signaling, San Jose, CA, USA). The secondary antibody was HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). β-actin was used as a protein loading control. Immunoreactivity was detected by the chemiluminescence method (Thermo Scientific, San Jose, CA, USA). Protein levels were obtained by sequential normalization to β-actin and experimental controls. The primary antibodies used in immunocytochemistry were rabbit anti-Iba-1, anti-VEGFR-C and anti-VEGFR3 (R&D Systems). DAPI (4′,6-diamidino-2-phenylindole) was used to stain nuclei.

Rat manipulations

Male Sprague–Dawley Rats (260–300 g) were obtained from the SLAC Laboratory Animal Co. Ltd (Shanghai, China). Rats were kept at 25°C, 50–60% humidity, and were fed on standard pellet chow and water. The TBI model was developed as described using a weight—drop device [14]. Briefly, a midline incision was made on the anaesthetized rats, after which the skull, right parietal craniotomy and dura were exposed with a high—speed microdrill. Then, a 40—G steel weight fell freely through a vertical tube from 2.5 m onto the motor cortex to induce TBI. Following injury, the bone flap was placed *in situ* and the scalp was sutured. Control rats (CTL) received a sham operation without TBI. Recombinant VEGF-C was injected intracranially at a dose of 5 μg.

Rotarod task

A rotarod was used to assess motor function as previously described (15). Briefly, a 7 cm diameter cylinder was positioned 1.2 m above a foam pad while speed and acceleration were controlled by computer interface (San Diego Instruments, San Diego, CA, USA). For each trial, the rat was placed on the rotating barrel, the speed was accelerated from 4 to 40 rpm over a period of 5 min, and the latency to fall was recorded. Pre-training occurred once a day for the 3 days preceding injury. Following injury, rats were re-tested on the rotarod task on days 1, 3, 5, 7, and 14 post-TBI. Each rat underwent four test trials per day.

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The average latency of the total of the four trials was calculated.

Neurological scores

Neurological function was evaluated on the basis of Modified Garcia Scale [21].

Brain water content

Rat brains were rapidly removed and weighed immediately at sacrifice (wet weight). The brains were then dried in an oven at 105°C for 24 h and weighed again (dry weight). Brain water content was calculated as [(wet weight - dry weight)/wet weight] × 100%.

Determination of BBB permeability

BBB permeability was quantitatively evaluated by Evans blue (EB; Sigma-Aldrich) extravasation analysis. Briefly, rats were injected intravenously with EB at a dose of 2% wt/vol in saline; 4 mL/kg body weight. EB was allowed to circulate in each animal for 40 min prior to determination of sample EB concentration using external standards ranging from 50 to 1000 ng/mL, prepared in the same solvent. The supernatant was quantified for absorbance of EB dye using a spectrophotometer (excitation 620 nm, emission 680 nm).

Statistical analysis

All of the statistical analyses were performed using the GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Statistical analysis of group difference was carried out using a one-way analysis of variance (ANOVA) test followed by Turkey multiple comparison *post-hoc* analysis. All values represent the mean ± standard deviation (SD). A value of $p < 0.05$ was considered significant after Bonferroni correction.

RESULTS

BV-2 microglia cells express both VEGF-C and its receptor VEGFR3

The BV-2 cell line is a broadly used murine microglia cell line. First, we analyzed the properties of the BV-2 cells. By flow cytometry, we found that BV-2 cells expressed both F4/80 and CD11b, two surface markers for macrophages (Fig. 1A). Moreover, by immunocytochemistry, we found that BV-2 cells expressed Iba-1, a specific marker for microglia in the brain (Fig. 1B). Since Iba-1 was not expressed by macrophages with a non-brain origin, our data suggest that BV-2 is a specific microglia cell line, which is distinguishable from normal macrophages.

Since a previous study has shown that VEGF-C and its unique receptor VEGFR3 are expressed by glial cells [18], we examined whether BV-2 cells also express VEGF-C and VEGFR3. We found that nearly all BV-2 cells are positive for VEGFR3, and the majority of BV-2 cells also expressed VEGF-C (Fig. 1B), consistent with previous reports that suggest an autocrine secretory pattern for VEGF-C/VEGFR3 in glial cells [18].

VEGF-C induces M2 polarization of BV-2 cells in a VEGFR3-dependent manner

Next, BV-2 cells were used as microglia cells to be tested for the possibility of VEGF-C-induced cell polarization. First, BV-2 cells were transfected with shVEGFR3, resulting in about 80% knockdown of VEGFR3 (Fig. 2A). Next, a co-culture system was applied. In the first condition, which was called BV (1), the BV-2 cells were cultured with media control. In the second condition, which was called BV (2), the BV-2 cells were cultured with recombinant VEGF-C. In the third condition, which was called BV (3), the BV-2 cells were cultured with recombinant VEGF-C in the presence of a specific VEGFR3 inhibitor, SAR131675. In the fourth condition, which was called BV (4), the BV-2-shVEGFR3 cells were cultured with recombinant VEGF-C (Fig. 2B). To assess for M2 macrophage polarization by VEGF-C, we checked the mRNA levels of Arginase 1 (Arg-1), Ym-1, and Fizz-1, three M2 macrophage markers. We found that VEGF-C induced an approximately 25 times' increase in Arg-1 mRNA (Fig. 2C), approximately 250 times' increase in Ym-1 mRNA (Fig. 2D), and approximately 13 times' increase in Fizz-1 mRNA (Fig. 2E). A typical characteristic of M2 macrophages is their potential role in converting L-arginine to ornithine and urea through the action of Arg-1. Thus, arginase activity was determined using a urea-based assay, showing significant increases in VEGF-C-treated macrophages (Fig. 2F). CD206 is a surface marker exclusively expressed by M2 macrophages. We found that CD206 was induced in VEGF-C-treated-macrophages by flow cytometry (Fig. 2G). All these M2-like changes in BV-2 cells

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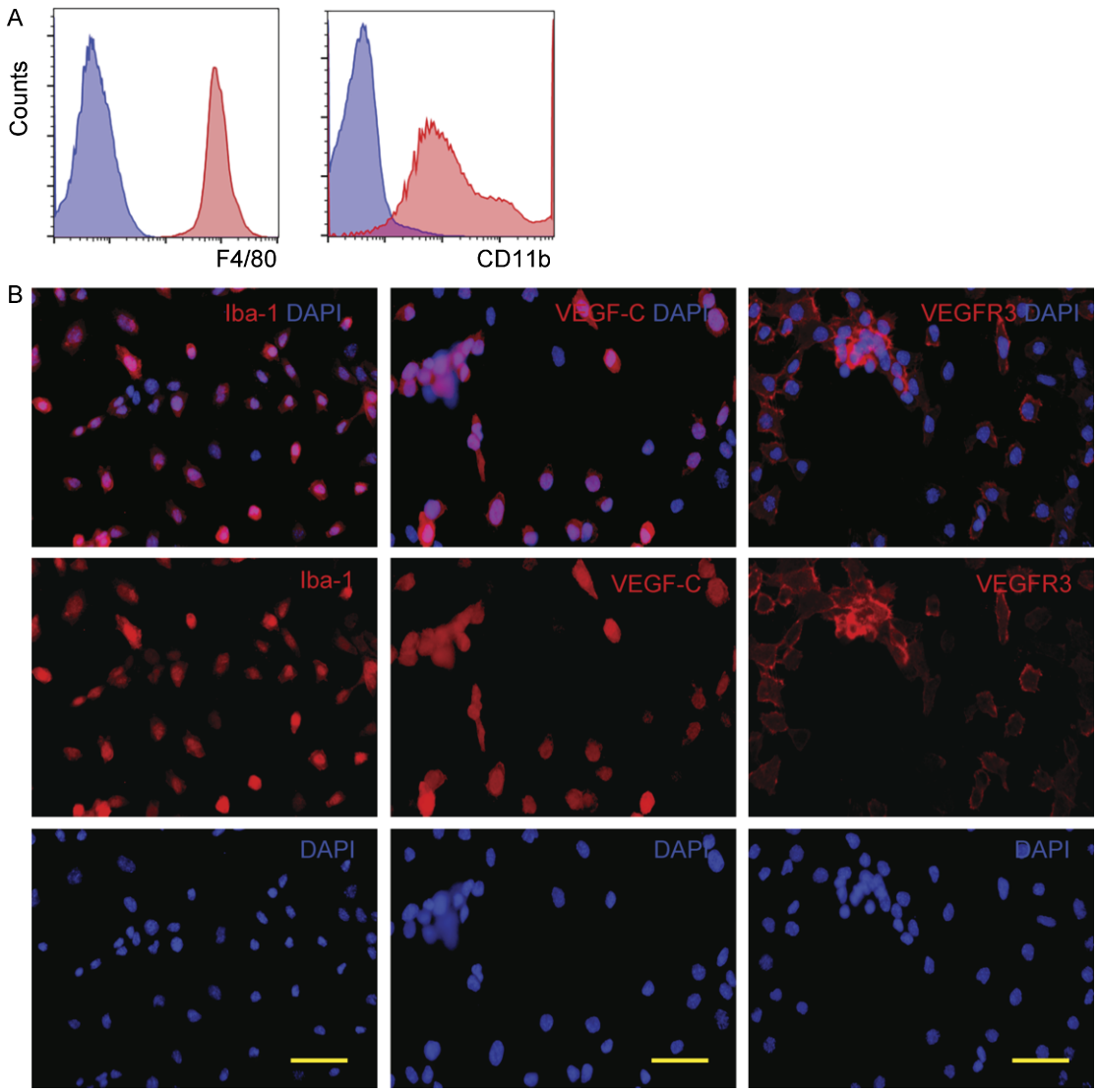

Fig. 1. BV-2 microglia cells express both VEGF-C and its receptor VEGFR3. A) We analyzed the expression of F4/80 and CD11b on BV-2 cells by flow cytometry, shown as representative flow charts. Blue: isotype control. Red: with antibody. B) Immunocytochemistry for Iba-1, VEGF-C and its unique receptor VEGFR3 in BV-2 cells. Scale bars are 20 μm.

were significantly attenuated by either the presence of SAR131675, or using VEGFR3-depleted BV-2 cells (Fig. 2C–G), suggesting that VEGF-C induces M2 polarization of BV-2 cells in a VEGFR3-dependent manner.

VEGF-C administration improves motor deficits after experimental TBI

TBI was induced in mice. VEGF-C or control saline were intracranially injected into the rat brain. The rats were separated into 3 groups. Group 1 comprised a sham operation with saline injection (CTL). Group 2 comprised of TBI with saline injection (TBI). Group 3 comprised of TBI with VEGF-C injection (TBI + VEGF–C). Brain function was assessed by a timed rotarod task method, and neurologic score was evaluated at 14 days post-TBI (Fig. 3A). We found that the latency to fall was significantly shortened in rats after TBI, a finding which was significantly attenuated in TBO-rats that had received VEGF-C (Fig. 3B). Thus, VEGF-C

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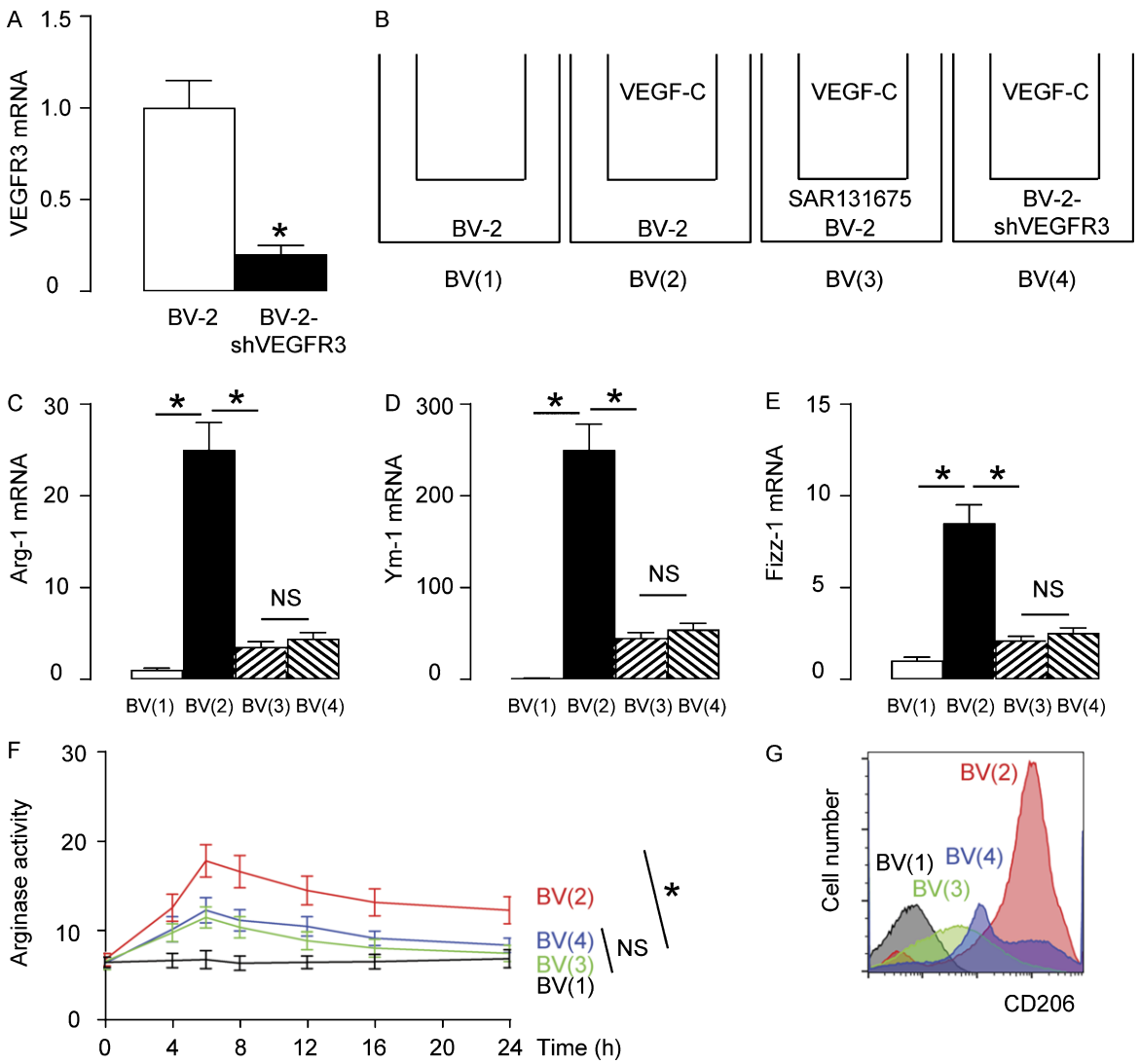

Fig. 2. VEGF-C induces M2 polarization of BV-2 cells in a VEGFR3-dependent manner. A) RT-qPCR for VEGFR3 on BV-2 cells transfected with plasmids carrying scrambled sequence (BV-2) and BV-2 cells transfected with shVEGFR3 (BV-2-shVEGFR3). B) Schematic of a co-culture system. In the first condition, which was called BV (1), the BV-2 cells were cultured with media control. In the second condition, which was called BV (2), the BV-2 cells were cultured with recombinant VEGF-C. In the third condition, which was called BV (3), the BV-2 cells were cultured with recombinant VEGF-C in the presence of a specific VEGFR3 inhibitor, SAR131675. In the fourth condition, which was called BV (4), the BV-2-shVEGFR3 cells were cultured with recombinant VEGF-C. C-E) RT-qPCR for Arginase 1 (Arg-1; C), Ym-1 (D) and Fizz-1 (E) on BV (1), BV (2), BV (3), and BV (4). F) Measurement of arginase activity. G) CD206 levels on BV (1), BV (2), BV (3), and BV (4) by flow cytometry. $^{*}p < 0.05$. NS, non-significant. N = 5.

administration improves motor deficits after experimental TBI.

VEGF-C administration improves brain function after experimental TBI

At 14 days after TBI, the neurological score in TBI rats was significantly reduced, a finding which was significantly attenuated in TBO-rats that had received VEGF-C (Fig. 4A). We then assessed the brain water content. We found that TBI significantly increased the brain water content, a finding which was significantly attenuated by VEGF-C (Fig. 4B). BBB permeability was also evaluated using an EB extravasation assay. We found that TBI significantly increased EB extravasation, a finding which was

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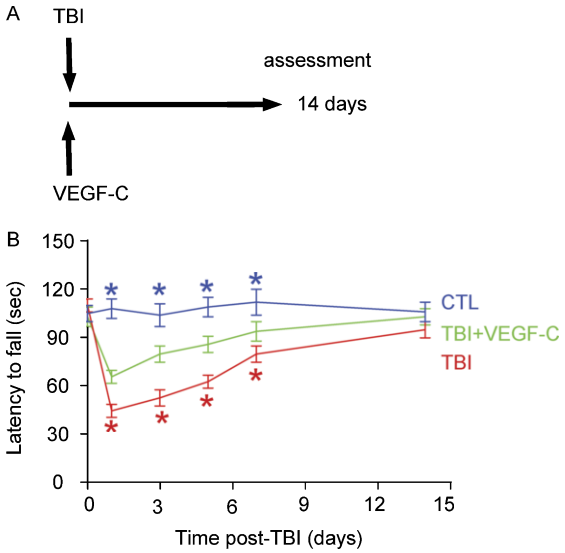

Fig. 3. VEGF-C administration improves motor deficits after experimental TBI. A) Schematic of the experiment. TBI was induced in mice. VEGF-C or control saline was intracranially injected into rat brain. The rats were separated into 3 groups. Group 1 comprised a sham operation with saline injection (CTL). Group 2 comprised TBI with saline injection (TBI). Group 3 comprised TBI with VEGF-C injection (TBI+VEGF-C). Brain function was assessed by a timed rotarod task method, and the neurologic score was evaluated at 14 days post-TBI. B) Measurement of the latency to fall in the rotarod task. Red $^{*}p < 0.05$, TBI versus TBI + VEGF-C; Blue$^{*}p < 0.05$, CTL versus TBI + VEGF-C; N = 10.

significantly attenuated by VEGF-C (Fig. 4C). BDNF is a marker for neural regeneration and high BDNF levels are often associated with preservation of neural function. We found that BDNF levels in the TBI-rat brain were significantly upregulated by VEGF-C (Fig. 4D). Together, these data suggest that VEGF-C administration improves brain function after experimental TBI.

VEGF-C reduces cell apoptosis in the TBI-brain

The effects of VEGF-C on cell apoptosis were then assessed by Annexin V assay on dissociated brain cells. We found that TBI significantly increased apoptotic cells in the brain, which was significantly attenuated by VEGF-C administration, shown by representative flow charts (Fig. 5A), and by quantification (Fig. 5B). Moreover, a key protein for apoptosis, cleavage caspase 3, was significantly upregulated in the TBI-brain, which was found to be significantly attenuated by VEGF-C (Fig. 5C), while a key anti-apoptotic protein, Bcl-2, was significantly downregulated in the TBI-brain, which was significantly attenuated by VEGF-C (Fig. 5D). These data suggest that VEGF-C administration may improve the function of the TBI-brain through reduction in cell apoptosis.

VEGF-C reduces microglia polarization to M2 in vivo

Finally, we examined the effects of VEGF-C on microglia polarization *in vivo*. The microglia cells were purified from the dissociated rat brain post-TBI by double positivity for F4/80 and Iba-1 (Fig. 6A). First, we examined expression of CD206 on those microglia cells in three conditions (CTL, TBI, TBI + VEGF-C). We found that CD206 was induced in the microglia cells from VEGF-C-treated-TBI-rats by flow cytometry (Fig. 6B). Moreover, significant increases in Arg-1 mRNA (Fig. 6C), in Ym-1 mRNA (Fig. 6D), and in Fizz-1 mRNA (Fig. 6E) were also induced in the microglia cells from VEGF-C-treated-TBI-rats. Together, these data suggest that VEGF-C reduces microglia polarization to M2 *in vivo*.

DISCUSSION

The increasing prevalence of TBI correlates with increasing societal burden, though there is currently a lack of protective or therapeutic strategies for alleviating the damage after TBI and for preserving neurological functionality [5, 6]. Microglia cells play a pivotal role in the post-TBI inflammatory response [8]. Microglia cells express a large diversity of cell surface markers, which allow them to be distinguished from peripheral macrophages. Unlike peripheral macrophages, microglia cells express low CD45 [22], high CX3CR1 [23], and specially ionized calcium binding adapter molecule 1 (Iba-1) [24]. The latter was used in the current study to isolate microglia cells from the rat brain.

Microglia cells often display a prolonged and persistent pattern of activation after TBI, and may have both beneficial and detrimental effects on the recovery of brain tissue [9]. Of note, these multiple effects of microglia cells largely stem from their differentiation potential [25]. Indeed, as a specific tissue-resident macrophage in the brain, microglia cells share a lot of common properties with peripheral macrophages, including M1/M2 polarization. The microglia response in TBI highly depends on the timing and nature of the

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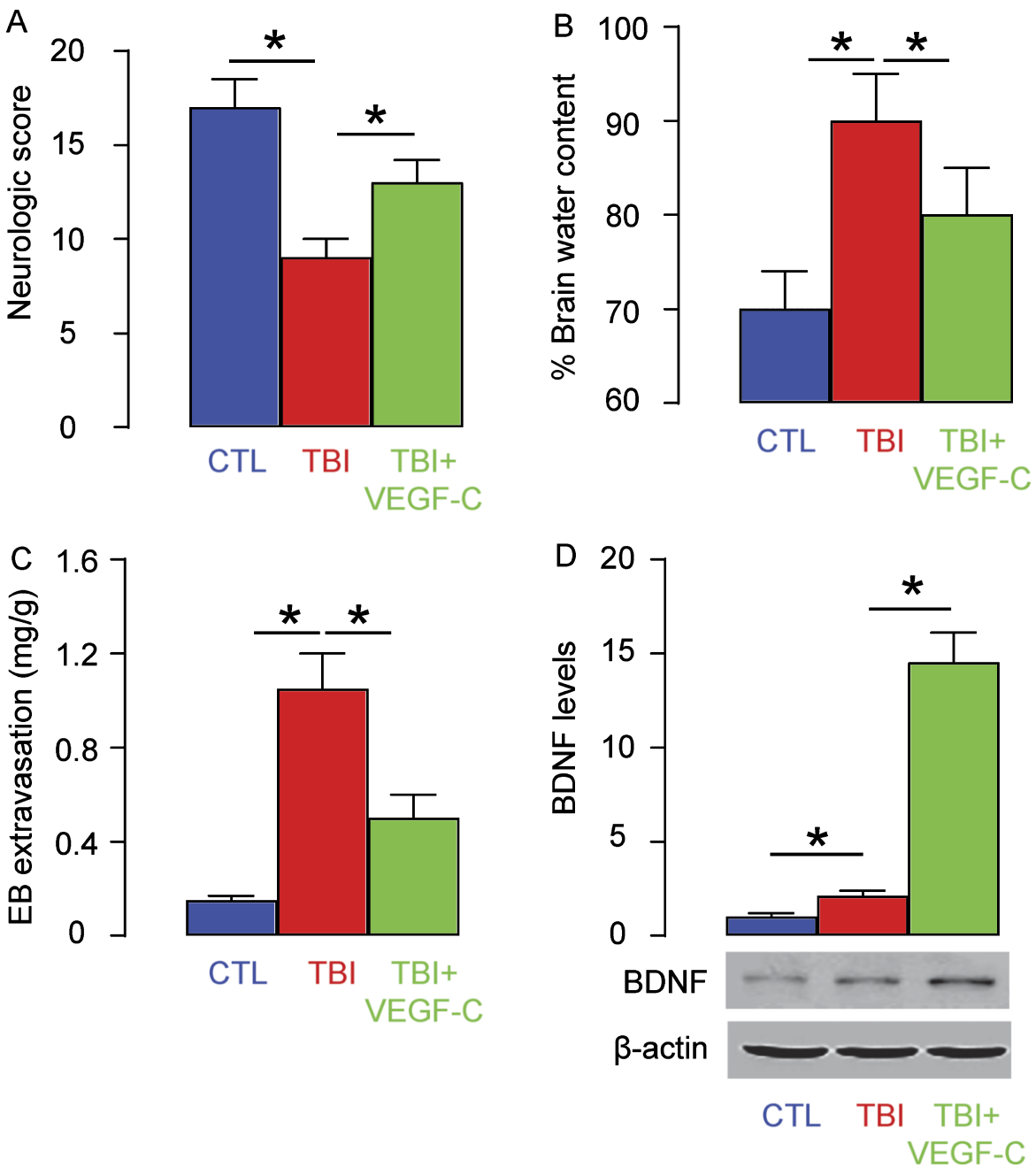

Fig. 4. VEGF-C administration improves brain function after experimental TBI. A) The neurological score in TBI rats was assessed at 14 days after TBI. B) Brain water content at 14 days after TBI. C) EB extravasation assay. D) Western blotting for BDNF levels in the rat brain. $^{*}p < 0.05$. N = 10.

injury, in which microglia acquire heterogeneous phenotypes in response to insults with certain environmental cues. As in macrophages, M1 microglia exhibit some phagocytosing capability, while M2 microglia are considered neuroprotective [26]. However, in previous studies, VEGF-C has not been shown to be a trigger for M2 polarization of microglia.

Here, consistent with a previous study which showed both VEGF-C and VEGFR3 expression on glial cells [18], we showed that VEGF-C and VEGFR3 were both expressed in a microglia cell

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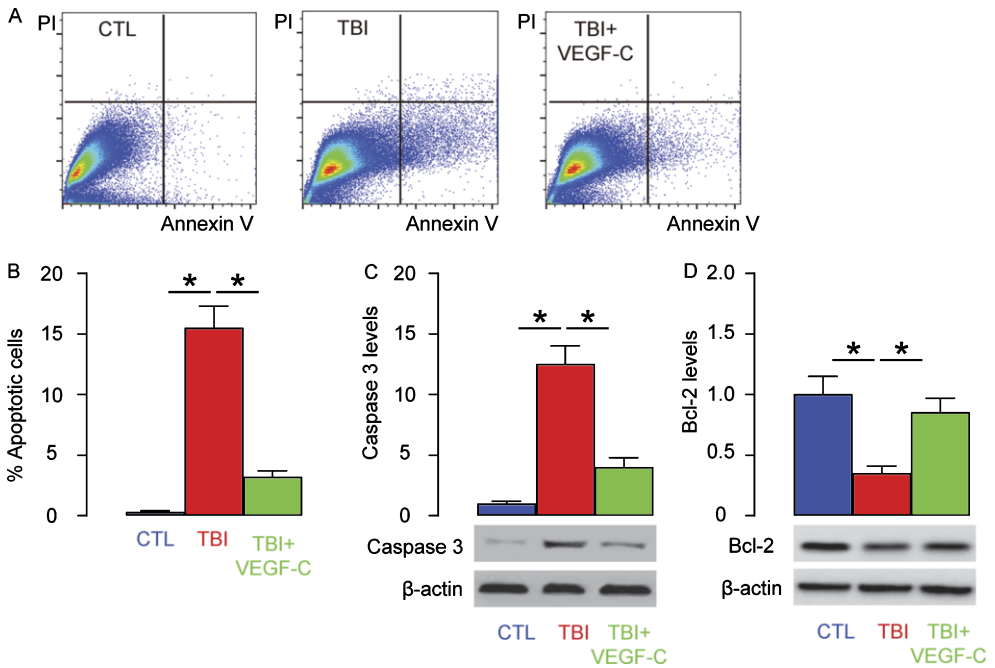

Fig. 5. VEGF-C reduces cell apoptosis in the TBI-brain. The effects of VEGF-C on cell apoptosis were then assessed by Annexin V assay on dissociated brain cells, shown by representative flow charts (A), and by quantification (B). Western blotting for cleavage caspase 3 (C) and Bcl-2 (D) in dissociated brain cells. $^{*}p < 0.05$. N = 10.

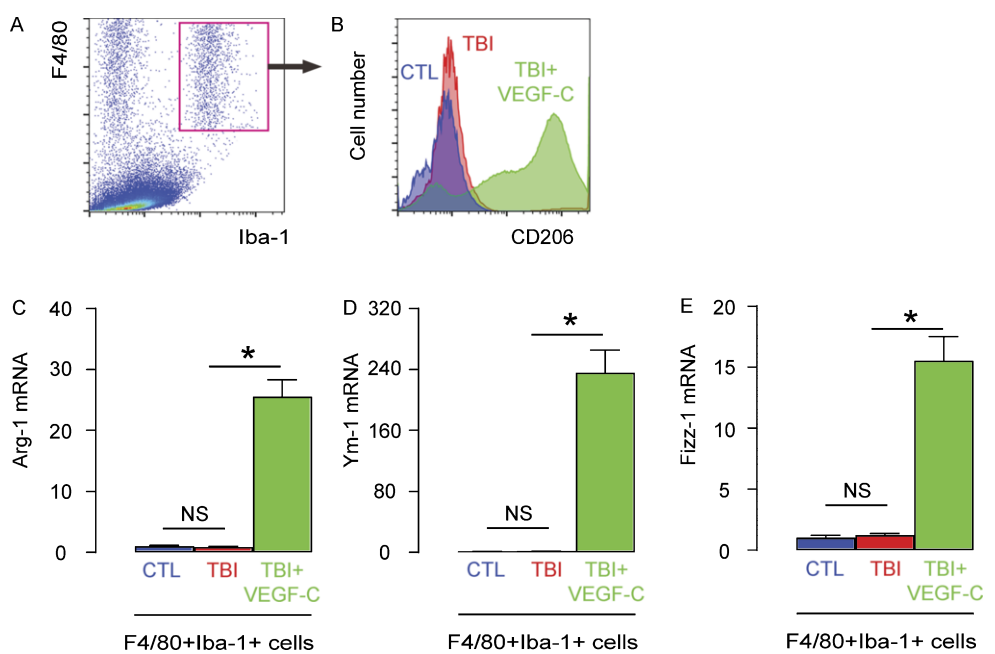

Fig. 6. VEGF-C reduces microglia polarization to M2 *in vivo*. A) The microglia cells were purified from the dissociated rat brain post-TBI by double positivity for F4/80 and Iba-1, shown by a representative flow chart. B) Flow cytometry for CD206 on these microglia cells in three conditions (CTL; TBI; TBI+VEGF-C). C-E) RT-qPCR for Arginase 1 (Arg-1; C), Ym-1 (D) and Fizz-1 (E) on F4/80 + Iba-1 + microglia cells. $^{*}p < 0.05$. NS, non-significant. N = 10.

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line. Since VEGFR3 seemed to be expressed by all the cells, while VEGF-C was only produced by some cells, we hypothesized that VEGF-C expression may represent a more active status of microglia cells, and that this VEGF-C/VEGFR3 signaling may function in an autocrine manner to amplify the response to VEGF-C. Most interestingly, triggering of this pathway by an external source of VEGF-C induced M2-like polarization of microglia cells *in vitro*, supporting our hypothesis.

To confirm our hypothesis, we used a TBI model in rats to assess the effects of VEGF-C administration. In order to analyze a clean microglia cell population, we had to use F4/80 and Iba-1 as markers for purification, since F4/80 is able to isolate peripheral macrophages and microglia cells from other cell types in the brain, while Iba-1 is only expressed on microglia cells, but not macrophages [24]. Iba-1 is not a surface marker, so we had to fix and perform penetration of the cells before analysis. Therefore, we were not able to perform an arginase activity assay on these fixed cells. However, the RT-qPCR data and results for expression of the cell surface marker CD206 supported an M2 polarization of microglia cells by VEGF-C. Of note, the beneficial effects of VEGF-C on protection and recovery of brain function, as well as on reduction in cell apoptosis, may result from both M2 polarization of microglia cells and augmentation of lymphangiogenesis [27], a classical function of VEGF-C, although so far there is no evidence that lymphangiogenesis plays a role in the pathological events post TBI [28].

To the best of our knowledge, the current study is the first to demonstrate a beneficial role of VEGF-C in treating TBI, which also suggests a possible mechanism of M2 polarization of microglia cells. Future studies may be designed to further characterize this model, in order to assess the possible use of VEGF-C for therapy in humans.

DISCLOSURE STATEMENT

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/19-0063r1).

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Perfusion Neuroimaging Abnormalities Alone Distinguish National Football League Players from a Healthy Population

Daniel G. Amen[a], Kristen Willeumier[a], Bennet Omalu[b], Andrew Newberg[c], Cauligi Raghavendra[d] and Cyrus A. Raji[e,*]
[a]*Amen Clinics, Inc., Costa Mesa, CA, USA*
[b]*Department of Pathology, University of California, Davis, CA, USA*
[c]*Myrna Brind Center of Integrative Medicine, Thomas Jefferson University, Philadelphia, PA, USA*
[d]*Department of Computer Science, University of Southern California, Los Angeles, CA, USA*
[e]*Department of Radiology, University of California Los Angeles Medical Center, Los Angeles, CA, USA*

Abstract.
Background: National Football League (NFL) players are exposed to multiple head collisions during their careers. Increasing awareness of the adverse long-term effects of repetitive head trauma has raised substantial concern among players, medical professionals, and the general public.
Objective: To determine whether low perfusion in specific brain regions on neuroimaging can accurately separate professional football players from healthy controls.
Method: A cohort of retired and current NFL players ($n = 161$) were recruited in a longitudinal study starting in 2009 with ongoing interval follow up. A healthy control group ($n = 124$) was separately recruited for comparison. Assessments included medical examinations, neuropsychological tests, and perfusion neuroimaging with single photon emission computed tomography (SPECT). Perfusion estimates of each scan were quantified using a standard atlas. We hypothesized that hypoperfusion particularly in the orbital frontal, anterior cingulate, anterior temporal, hippocampal, amygdala, insular, caudate, superior/mid occipital, and cerebellar sub-regions alone would reliably separate controls from NFL players. Cerebral perfusion differences were calculated using a one-way ANOVA and diagnostic separation was determined with discriminant and automatic linear regression predictive models.
Results: NFL players showed lower cerebral perfusion on average ($p < 0.01$) in 36 brain regions. The discriminant analysis subsequently distinguished NFL players from controls with 90% sensitivity, 86% specificity, and 94% accuracy (95% CI 95-99). Automatic linear modeling achieved similar results. Inclusion of age and clinical co-morbidities did not improve diagnostic classification.
Conclusion: Specific brain regions commonly damaged in traumatic brain injury show abnormally low perfusion on SPECT in professional NFL players. These same regions alone can distinguish this group from healthy subjects with high diagnostic accuracy. This study carries implications for the neurological safety of NFL players.

Keywords: Brain imaging, cognition, concussion, football, NFL, SPECT, traumatic brain injury

INTRODUCTION

The current public dialogue regarding the possible risks of repetitive head trauma playing football is not unlike the sport itself, with intense exchanges of

*Correspondence to: Cyrus A. Raji, MD, PhD, UCLA Medical Center, Los Angeles, CA, USA. E-mail: cyrusraji@gmail.com.

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opposing perspectives [1]. The genesis of this recent debate was the discovery of neuropsychiatric symptoms linked to a distinct neuropathological entity in former National Football League (NFL) players, chronic traumatic encephalopathy (CTE) [2, 3]. Noticeably lacking in this dialogue are assertions weighted in data from living professional football players. Several small studies have described structural and functional brain abnormalities in this group. One study of 28 former NFL players found lower hippocampal volumes in those with a history of concussion leading to loss of consciousness compared to 27 controls [4]. This same cohort was found to have white matter tract abnormalities on diffusion tensor imaging correlating to increased symptoms of depression [5]. A separate functional MRI study of 54 retired NFL players compared to 53 controls found hypoconnectivity and hyperactivation in the frontal lobes suggestive of executive dysfunction [6].

Other studies have shown increased structural abnormalities [7] and neuropsychiatric deficits in NFL players. A study of 30 retired players found a lifetime history of concussions correlated to increased depressive symptoms on the Beck Depression Inventory II test [8]. In a larger group of 1,044 former players surveyed, the increased risk of depression persists at least up to 9 years after retirement [9] and this association was independent of declining physical health. Currently, there is a relative lack of data on i) What regions are neurophysiologically impaired in living NFL players compared to healthy controls; ii) How well these brain abnormalities distinguish possible repetitive concussive and subconcussive pathology in NFL players from healthy individuals; and iii) What specific brain areas are most predictive of such a classification. The purpose of this work is to address these questions in a large NFL cohort with functional imaging data and predictive data analytics. We specifically hypothesize that areas of abnormally low cerebral perfusion on single photon emission computed tomography (SPECT) imaging in NFL players compared to controls will reliably separate these groups with high accuracy.

METHODS

Study participants

Starting in 2009, we recruited a cohort of retired and current NFL players ($n = 161$; mean age = 52 $\pm$ 14.2 years) as described in prior work [10] along with a control group of healthy individuals ($n = 124$; mean age = 44 $\pm$ 16.6 years, M:F 44%:56%). Players were recruited from 27 teams and all positions (Integ Review IRB Certificate Number: 004). All recruitment was done in accordance with Institutional Review Board approval. Inclusion criteria were being on an active NLF roster for a minimum of 1 year. Exclusion criteria were any subjects that could not discontinue psychoactive medications for an appropriate period prior to functional neuroimaging. Each study participant was interviewed by a board certified psychiatrist, completed a 315 intake questionnaire of mental and physical health, and was given either the Microcog Assessment of Cognitive Functioning [11] or WebNeuro [12] computerized neuropsychological battery. Concussion history from high school through NFL play, including loss of consciousness, was obtained from each study participant. Concussions were defined using the Center for Disease Control and Prevention (CDC) [13] criteria "conditions of temporarily altered mental status as a result of head trauma" that may or may not involve a loss of consciousness.

Functional perfusion neuroimaging

All subjects ($n = 285$) received functional perfusion neuroimaging with SPECT as described in previous studies [14, 15]. Briefly, each participant received an appropriate dose of technetium-99 m hexamethylpropyleneamine (HMPAO) intravenously. The injection was done in normal room lighting while each subject performed the Connors' Continuous Performance Test II [16]. Regional cerebral blood flow was modeled in 128 regions using a standard neuroanatomical atlas [17] as previously described [14, 15]. Age, gender, and race corrections were done in the analyses.

Predictive data analytics

All statistical analyses were performed in Statistical Package for Social Science (SPSS, version 23, IBM, Armonk, NY) and were controlled for both age and gender. To identify specific brain regions we hypothesize are predictive in distinguishing NFL players from controls, a one-way ANOVA was conducted to identify regions of low perfusion in NFL players compared to controls. In all, 36 regions out of the 128 tested met our cutoff level for statistical significance ($p < 0.01$). These regions were then used to classify NFL players from controls with two

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separate predictive data analytic methods. The first was discriminant analysis using a leave-one-out cross validation [18] followed by extraction of predicted probabilities from this model. These probabilities were then used to determine diagnostic sensitivity, specificity, and accuracy from input into receiver operating characteristic (ROC) and Area Under the Curve (AUC) analyses. The second approach was the automatic linear modeling (ALM) approach in SPSS [19] done to verify the diagnostic accuracy metrics obtained from the discriminant analysis and to provide a ranking of the most predictive features from the 36 brain regions initially selected for the analysis.

RESULTS

All NFL players were male, while 56% of the control group were women. The control group was 56% Caucasian, while the NFL group was 53%. In the NFL group, baseline neuropsychological assessments showed 92% of players had decreased general cognitive proficiency, 86% had decreased information processing speed, 83% had memory loss, 83% had attentional deficits, and 85% had executive function impairment. The most common psychiatric co-morbidities in the NFL players were depression (29%), anxiety (22%), and attention deficit hyperactivity disorder (ADHD) (13%). In the NFL cohort, 3% had a history of substance abuse. In total, 36 brain regions were found to have abnormally low blood flow on functional perfusion neuroimaging on ANOVA ($p < 0.01$, average F-value 29.51 $\pm$ 16.76. These regions are listed in Table 1. Figure 1 shows a visual 3-D rendering of a SPECT scan of a 12-year NFL player compared to a control subject.

Table 1
Brain regions with hypoperfusion in NFL players compared to healthy controls

Brain Area	F-value	*p*-value
Amygdala Left	26.676	0.0000
Amygdala Right	24.279	0.0000
Cingulum Ant Left	38.716	0.0000
Cingulum Ant Right	14.611	0.0000
Cingulum Mid Left	8.101	0.0050
Cingulum Post Right	13.308	0.0000
Caudate Left	16.417	0.0000
Caudate Right	12.437	0.0000
Frontal Inferior Opercular Left	33.461	0.0000
Frontal Inferior Opercular Right	23.21	0.0000
Frontal Inferior Orbital Left	22.678	0.0000
Frontal Inferior Orbital Right	25.082	0.0000
Hippocampus Left	9.739	0.0020
Hippocampus Right	17.07	0.0000
Insula Left	22.26	0.0000
Insula Right	59.181	0.0000
Paracentral Lobule Left	29.503	0.0000
Paracentral Lobule Right	26.7	0.0000
Postcentral Left	26.032	0.0000
Postcentral Right	19.48	0.0000
Precuneus Left	40.399	0.0000
Precuneus Right	40.882	0.0000
Rectus Left	8.556	0.0040
Rectus Right	14.49	0.0000
Temporal Pole Sup Left	62.102	0.0000
Temporal Pole Sup Right	51.886	0.0000
Temporal Sup Ant Left	50.16	0.0000
Temporal Sup Ant Right	70.902	0.0000
Vermis 10	37.222	0.0000
Vermis 45	7.501	0.0070
Cerebellum 10 Left	17.694	0.0000
Cerebellum 10 Right	31.926	0.0000
Rolandic Opercular Left	40.464	0.0000
Rolandic Opercular Right	60.956	0.0000
Olfactory Left	19.853	0.0000
Olfactory Right	38.418	0.0000

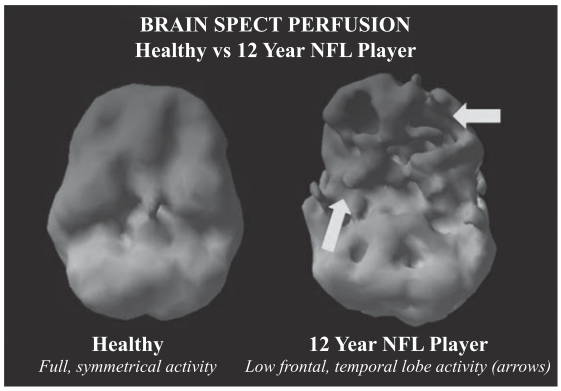

Fig. 1. Example of a volume rendered of the undersurface of the brain in normal control compared to a 12-year NFL player. While the control subject has symmetric activity, the NFL player has multiple defects in the inferior frontal and temporal lobes suggestive of concussion related hypoperfusion.

Predictive modeling

The discriminant analysis yielded a correct classification rate of 87% with the leave-one-out cross validation at 82%. The corresponding accuracy of the linear discriminant probabilities in identifying NFL players from controls was 94.4% based upon AUC analysis with 90% sensitivity and 86% specificity. The automatic linear modeling analysis produced essentially identical results with 95% accuracy (95% CI: 92.6-97.6), 90% sensitivity and 87% specificity. The diagnostic metrics of these two models do not change when adding demographic co-variates such as age, gender, and race or co-morbidities such as depression or ADHD. Based upon the automated linear modeling, the most important regions in

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predicting NFL players from controls were the i) anterior superior temporal lobes, ii) rolandic operculum, iii) insula, iv) superior temporal poles, v) precuneus, and vi) cerebellar vermis. The accuracy estimates from these models did not change when accounting for age, gender, or co-morbidities such as anxiety, depression, or ADHD by including them as co-variates in both discriminant and automatic linear regression models. Additionally, the results of these predictive models were not significantly different when female subjects were entirely excluded from the analysis. For example, in the discriminant analysis, the accuracy was 92%, sensitivity was 86%, and specificity was 81%. The correct classification was 86% and 80% with a leave-one-out validation with females completely excluded from the analysis.

DISCUSSION

Former and current NFL players have lower cerebral perfusion and impaired neuropsychological function compared to healthy controls. The regional perfusion abnormalities alone reliably separate these individuals from controls with high accuracy in two separate predictive analytic models. The result of our study advances the literature in living NFL players by characterizing regional abnormalities and their ability to distinguish players from controls in the largest known imaging study of this population. The main caveats to our data are its retrospective design and lack of NFL players with no traumatic brain injury (TBI) to serve as an NFL control group thus necessitating our use of a non-NFL control group.

Playing professional football has been correlated with alterations on structural and molecular neuroimaging [20] and our study adds several key insights. First results of low perfusion in the frontal and temporal lobes match what is known from a prior systematic review of the literature identifying the most common abnormalities on SPECT in mild TBI [21]. Second, involvement of the hippocampus and precuneus suggest neural correlates for poor memory function in our NFL subjects and increased risk for dementia [22]. Abnormally low flow in the insula is consistent with literature correlating perfusion decreases in this structure with depression, a common co-morbidity in our study [23]. Low flow in the cerebellar vermis is relevant as mild TBI involving this structure in diffusion tensor imaging is related to anxiety, also a common co-morbidity in our population [24]. From a network perspective, the abnormal brain regions in this study are most commonly found in the default mode network and frontal-parietal network, both of which are implicated in Alzheimer's disease and psychiatric disorders [25]. We had previously used default mode network regions to separate veterans with histories of TBI from healthy controls with 94% accuracy [15]. Also of note is the correlation between the functional abnormalities seen in our cohort and the *in vivo* characterization of CTE on PET tau imaging with increased deposits in frontal regions, amygdala, and anterior cingulate [26, 27].

There are several implications of our study to consider. In this and other studies, it is becoming increasingly suggestive that playing football at the professional level is associated with brain abnormalities in players. Longitudinal studies are required at the pre-teen, high school, and college level to better characterize the time course of such alternations.

DISCLOSURE STATEMENT

Authors' disclosures available online (http://j-alz.com/manuscript-disclosures/16-0207r1).

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White Matter and Cognition in Traumatic Brain Injury

Christopher M. Filley[a,b,c,d,*] and James P. Kelly[a,b,d]
[a]*Behavioral Neurology Section, University of Colorado School of Medicine, Aurora, CO, USA*
[b]*Department of Neurology, University of Colorado School of Medicine, Aurora, CO, USA*
[c]*Department of Psychiatry, University of Colorado School of Medicine, Aurora, CO, USA*
[d]*Marcus Institute for Brain Health, University of Colorado School of Medicine, Aurora, CO, USA*

Abstract. Traumatic brain injury (TBI) is a leading cause of disability and produces a wide range of cognitive, emotional, and physical consequences. The impact of TBI on cognition is among the most important questions in this field but remains incompletely understood. The immediate cognitive effects of concussion, while usually short-lived, may be profound and lasting in some individuals, and long-term sequelae of TBI may include dementia of several varieties including post-traumatic leukoencephalopathy, chronic traumatic encephalopathy, and Alzheimer's disease. Whereas the etiopathogenesis of cognitive dysfunction after TBI remains uncertain, a reasonable point to begin is a focus on the white matter of the brain, where the neuropathological lesion known as diffuse axonal injury (DAI) is routinely identified. White matter is not typically accorded the significance granted to cortical gray matter in discussions of cognitive dysfunction and dementia, but increasing evidence is accumulating to suggest that cognitive decline after TBI is a direct result of white matter injury, and that lesions in this brain component are crucial in the sequence of events leading ultimately to dementia of several types. In this review, we consider the topic of white matter and cognition in TBI, beginning with DAI and proceeding to the role of inflammation in the pathogenesis of cognitive dysfunction and dementia that can follow. A brief review of possible therapeutic options will also be offered, including the use of anti-inflammatory agents and the exploitation of white matter plasticity, to treat acute and post-acute injuries, and lower the incidence of dementia resulting from TBI.

Keywords: Alzheimer's disease, chronic traumatic encephalopathy, cognition, diffuse axonal injury, traumatic brain injury, white matter

INTRODUCTION

Traumatic brain injury (TBI) affects some 10 million people worldwide every year [1], and in the United States a recent estimate holds that some 42% of the population suffers at least one TBI during their lifetime [2]. Falls and road traffic accidents account for more than 50% of all cases, with peak TBI incidence found in adults more than 50 years old, but military service members represent a unique at-risk group because of TBI related to combat and training exposures [1]. Men tend to be at higher risk of TBI than women, yet women may endure more prolonged post-TBI symptoms [1]. Children and young adolescents appear to be at risk for slower recovery than adults, and early participation in contact sports may result in a higher likelihood of long-term cognitive sequelae; similarly, older people with TBI likely have a higher risk of dementia than younger adults

*Correspondence to: Christopher M. Filley, 12469 East 17th Place, Mail Stop F429, Aurora, CO 80045, USA. Tel.: +1 303 724 8225; Fax: +1 303 724 0985; E-mail: christopher.filley @ucdenver.edu.

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[1]. Cognitive dysfunction is a central clinical feature of all TBI, contributing significantly to both acute and chronic disability. Moreover, emerging associations between TBI and neurodegenerative disease, both international public health concerns, underscore the special urgency of this problem. Despite these observations, however, major questions remain unanswered regarding pathophysiology, long-term sequelae, and treatment. Typically classified as mild, moderate, or severe based on neurological assessment and neuroimaging, TBI may result from penetrating wounds, or, far more commonly, closed head injury related to blows to the cranium, whiplash mechanism injuries, or blast injury sustained in military settings [1, 3]. Whereas moderate and severe TBI often present as neurosurgical emergencies and usually produce clinically obvious cognitive sequelae, mild TBI, which accounts for 80–90% of all cases, also leads to cognitive dysfunction, albeit more subtle and often overlooked [1]. In this regard, the prognosis of mild TBI has been regarded as favorable, with full recovery within days to weeks, but recent data have questioned the veracity of this view, and these patients may experience cognitive symptoms lasting far longer. Most concerning is the potential for progressive cognitive dysfunction, and even dementia, as a result of single TBI, or repetitive mild TBI sustained in military service and various forms of contact sports [1].

In this review, we consider evidence for neuropathology in the white matter of the brain as the *primum movens* of cognitive dysfunction generated by TBI [1, 4]. While neuropathological changes in this brain component have been recognized for many years, concomitant neuropathology in the gray matter, particularly the cerebral cortex and hippocampus, is also well-known in TBI and naturally attracts much neuroscientific interest. However, converging evidence increasingly suggests that white matter neuropathology is most crucial in the pathogenesis of cognitive dysfunction across the entire range of TBI severity, both acutely and in the long term [1, 4]. Relevant terms used in this review are explained in the Table 1.

WHITE MATTER NEUROBIOLOGY

About half of the brain is comprised of white matter, the term used to refer to grossly visible collections of myelinated tracts coursing throughout the central nervous system, together with blood vessels and associated glial cells: myelin-producing oligodendrocytes, astrocytes, microglia, and oligodendrocyte progenitor cells (OPCs). White matter tracts are bundles of axons, many of which are surrounded by myelin, an insulating substance that confers much greater axonal conduction velocity than in unmyelinated axons, and concomitant increase in cognitive processing speed [5]. Most critical for cognition are the association and commissural tracts, which connect gray matter areas within and between the cerebral hemispheres, and other tracts link the hemispheres with deep structures including the thalamus, basal ganglia, brainstem, and cerebellum; white matter thus participates in all neural networks subserving cognition [5].

The operations of cognition rely heavily on the activity of the modulatory neurotransmitter acetylcholine, which is widely distributed within cerebral white matter tracts [6] and serves an essential role in the facilitation or inhibition of neuronal responses to other neurotransmitters including glutamate, γ-aminobutyric acid, and the monoamines [7]. All sectors of the cerebral cortex and the hippocampus receive dense cholinergic input, which arises from 8 distinct nuclear groups in the basal forebrain (Ch1-Ch8) from which cholinergic axons project to more rostral cerebral regions [7]. Most noteworthy for this review are the medial and lateral pathways coursing from Ch4 (the nucleus basalis of Meynert) to the cerebral cortex [8], and the fornix traveling from Ch1 and Ch2 to the hippocampus [7]. Cerebral cholinergic projections are particularly vulnerable to TBI, resulting in acute and chronic dysfunction, the latter being especially critical because cholinergic function remains chronically reduced despite the relative normalization of other neurotransmitters such as glutamate, catecholamines, and serotonin [9].

An important observation is that cerebral white matter has actually expanded more in evolution than cortical gray matter, implying a crucial contribution to the unique cognitive functions of *homo sapiens* [10]. Also noteworthy is that white matter undergoes developmental changes such that myelination is a slow process not complete until mid-life, and then, in late life, a decline in white matter volume appears to occur, implying differential vulnerability to acquired neuropathology as a function of age [11]. White matter is susceptible to a wide range of neuropathological insults beyond TBI, and in all clinical examples available for detailed study, neurobehavioral dysfunction of some kind—broadly considered, cognitive or emotional—has been observed [12]. In

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Table 1
Major concepts in the study of white matter and cognition in TBI

Diffuse Axonal Injury (DAI)	Traumatically-induced shearing injury of white matter tracts in the brain
Concussion	Short-lived TBI caused by biomechanical forces, with or without loss of consciousness; also called mild TBI
Diffusion Tensor Imaging (DTI)	The neuroimaging modality most commonly used for the assessment white matter microstructure
Fractional Anisotropy (FA)	A DTI measure that, when abnormally low, implies microstructural white matter injury
Mean Diffusivity (MD)	A DTI measure that, when abnormally high, implies microstructural white matter injury
Complicated Mild TBI	Mild TBI with neuroimaging evidence of trauma-related intracranial involvement
Improvise Explosive Devices (IEDs)	The most important weapon leading to TBI in the recent Middle East conflicts
Blast Injury	A major form of TBI in recent Middle East conflicts, typically related to nearby IED explosions
Post-Traumatic Stress Disorder (PTSD)	A common anxiety disorder that is often co-morbid with TBI
Post-Concussion Syndrome (PCS)	A cluster of persistent symptoms and signs that can follow concussion
Repetitive mild TBI	Frequent mild TBI that has been associated with the later development of CTE (see below)
Subconcussive Blows	Blows to the head that do not cause concussion but are postulated to injure white matter tracts
Post-Traumatic Leukoencephalopathy	A late sequel of TBI recognized primarily by neuroimaging and neuropathological studies
Amyloid-β Protein Precursor (AβPP)	A normal membrane-bound protein in the brain that is the parent molecule of Aβ
Aβ	A protein expressed in the brain after TBI, and the primary constituent of neuritic plaques
Tau	A normal intra-axonal protein in the brain associated with microtubules
Hyperphosphorylated tau	Abnormally phosphorylated tau, the major component of neurofibrillary tangles
Chronic Traumatic Encephalopathy (CTE)	A late life dementia syndrome putatively associated with repetitive mild TBI
Alzheimer's Disease (AD)	The most common dementia of older people, which has been associated with moderate to severe TBI

the tradition of Norman Geschwind and the legacy of his work on disconnection in the brain [13], white matter lesions of all types can be conceptualized as disrupting distributed neural networks mediating cognition, and the study of white matter in health and disease has direct relevance to modern ideas on brain connectivity as well as clinical practice in behavioral neurology and allied disciplines [5, 12].

DIFFUSE AXONAL INJURY

The concept of diffuse axonal injury (DAI) was introduced in 1982 when neuropathological studies of humans [14] and monkeys [15] with non-penetrating TBI documented widespread focal areas of damage in the cerebral white matter. Some years later, the term traumatic axonal injury was proposed in an effort to be more specific about etiology [16], but DAI remains widely popular in discussions of this phenomenon. DAI is thought to result from rotational and translational injury to the brain exerted by the mechanical force imparted by head impacts, and while DAI denotes injury to axons, myelin damage is also prominent [14, 15]. The effects of DAI are most apparent in the cerebral hemispheres near gray-white junctions, corpus callosum, fornices, and rostral brainstem, and associated findings include microhemorrhages, axonal varicosities with amyloid-β protein precursor (AβPP), microtubular disruption, and activated microglia [1]. Rotational force is considered more injurious to white matter than translational force, and a direct relationship has been shown between the amount of rotational head movement and the severity of DAI, the duration of coma, and the level of neurological impairment [15]. DAI was a novel term when first proposed, but similar observations were made in postmortem human brains years before the modern terminology was introduced; in 1968, Strich documented "shearing injury" of white matter in association with dementia after severe TBI [17], and in 1968 Oppenheimer described "shearing" and "stretching" of white matter fibers after mild TBI [18]. Today, the neuropathological lesion of DAI is accepted as a core neuropathological lesion in TBI that is present with or without other traumatically-induced lesions [1, 4, 19–21].

A critical issue in the study of TBI, however, is the heterogeneity of neuropathology, a problem

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considered one of the most important barriers to finding effective therapeutic strategies [22]. A recent review pointedly commented that TBI "remains one of the most complex diseases known in the most complex of all organs in the body" [23]. In this light, it is appropriate to consider the degree to which other injuries that can occur in addition to DAI—focal cortical contusions, intracranial hematomas (epidural, subdural, and parenchymal), and subarachnoid hemorrhage—contribute to cognitive dysfunction and dementia following TBI [22]. This question remains under study and without a definitive answer, but as clinical, neuroimaging, and neuropathological techniques continue to improve, a more precise understanding of the cognitive sequelae of various TBI lesions is emerging. This review focuses on DAI, for which neuropsychological [24–26], neuroimaging [27], and neuropathological [23, 28, 29] evidence exists to support the generation of cognitive deficits even when DAI is unaccompanied by any other TBI-related brain lesion. Although the cognitive importance of other lesions cannot be neglected, a plausible case can be made that DAI is universal in TBI, and that levels of post-TBI cognitive loss can be securely linked with the degree of DAI sustained [1, 4, 19–21, 23–29]. As will be discussed in detail below, DAI can also be conceptualized as a lesion that is crucial for the pathogenesis of acute and chronic cognitive dysfunction [1, 4, 19–21].

CONCUSSION

The idea of concussion has existed in medical thinking for centuries and is understood as a general concept in clinical practice, but a secure and widely accepted definition has proven remarkably elusive. Current consensus opinion holds that concussion is a short-lived TBI caused by biomechanical forces, with or without loss of consciousness, that may involve neuropathological changes or a functional disturbance of brain tissue [30]. A distinction between concussion and mild TBI has often been suggested, but as no differentiation has been conclusively established [30], the two terms will be used interchangeably herein. Concussion is presently characterized as featuring loss of consciousness for 0–30 minutes, confusion for less than 24 hours, posttraumatic amnesia for up to one day, normal structural neuroimaging, and a Glasgow Coma Scale score of 13–15 within 24 hours of the injury [1]. Cognitive symptoms are typical, including poor concentration and impaired memory, but disrupted sleep, headache, nausea, vomiting, dizziness, anxiety, and irritability are very common [1]. A favorable prognosis of concussion within days to weeks has long been taught, and remains largely supportable in most cases, but it has become clear that prolonged symptoms and signs may plague some individuals for extended periods [31]. In view of evidence of DAI in the brains of concussed patients as demonstrated by autopsy studies [18, 32], white matter involvement appears to be clinically significant in concussion. In recent years, diffusion tensor imaging (DTI) has added valuable new microstructural information by allowing the study of tract integrity with the measures of fractional anisotropy (FA) and mean diffusivity (MD), and white matter damage can also be shown by this technique in concussion [33, 34]. Thus, even at what has been considered the mildest end of the TBI spectrum, the potential importance of white matter injury in the pathogenesis of lasting neurobehavioral dysfunction assumes increasing importance.

COMPLICATED MILD TBI

A subgroup of patients with concussion presents with a trauma-related intracranial abnormality on computed tomography (CT) of the head, or brain magnetic resonance imaging (MRI), along with clinical evidence of the injury. This group has been designated as having sustained complicated mild TBI [35], and typical lesions include microhemorrhages on susceptibility-weighted imaging, T2 hyperintensities, and small intracerebral or extra-axial hemorrhages. The prevalence of this condition varies widely, with a reported range of 5–40% among all mild TBI cases [36]. Not unexpectedly, white matter is damaged in these patients as in those with uncomplicated mild TBI, but may be more severely injured, as one DTI study demonstrated [36]. Currently, the presence of an intracranial CT or MRI lesion after mild TBI is clinically concerning and may be associated with more widespread microstructural white matter damage but does necessarily imply a worse prognosis [36]. Further study will be required to understand the implications of this added insult to the brain as a consequence of concussion.

BLAST INJURY

The recent military conflicts in the Middle East have brought to light a new form of TBI known as

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blast injury. Soldiers in these wars are relatively well protected from fatal wounds by body armor and helmets, but the head is nevertheless left susceptible to injuries from the detonation of nearby improvised explosive devices (IEDs) that produce a high-velocity air wave with associated shrapnel, sand, and dust [37]. In addition to the blast injury itself, individuals affected may sustain additional TBI as a result of being thrown by the explosion [37]. More than 60% of the U.S. combat casualties in Iraq and Afghanistan have in fact been attributed to explosive blasts related to IEDs [37]. Similar to blunt head trauma, blast injury is classified as mild, moderate, and severe, with most injuries being mild, and a post-concussion syndrome (see below) after blast injury is also recognized [37]. Blast injury is thought by many to be similar to concussion in its clinical effects and pathophysiology, although the frequent co-morbid state of post-traumatic stress disorder (PTSD) in military personnel complicates analysis [37]. Whereas previous postmortem reports of individuals with blast exposure disclosed DAI and petechial hemorrhages in the white matter, intriguing new data have recently been presented from five cases that were found to have distinctive astroglial scarring at gray-white junctions as well as U fiber DAI [38]. The involvement of U fibers at the gray-white matter junction in the cerebral hemispheres may underlie cognitive dysfunction, while astroglial scarring in the orbitofrontal cortex, amygdala, and hippocampus may explain PTSD symptoms [38]. Thus, in contrast to concussion from blunt impact, white matter appears to be variably damaged in blast injury, suggesting a distinct neuropathology that may have unique pathophysiological origins. Evidence is also accumulating that that the effects of this kind of injury may be lasting, as shown by DTI studies that found white matter changes in veterans more than one year after blast injury [39], and a dose-response relationship between the number of blast exposures and decreased white matter integrity [40].

POST-CONCUSSION SYNDROME

As mentioned above, a small proportion of mild TBI patients do not experience a prompt recovery, and instead develop the post-concussion syndrome (PCS), currently defined as the presence of symptoms persisting for more than 3 months after the traumatic injury [41]. Some 10–15% of mild TBI patients are thought to go on to PCS [41], which features a variety of symptoms including poor concentration, memory loss, sleep disturbance, fatigue, headache, dizziness, anxiety, irritability, and depression [1]. The pathogenesis of the PCS is poorly understood, and psychological factors complicate the study of this disorder [41], at times leading to much litigation and psychosocial distress. A neurobiological basis for PCS finds support in a controlled DTI study of mild TBI patients which disclosed microstructural injury in several association tracts and the corpus callosum that correlated with severity of PCS symptoms [42]. Of note, the individuals with mild TBI had a mean Mini-Mental State Examination [43] score of 27 and no evidence of no macrostructural white matter changes on conventional MRI, suggesting that, while the PCS can be disabling, it may reflect subtle white matter damage that often escapes detection by standard clinical and neuroradiological assessment. It is also of interest that the symptoms of PCS overlap considerably with those of more prolonged cognitive disorders, most often associated with repetitive mild TBI. Thus the cognitive dysfunction of PCS can be interpreted as a harbinger of more lasting dysfunction that may result from repeated mild TBI. White matter neuropathology may play a critical role in the pathophysiology of PCS and may exert an influence on whether any further cognitive dysfunction occurs in the months or years that follow the onset of the syndrome.

REPETITIVE MILD TBI

In military service and in sports, mild TBI can often be repetitive, and affected individuals may sustain hundreds or even thousands of injuries. Figures such as these suggest that the brain may simply not possess the capacity to recover fully from such frequent injury. One of the early advances in repetitive mild TBI associated with sports was the recognition of the second impact syndrome with a fatal outcome [44], and effective return-to-play guidelines in sport are critical to prevent severe or catastrophic sequelae [45, 46]. It is clear, however, that the accumulation of these injuries over time may have much more gradual effects on cognitive function, as will be considered below. As the discussion proceeds, some epidemiological data will be helpful to keep in mind. Repetitive mild TBI occurs most often in military service with combat exposure, and in sporting contests involving the potential of frequent head blows such as American football, ice hockey, and combat sports such as

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boxing [47]. It has been difficult to quantitate how many impacts are sustained and over what time periods, but recent studies from high school and collegiate athletics have shed some light on these questions. The use of helmet-based accelerometers has permitted estimates of 170–652 impacts per season in football and hockey players, with a maximum of 2,235 impacts recorded in football [47]. These impacts result in some 300,000 sport-related concussions in the U.S. each year, and prospective study has shown not only that players with a history of concussion are more likely to have future concussions, but also that previous concussions may lead to slower recovery [48]. As might be expected, DAI has been implicated in repetitive mild TBI, and cognitive consequences can result. Evidence for white matter injury has been adduced from study of Olympic boxers within six days of a bout, of whom more than 80% had elevated cerebrospinal fluid biomarkers suggestive of subcortical myelinated axon injury (neurofilament light) and cortical axonal damage (total-tau) [49]. As for cognitive sequelae of repetitive mild TBI, a DTI investigation of 80 collegiate football and ice hockey players over a single season found a relationship between the number of head impacts, white matter diffusion measures, and cognitive function [50]. Intriguingly, concussion was not diagnosed in these subjects, raising the possibility that blows to the head can be cognitively significant even without inducing what is regarded as mild TBI [50]. These kinds of blows, however, remain controversial, as the next section will discuss.

SUBCONCUSSIVE BLOWS

A particularly worrisome phenomenon that has recently been observed is that blows to the head may be harmful to the brain even when concussion is not produced. These impacts have been called subconcussive blows [52]. Also called repetitive subclinical brain trauma [52], a subconcussion is defined as a cranial impact that does not result in a concussion as typically diagnosed [51]. One of the most alarming implications of subconcussion is the suggestion that this kind of blow—which may occur many times more often than those impacts sufficient to induce concussion and be clinically inapparent—may lead to long-term detrimental effects on cognition, including neurodegenerative diseases [51]. Indeed, preliminary neuropathological evidence exists for cerebral white matter injury in former professional athletes who had no history of concussion [51]. However, the concept of subconcussion has come under significant criticism based on its imprecision (how does a subconcussive blow differ from a more severe concussive blow, and from any trivial blow to the head?), and from the absence of any clinical data that disclose short-term effects in exposed individuals [53]. Similarly, with respect to white matter involvement, the data are inconclusive. Some DTI evidence, for example, exists for lasting changes in white matter after impacts that do not lead to a concussion diagnosis [54], but other DTI work has shown that athletes with subconcussion have FA changes in both directions, that is, indicating both decreased and increased white matter integrity [53]. At this point in neuroscientific understanding, the idea of subconcussion is best considered imperfect [53] as the entity lacks a secure definition and is not well correlated with either clinical features or neuroimaging data [53]. Attention has also been called to the need for caution in presenting information to the general public about the risk of neurodegenerative disease from subconcussions that is as yet unwarranted [53]. Still, the notion of subconcussion deserves further study with prospective, controlled studies using a clear definition and validated outcome measures to determine its utility in the study of TBI. If more solid data emerge from such investigation, the role of white matter injury associated with these blows surely merits careful consideration.

NEUROPSYCHOLOGICAL OVERVIEW OF LONG-TERM COGNITIVE SEQUELAE

The long-term cognitive effects of mild, moderate, and severe TBI have received increasing scrutiny, and studies have examined both single incidents and repetitive injuries. We now turn to these long-term sequelae, beginning with a brief consideration of what is known about cognitive outcome without regard for its neuropathological basis, and then proceeding to the role of white matter involvement within specific categories of TBI.

Whereas most people with mild TBI make a full cognitive recovery, this outcome can be expected in just 60% of those moderately injured and in 15–20% of those with severe TBI [55]. Cognitive deficits in all groups typically become most evident in the domains of executive function, processing speed, attention, and episodic memory [56]. Whereas other comorbidities such as depression, PTSD, epilepsy, and

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physical injury can impact overall outcome, systematic review of the neuropsychological literature has found that severity of the inciting injury is a good predictor of cognitive outcome after TBI, as a strong dose-response relationship exists between TBI severity and the extent of neuropsychological impairment 6 months or longer after injury [56]. Other work has extended cognitive follow-up to 1-2 years with similar results [56], but it is important to note that observation of cognitive outcome over many years or decades is limited. Whereas many case-control studies are available, the relationship of TBI to later cognitive decline or dementia has rarely been investigated in a prospective longitudinal study. One of the most urgent questions facing the research communities in both TBI and dementia—in what way does early TBI affect subsequent cognitive decline—must be approached by more indirect investigation using methods presently available.

This discussion leads to a consideration of what is meant by the term dementia. This question is critical, as much of the medical and neuroscientific literature tends to confine the scope of dementia to the common neurodegenerative diseases of the cortical and deep gray matter while neglecting the many other causes of the syndrome. Dementia, however, simply considered as an acquired disorder of cognition that affects usual social and occupational activities [57], carries no implications about etiology, pathogenesis, or natural history, and TBI can certainly produce cognitive dysfunction of sufficient severity to qualify as dementia [17, 58, 59]. This syndrome has naturally been better appreciated after moderate and especially severe TBI, but concern is rapidly growing with respect to dementia after repetitive mild TBI. The recent adoption of the term "major neurocognitive disorder" by the widely influential DSM-5 [60] may offer a more suitable descriptor for the concept of dementia, helping avert the tendency to assume that all demented individuals harbor a recognized neurodegenerative disease, but the more familiar term will be used here. The specific types of dementia associated with TBI are unclear [1], and much controversy pervades research addressing this question [58, 59], but the issue highlights the pressing public health implications of both problems, and considering dementia in its most fundamental sense as a clinical syndrome is vital for maintaining an appropriate framework with which to consider its association with TBI. As will be discussed below, dementia syndromes that follow TBI may indeed implicate neurodegeneration in the cerebral cortex, but a broadly inclusive conceptualization of dementia leads to the recognition that a prominent contribution of white matter is increasingly compelling in the understanding of how dementia evolves from TBI.

CONCUSSION, DEMENTIA, AND WHITE MATTER

When TBI occurs, it is most often mild, and the effects of single incident concussion have been most often studied. The commonly assumed dictum that most patients with concussion recover promptly and without sequelae remains appropriate in clinical practice, but new data have begun to introduce the notion that dementia may be a long-term outcome in some individuals. A recent retrospective Swedish study that included all individuals in that country aged 50 and older (well over 3,000,000 people) found that a single mild TBI increased the risk of dementia, including Alzheimer's disease (AD), vascular dementia, and unspecified dementia [61]. The risk was highest in the first year after injury but was still evident more than 30 years later [61]. It should be recognized that dementia was diagnosed clinically in this study, and the specific cause was not confirmed by autopsy, highlighting the problem of diagnostic uncertainty that besets the literature on this topic in general. Another issue is that identification of dementia may have been erroneous because the individuals were experiencing PCS, which is presumably transient, or because of reverse causality (i.e., that dementia caused the TBI rather than the opposite). However, the results from Sweden showing increased risk over more than 30 years cannot be explained by these factors, and the Swedish data are consistent with prior work suggesting a similar small increase in risk of dementia, including AD, after concussion [62]. This arresting notion requires further study, as clinical experience would suggest that mild TBI is a self-limited disorder with no chronic sequelae. If verified, however, this increased risk of dementia may be related to persisting white matter injury. As mentioned above, concussion has been associated with white matter injury in both autopsy [18, 32] and DTI [33, 34] studies. More recently, moreover, a multiparametric MRI study of adolescent hockey players with a single concussion disclosed abnormalities in multiple white matter tracts after all clinical scores had returned to normal and the players had been cleared to play [63]. While much remains to be learned, the

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idea has been raised that even a single concussion may lead to ongoing white matter injury that later contributes to dementia.

These concerns are worrisome indeed, but it should be noted that the great majority of work on the long-term cognitive sequelae of TBI has been done on repetitive mild TBI and single incident moderate or severe TBI. Three dementia syndromes with prominent white matter neuropathology emerge from a consideration of these forms of TBI, and to begin, we consider a disorder seldom recognized clinically but potentially of great importance.

POST-TRAUMATIC LEUKOENCEPHALOPATHY

Leukoencephalopathy refers to a disorder of white matter, and whereas this term is generally used to describe white matter disease irrespective of etiology, a growing body of evidence has appeared to support the existence of a syndrome of static or even progressive leukoencephalopathy in the years that follow TBI [64]. It is important to emphasize that this syndrome differs from the more familiar dementias being studied as late sequelae of TBI. As discussed above, these syndromes are widely considered neurodegenerative diseases that are presumed to involve the cerebral cortex primarily. Post-traumatic leukoencephalopathy, in contrast, is proposed to be a disorder in which the white matter is selectively damaged without concomitant gray matter neuropathology of comparable significance. Some background information will help introduce this idea.

Despite Geschwind's seminal work on disconnection in 1965 establishing the relevance of white matter to cognition in the context of behavioral neurology [13], myelinated systems of the brain have not been as readily incorporated into the thinking of the dementia research community. Yet it is clear that dementia does result from disorders of white matter as surely as from those that affect the cortical or subcortical gray matter [65–68]. The term "white matter dementia" has been introduced to all attention to the capacity of selective white matter involvement to produce often devastating dementia syndromes [65–68]. TBI is a plausible example of this principle, as it may clearly lead to dementia, an acquired state of cognitive dysfunction that interferes with usual activities, and manifests equally clear white matter neuropathology [65–68]. Although work has not yet advanced to support an unequivocal link between post-TBI dementia and white matter involvement, the evidence supporting a syndrome we term post-traumatic leukoencephalopathy is increasingly intriguing. We will consider this topic by developing a synopsis of selective, chronic white matter involvement in TBI, as demonstrated by neuropathology, experimental studies, and neuroimaging, together with correlations of these data with cognitive dysfunction.

In 1956, Sabina Strich published a seminal neuropathological report in which five patients with severe TBI had "extreme dementia" that was unequivocally attributed to diffuse degeneration of the white matter [17]. Cortical involvement was minimal, and in language presaging modern connectionist thinking, Strich described ". . . severe diffuse degeneration of the white matter in the cerebral hemispheres, which had interrupted. . . connexions between different areas of cortex," adding that ". . . it is not enough to examine the brain-stem reticular formation or the cerebral cortex when investigating cases of severe disturbance of consciousness after head injury" [17]. This remarkable work was eventually followed by more extensive studies finding similar results in larger numbers of severe TBI in humans [14] and experimental animals [15], and in concussion [18, 32].

What Strich found in 1956 was most likely DAI, and this lesion has since become widely accepted as a consistent feature of all TBI [17–29, 69–71]. DAI produces widespread damage to white matter [17–29, 69–71], and unmyelinated fibers within white matter tracts may be more vulnerable to this kind of injury than those fibers invested with myelin [72]. Recent information from animal and human studies of concussion has identified a neurometabolic cascade after DAI involving ionic flux, glutamate release, oxidative stress, mitochondrial dysfunction, cytoskeletal damage, altered neurotransmission, and inflammation, all of which contribute to axonal dysfunction that disrupts the function of multiple neural networks subserving cognition [73]. Damage to both myelinated and unmyelinated axons leads to cognitive impairment in TBI, but network efficiency is particularly compromised by DAI impacting myelinated fibers, as these lesions dramatically reduce conduction velocity to produce slowed cognition and related neurobehavioral deficits [73]. The ultimate result of white matter neuropathology is therefore network disruption with multiple cognitive domains affected.

With the advent of modern neuroimaging, researchers have increasingly turned to *in vivo* studies for the assessment of white matter neuropathology. MRI has been and remains the standard modality for

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imaging of macrostructural TBI white matter lesions, the most important of which, for our purposes, is volume loss. As MRI is insensitive to microstructural injuries, however, other techniques, most notably DTI, have been exploited to examine white matter integrity in regions that appear normal on MRI. We will consider studies using MRI and DTI in turn, and conclude this section with a discussion of how post-traumatic leukoencephalopathy may be a progressive disorder in some patients.

MRI has primarily been employed in severe TBI patients, in whom gross volume reductions detectable with volumetric techniques can be predicted. Studies of severe TBI have shown prominent volume reductions in white matter structures including the corpus callosum, fornix, cerebellar peduncles, internal capsule, external capsule, inferior longitudinal fasciculus, superior longitudinal fasciculus, and corona radiata [74–76]. Whereas these studies all found reduced cerebral volumes that included concomitant gray matter atrophy, white matter changes tended to be more critical for cognitive impairment, and in one study, lower global white matter volume was correlated with impaired performance on tests of psychomotor speed, attention, memory, and executive function, while gray matter volume loss produced no such effect [76].

In contrast to the macrostructural measurements of white matter volume suitable for MRI, the microstructure of white matter is accessible to DTI, a technique with the capacity to assess the normal-appearing white matter. Whereas DTI is very sensitive to subtle changes in white matter, some caution is warranted in data interpretation as studies of acute mild TBI have shown either increased or decreased white matter integrity, likely related to issues such as timing after injury, patient age, injury severity, regions analyzed, and specifics of the method used [73]. As more data are reported, however, DTI evidence is accumulating to support the notion that white matter involvement with cognitive decline is prominent at all levels of TBI severity.

DTI has been conducted in combination with neuropsychological evaluation, and all levels of TBI severity have been analyzed [42, 76–80]. In general, microstructural changes as indexed by decreased FA and increased MD have been found in a variety of white matter tracts, including the corpus callosum, corona radiata, inferior longitudinal fasciculus, superior longitudinal fasciculus, sagittal striatum, uncinate fasciculus, cingulum, and fornix [42, 76–80]. Cognitive dysfunction has been clearly associated with DTI white matter involvement, and the domains of attention, memory, executive function, and processing speed tend to be most strongly correlated [42, 76–80]. In most studies, aggregate measures of white matter disruption and neuropsychological dysfunction have been used to detect correlations, and both of these parameters worsen as injury severity level increases [42, 76–80]. One study found that the severity of PCS symptoms after mild TBI also correlated with widespread microstructural white matter changes [42]. Specific correlations between damage to a single tract and impairment in one cognitive domain are uncommon, but in one study, disruption in the fornix predicted declines in learning and memory while damage to frontal lobe white matter connections predicted executive dysfunction [78], consistent with what is known with respect to white matter-behavior relationships [5, 68]. Injury to the fornix may even be the primary lesion leading to the often-observed hippocampal atrophy that occurs in TBI, as the fornix is the major tract associated with this structure [64].

This account of post-traumatic leukoencephalopathy would not be complete without a discussion of longitudinal white matter changes that have been identified years after TBI [64]. Whereas cross-sectional data, reviewed above, showing loss of white matter concur with the plausible clinical assumption that white matter changes after TBI would simply represent static leukoencephalopathy, the possibility of progressive white matter neurodegeneration is being raised by neuroimaging data from patients whose injury occurred many years before. The recognition that ongoing damage to white matter tracts may occur over time suggests a locus for neurodegeneration specific to TBI that does not involve the cerebral cortex or deep gray matter. Gray matter damage, when it does occur, may be secondary to the tract injury, as has been suggested in the case of hippocampal atrophy deriving from primary DAI in the fornix [64].

One of the most notable long-term studies in this context reported neuropathological evidence of ongoing corpus callosum atrophy for up to 18 years after a single moderate or severe TBI [71]. In this autopsy study, white matter deterioration was thought to be driven primarily by ongoing neuroinflammation [71]. This conclusion has found support from neuroimaging studies, using either MRI volumetrics [81, 82] or DTI [83–86], for long-term degradation of white matter after moderate to severe TBI. Some studies have also found correlations between ongoing

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white matter injury and declines in cognition, typically in memory, processing speed, and executive function [83–86]. The corpus callosum has thus far been the tract most often identified to undergo progressive volume loss, and in the longest follow-up TBI study available using structural neuroimaging, the corpus callosum showed continued atrophy over 8 years while the hippocampus did not [82]. The predilection of the corpus callosum for this kind of degeneration may be explained by the presence of extensive, densely packed reactive microglia in this structure that strongly suggests a chronic inflammatory state as a potential causative mechanism [71]. This leukoencephalopathy may not occur in all TBI patients, as only about 50% of moderate to severe pediatric TBI patients in one study were found to have impaired callosal organization 13–19 months post-TBI [86]. These data suggest that as yet unidentified factors—most notably genetic variation—may be protective in some individuals with TBI, resulting in variable outcome with respect to leukoencephalopathy. Taken together, these studies suggest that a progressive inflammatory post-traumatic leukoencephalopathy can develop in some patients after moderate to severe TBI.

CHRONIC TRAUMATIC ENCEPHALOPATHY

The previous section considered the evidence supporting a unique leukoencephalopathy that follows TBI, and may even be progressive over time, but a far more familiar cognitive sequel of TBI is the disorder known as chronic traumatic encephalopathy (CTE) [87]. This disease has been widely publicized in recent years because of alarming suggestions that repetitive mild TBI in athletes and military combatants may lead to a progressive dementia with possibly high prevalence in the general population. Whereas concern is appropriate with respect to any dementia syndrome that may have its origin in environmental insults, undue public anxiety is unwarranted given how much is still unknown in this area. We will approach CTE in the context of how TBI and its signature lesion of DAI may help inform the many areas of uncertainty surrounding this topic.

The history of the disorder now referred to as CTE deserves brief review to highlight major gaps in our knowledge as well as what is known [88]. The first report of a disease suggestive of CTE dates from 1928, when Martland described the "Punch Drunk" syndrome in boxers [89]. Nine years later, Millspaugh reported on similar patients, using the somewhat less derisive term "dementia pugilistica" [90]. A treatise on progressive dementia in boxers appeared in 1949, when Critchley used the term CTE [91]. In the late 1960 s, due to concern about how prevalent this disorder might be, the Royal College of Physicians in London sponsored a cross-sectional clinical follow-up study of British boxers who had competed several decades previously [88]. This project, which remains the only clinical outcome study of TBI in boxers over many years, found that 17% of these athletes had central nervous system lesions attributable to boxing, although only 6% had a clinical picture consistent with dementia pugilistica [92]. Then, in 1973, Corsellis and colleagues presented a detailed neuropathological study of 15 boxers who died many years after their careers, identifying neurofibrillary tangles with limited numbers of neuritic plaques [93]. The modern introduction of CTE occurred in 2005, when Omalu and colleagues described the autopsy of a former National Football League player [94], and together with another case in the following year [95], CTE came to be conceptualized as a tauopathy distinct from AD mainly because of sparse or absent amyloid neuropathology in CTE brains. Since then, interest in CTE rapidly accelerated as the neuropathology was found in many other former contact sports athletes and in military service members who had been exposed to combat [87].

From the perspective of this review, it is noteworthy that, in addition to the tauopathy, all four neuropathological categories of CTE involve white matter changes consistent with DAI [87]. Whereas the prominence of tau in its unique distribution surely deserves attention, the importance of DAI should not be overlooked, as the significance of tau in TBI is still uncertain. Tau is a normal component of axons, promoting microtubule assembly and stabilization, and while this protein may prove to be toxic, it may alternatively represent normal physiology or even play a protective role [96]. However, tau deposition is interpreted, the centrality of DAI in CTE is apparent, suggesting that white matter damage may be the inciting event in this disorder, with all the clinical implications implied by such a perspective.

Recent neuropathological studies have added support to the role of white matter damage as a direct precursor of CTE. A novel approach involved the post-mortem study of chronic schizophrenic patients (mean age 78 years) who had been subjected to prefrontal leukotomy during the era of psychosurgery

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[97]. This procedure, now long abandoned, was intended to treat psychosis by disconnecting the frontal lobes from the remainder of the brain, and, as the target was frontal lobe white matter, prefrontal leukotomy generated a useful clinical model for examining the effects of DAI over time [97]. Remarkably, there was a clear predilection of hyperphosphorylated tau in the depths of sulci, but only in areas adjacent to the site of the leukotomy, implying a direct effect of DAI on the development of tauopathy [98]. As this leukotomy-induced lesion is comparable to that described in CTE, repetitive mild TBI can be postulated to have a similar effect. In support of this idea is an autopsy study of four teenage athletes with repeated mTBI and subconcussive blows who died from other reasons and were found to have phosphorylated tauopathy – one case even meeting criteria for early-stage CTE – in association with myelinated axonopathy [98].

ALZHEIMER'S DISEASE

AD is the prototype cortical dementia, and its pathogenesis is widely held to involve degeneration of the cerebral cortex and hippocampus in association with the deposition of amyloid-β (Aβ) in extracellular neuritic plaques and hyperphosphorylated tau within intracellular neurofibrillary tangles [99]. Yet this pathogenetic formulation has never been indisputably established [100], and all efforts to treat the disease by targeting its signature proteins have thus far failed [101]. This discouraging reality raises the question whether Aβ and hyperphosphorylated tau are invariably toxic to the brain, and indeed, it is well known that their precursors—AβPP and intra-axonal tau—are normal brain constituents whose role in neuropathology remains obscure. In this light, the myelin model of AD has been proposed [11], which proposes that the initial neuropathology takes hold in white matter, preceding the appearance of plaques and tangles. This innovative model posits that late-life decline in white matter volume, a process often termed retrogenesis [102], is already underway in older people, and acquired insults hasten this decline and lead to the deposition of cortical proteins as secondary phenomena [11]. One of the insults to the brain is thought to be TBI (clinically apparent or silent) that involves white matter lesions disrupting distributed neural networks and may lead ultimately to AD as well as to other dementias [11, 68, 103].

The relationship of TBI to AD remains somewhat uncertain because while many studies document an association, some do not [58]. A large prospective historical study, however, merits special mention in the regard, as World War II veterans studied 50 years after combat showed a 2.3-fold increased risk of AD following single-incident moderate TBI, and a 4.5-fold increased risk after severe TBI [104]. These data serve to help build the argument for white matter involvement preceding the late-life neurodegeneration of AD. The presumed mechanism of this development is thought to begin with DAI as the inciting insult, which then leads to the deposition of protein aggregates in the brain parenchyma [52, 105]. Within a few hours of single-incident severe TBI, for example, AβPP is visible in damaged axons and cell bodies, and whereas this change resolves over time in many individuals, some 30% of severely-injured TBI patients develop diffuse Aβ plaques [52]. DAI and parenchymal Aβ can be putatively linked by postulating that TBI produces a large pool of intra-axonal Aβ that is released by lysis or leakage of damaged axons into the cerebrospinal fluid, where high levels have been described [105]; this process would enable Aβ to gain access to remote brain areas where it is deposited in the parenchyma [105] and detected as Aβ plaques many years later [106]. Tauopathy also develops as a consequence of DAI, as the shear-strain force from TBI leads to hyperphosphorylation, misfolding, and tau accumulation, thus completing the standard microscopic neuropathology of AD [52, 99].

Whereas the still dominant amyloid cascade hypothesis of AD has undergone significant criticism in view of the frequent presence of AβPP and tau in normal older brains, and the failure of all anti-amyloid and anti-tau drugs to treat AD dementia [101], the relationship of DAI to Aβ and hyperphosphorylated tau merits consideration. As mentioned above, both proteins may represent salutary host responses in some individuals [96, 100], and if so, they only become toxic in those individuals in whom the proteins persist and accumulate [67, 68]. As the common feature of all cases, DAI in the white matter may be the initiating event leading to cognitive impairment in AD, with a potential additive component of amyloid and tau neuropathology as time progresses and neurodegeneration develops [67, 68]. Thus, the focus on white matter as the initial site of TBI-related dementia offers a more nuanced approach to amyloid and tau in AD pathogenesis.

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PATHOPHYSIOLOGY: THE ROLE OF INFLAMMATION

In an earlier section, neuropathological data were cited showing ongoing white matter inflammation and corpus callosum atrophy for up to 18 years after a single moderate or severe TBI [71]. This study echoes others in which ongoing inflammation within white matter has been observed many years after TBI [107, 108]. This inflammatory process likely implicates activated microglia, which are normal microglia modified by the external stimulus of TBI to undergo morphological change that results in cytokine release and a sustained inflammatory response [108]. Some evidence exists from study of normal brains that microglia are significantly more numerous in white than gray matter [109], which may be a factor explaining why DAI may lead to greater microglial activation in white matter tracts [110]. The inflammatory cascade facilitated by activated microglia and cytokine release may then lead to the neurodegenerative dementias of CTE and AD [1, 52, 58, 67].

Although this review is focused on clinical aspects of TBI, the human data on TBI-related white matter inflammation [71, 107, 108] finds critical support in an intriguing experimental study demonstrating long-standing inflammation and atrophy in the corpus callosum, in association with cognitive dysfunction, after repetitive mild TBI [110]. Of particular interest in this study is that neither Aβ nor hyperphosphorylated tau were present in the cerebral cortex or hippocampus of the animals, and that the white matter neuropathology was considered responsible for the observed neurobehavioral deficits [110]. A chronic traumatic inflammatory encephalopathy is thus a plausible pathophysiological mechanism that contributes to the series of events leading from TBI to dementia [111], and this sequence may proceed from TBI to one of the three major outcomes discussed above: post-traumatic leukoencephalopathy, CTE, or AD. The specific cognitive outcome of TBI in any given individual is unknown, but presumably results from the effects of as yet undetermined factors unique to each injured person.

THERAPEUTIC IMPLICATIONS

The preceding sections have constructed the case for white matter injury after TBI as the common denominator of a wide range of cognitive deficits that may follow. The next task is to consider how this insight, if accurate or even partially so, can inform attempts at treatment. The lack of successful therapy for DAI thus far [112] should not deter further treatment efforts, as it is clear that white matter is a biologically active component of the brain, likely under the variable influence of ongoing inflammation after TBI, that may well be amenable to various disease-modifying and rehabilitative modalities. After some general comments on white matter as a target of treatment, some medical interventions centered on inflammation and the concept of white matter plasticity will be considered.

Two principles of white matter structure and function are pertinent to a discussion of possible treatment of DAI. First, the prognosis of white matter injury depends to a large extent on the relative degree of injury sustained by axons and myelin. In central nervous system disorders such as TBI that include a prominent degree of white matter damage, such as spinal cord injury, multiple sclerosis, leukodystrophies, central pontine myelinolysis, acquired immunodeficiency syndrome (AIDS), and subcortical ischemic disease, the prognosis is clearly more favorable if axons are preserved [113, 114]. That is, if the axonal scaffolding on which myelin can regain its integrity is preserved, tract structure and function have a far greater chance of recovering so that network efficiency and cognitive function may be restored. In the case of TBI with its typical DAI, the prompt treatment of inflammation would presumably have the potential to preserve both myelin and axons before more significant damage is sustained.

The second principle is that white matter possesses the capacity of plasticity, which is most simply conceptualized as the modification of brain structure by experience [115]. Plasticity is not confined to the gray matter, where synaptic function has long been known to manifest physiological and structural changes associated, for example, with learning and memory, but can readily be observed in white matter as well [116, 117]. White matter is dynamic tissue no different in this respect than its gray matter counterpart, and, to illustrate, recent data have shown that neuronal activity can produce plastic changes in white matter via activity-dependent myelination [117] and enhanced oligodendrogenesis [118]. In addition to the restoration of myelin implied by these data, another means of plasticity may be axonal sprouting, which has not been demonstrated in brain white matter but which does occur in spinal cord white matter after injury [116]. Cellular events following changes

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in experience thus indicate that many possibilities for restoring white matter structure and function after TBI may be found useful.

A wide range of pharmacological interventions are potentially conceivable to suppress inflammation after TBI [112, 119], the details of which are beyond the scope of this review. However, the most familiar agents with anti-inflammatory effects—the hormones with glucocorticoid actions widely known as corticosteroids—deserve some attention in view of their very common clinical use. Although corticosteroids have not been shown effective in the acute treatment of moderate and severe TBI, patients with these injuries are critically ill and typically have many other issues, such as cerebral edema, that complicate the pathophysiology [120]. With respect to white matter neuropathology, encouraging experimental evidence shows that estrogen and progesterone may have effects on activated microglia after TBI [121]. Moreover, the neurosteroid allopregnanolone, under study for AD, appears to act in the white matter, upstream of Aβ generation, to increase proliferation of OPCs and axonal myelination [122]. Theoretically, while such treatment is underway, the use of cholinesterase inhibitors such as donepezil could help potentiate the function of still viable axons coursing within the injured white matter [7, 123].

A related line of investigation relevant to future neurodegeneration pertains to potential immunotherapy focused on the prevention of tauopathy. In experimental studies, the tau epitope *cis* P-tau is robustly expressed in axons acutely after TBI, and treatment with a monoclonal antibody against *cis* P-tau blocks the development and spread of tauopathy, while restoring behavioral deficits and long-term potentiation, and preventing brain atrophy [124]. As tauopathy is a central feature of both CTE and AD, any intervention that could abort the accumulation of tau after TBI has much appeal [124].

Another therapeutic possibility for which supportive evidence is rapidly accumulating involves the exploitation of white matter plasticity. In contrast to medical intervention intended to reverse microstructural dysfunction such as inflammatory degradation, or prevent the development and spread of tauopathy, this approach begins with the notion that white matter is damaged but not destroyed, particularly if axons are preserved, and thus has the capacity to repair itself under the influence of appropriately designed therapeutic interventions. As white matter tracts are essential components of distributed neural networks subserving cognition [5, 65–68], this strategy fundamentally involves the restoration of connectivity and resultant improvement in cognition [125].

An appealing and readily available therapeutic approach is the traditional triad of physiatric treatments: physical therapy, occupational therapy, and speech therapy. These familiar treatments may exert significant effects on white matter structure and function by stimulating plasticity within injured tracts. To illustrate, a recent meta-analysis of 29 studies of aging individuals reported significant correlation between physical activity and white matter structure in aging [126], and in patients with Broca's aphasia who had left frontal lesions, melodic intonation therapy not only improved language output but was also associated with increased size of the right arcuate fasciculus [127]. Related to physiatric treatment is the emerging field of non-invasive transcranial brain stimulation, which aims to exploit modalities such as transcranial magnetic stimulation, transcranial direct current stimulation, and transcranial alternating current stimulation. Whereas this kind of stimulation is thought to promote brain plasticity through its influence on synaptic function in the cerebral cortex [128], effects on the white matter subjacent to the cortex may also be possible. In a study of patients with chronic left hemisphere cerebral infarcts, for example, transcranial direct current stimulation was found to be associated with increased FA in ipsilesional white matter of the internal capsule and upper brainstem [129]. Data such as these indicate that it may become feasible with advancing technology to influence plasticity in white matter tracts devoted to cognitive function. Last, a more remote but still intriguing possibility is the use of cell-based therapy to regenerate white matter. This intervention could involve stimulation of OPCs to form functional oligodendrocytes [118], or the use of stem cells (endogenous or exogenous) to replenish oligodendrocytes and enhance remyelination [130].

In sum, the options for treating white matter damage after TBI may involve a wide range of therapeutic modalities, all of which show promise and need not be mutually exclusive. The goals of reducing inflammation, preventing tauopathy, and restoring myelination are all under consideration, and other ideas may also lead to treatment options. It hardly need be stated that, if the primacy of white matter involvement in TBI is unequivocally confirmed, the implementation of these methods would ideally occur as soon as possible after the injury is sustained.

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CONCLUSION

The purpose of this review has been to call attention to the prominence of white matter injury in TBI with the intention of focusing clinical and basic research on a component of the brain not always thought to be important for cognition. While we readily acknowledge the presence of neuropathology elsewhere in the brain that must be considered, the notion that DAI is a fundamental TBI lesion from which major acute and chronic cognitive effects develop has much support and warrants further detailed investigation. Much more study is needed to establish pathophysiological mechanisms, but a powerful hypothesis emerges from this perspective: a common neuropathology links all severity of TBI, leads to post-concussion symptoms and, in perhaps many cases, dementia, and offers a potentially useful avenue toward early intervention to restore brain health and avert long-term cognitive decline. We readily acknowledge that proposing a fundamental role of DAI in the causation of lasting or progressive cognitive dysfunction—including CTE and even AD—is fraught with uncertainty. Nevertheless, the public health importance of TBI and dementia, combined with major gaps in information that can serve to guide clinical practice and public policy, suggest that this point of view may be transformative. Clinical and basic science methods are becoming ever more effective in evaluating these ideas, and such work may offer untold benefits to the many individuals currently coping with TBI, as well as to the many more who will undoubtedly follow.

DISCLOSURE STATEMENT

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/18-0287r2).

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MRI Volumetric Quantification in Persons with a History of Traumatic Brain Injury and Cognitive Impairment

Somayeh Meysami[a], Cyrus A. Raji[b,*], David A. Merrill[c,d], Verna R. Porter[a,d] and Mario F. Mendez[a,c,e]
[a]*Department of Neurology, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA*
[b]*Mallinckrodt Institute of Radiology, Division of Neuroradiology, Washington University, St. Louis, MO, USA*
[c]*Department of Psychiatry and Biobehavioral Sciences, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA*
[d]*The John Wayne Cancer Institute and Pacific Neuroscience Institute, Providence and St. Johns Health Center, Santa Monica, CA, USA*
[e]*V.A. Greater Los Angeles Healthcare System, Los Angeles, CA, USA*

Abstract.
Background: While traumatic brain injury (TBI) is recognized as a risk factor for dementia, there is lack of clinical tools to identify brain changes that may confer such vulnerability. Brain MRI volumetric quantification can sensitively identify brain atrophy.
Objective: To characterize regional brain volume loss in persons with TBI presenting with cognitive impairment.
Methods: IRB approved review of medical records in patients with cognitive decline focused on those who had documented TBI histories and brain MRI scans after TBI ($n = 40$, 67.7 ± 14.5 years) with volumetric quantification by applying an FDA cleared software program. TBI documentation included head trauma mechanism. Brain volumes were compared to a normative database to determine the extent of atrophy. Correlations between these regions and global tests of cognition (MMSE in $n = 17$, MoCA in $n = 27$, $n = 14$ in both) were performed.
Results: Multiple regions demonstrated volume loss in TBI, particularly ventral diencephalon, putamen, and pallidum with smaller magnitude of atrophy in temporal lobes and brainstem. Lobar structures showed strongest correlations between atrophy and lower scores on MMSE and MoCA. The hippocampus, while correlated to tests of cognitive function, was the least atrophic region as a function of TBI history.
Conclusion: Persons with TBI history exhibit show regional brain atrophy. Several of these areas, such as thalamus and temporal lobes, also correlate with cognitive function. Alzheimer's disease atrophy was less likely given relative sparing of the hippocampi. Volumetric quantification of brain MRI in TBI warrants further investigation to further determine its clinical potential in TBI and differentiating causes of cognitive impairment.

Keywords: Magnetic resonance imaging, traumatic brain injury, volumetric quantification

INTRODUCTION

Traumatic brain injury (TBI) affects about 2.5 million persons per year in the U.S., resulting in an estimated annual cost of 76.5 billion dollars [1]. TBI of all severities, including mild TBI, are

*Correspondence to: Cyrus A. Raji, MD, PhD, Mallinckrodt Institute of Radiology, Division of Neuroradiology, Washington University, St. Louis, MO, USA. E-mail: cyrusraji@gmail.com.

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related to increased cognitive impairment as well as a higher risk for dementia [2]. Additionally, TBI may potentially reduce the age of onset and expedite the emergence of Alzheimer's disease (AD) [3]. Repetitive, mild TBIs are also associated with chronic traumatic encephalopathy (CTE), a neurodegenerative disorder which is neuropathologically distinct from AD [4, 5].

Imaging of TBI is utilized mostly in the acute clinical care setting with non-contrast computerized tomography (CT) brain scans used to identify critical findings such as hemorrhage, hydrocephalus, herniation, and vascular injury [6]. Some patients with a remote history of TBI may have overt brain atrophy, ventricular enlargement, a cavum septum pellucidum, white matter changes, or other residual findings from TBI; however, many of these patients have visually interpreted "normal" neuroimaging [7]. Consequently, individuals with the late cognitive consequences of TBI may not have identifiable measures of brain changes on conventional imaging. Clinicians need an objective neuroimaging measure to assess whether remote TBIs are associated with cognitive complaints in later life.

Quantitative volumetric MR brain imaging, which has been utilized for AD and cognitive disorders [8, 9], may be a potential tool for assessing the chronic, delayed effects of TBI. Given that TBI is thought to increase risk for cognitive decline such as AD and CTE, our purpose was to apply readily available quantitative volumetric MRI, with normative data, to persons presenting for cognitive complaints and having a history of TBI sometime in the past. We hypothesized that these patients would have a distinct neuroimaging profile, different from age-related changes and distinct from the usual patterns seen in common dementias such AD.

METHODS

Participants

As part of an IRB approved study at UCLA (IRB# 16-001491), we retrospectively evaluated 40 MRI scans from patients who were referred for cognitive complaints and had a documented history of a traumatic brain injury by hospital records. All subjects received referral to and/or evaluation by either internal medicine, neurosurgery, geriatricians, subspecialty cognitive and behavioral neurologist (VRP or MFM), or a geriatric psychiatrist (DAM). We did not include persons with a TBI history who lacked corresponding documentation of trauma mechanism. As the data and mechanism of trauma were extracted by history, severity of classification for mild, moderate, and severe TBI were not invoked for this study.

As part of their medical evaluation, subjects were screened for history of known dementia or other disorders that would influence cognition including metastatic cancer to the brain, primary brain malignancy, or large territorial ischemic infarcts. We also used the medical records to identify history of vascular disease such as hypertension and type 2 diabetes mellitus. For all subjects, time of MRI scan from injury was extracted from their medical records and ranged from as recent as 1 month to years. We defined four categories of time of MRI scan from injury: 1) less than 6 months, 2) 6 months but less than 1 year, 3) 1 year or longer but less than 5 years, and 4) 5 years or longer. Cognitive evaluations at intake included either the Mini-Mental State Examination (MMSE) [10] ($n = 17$), the Montreal Cognitive Assessment (MoCA) [11] ($n = 27$), or both ($n = 14$). The 10 subjects who did not receive MMSE or MoCA received heterogeneous neuropsychological tests that included both commercial vendors (CNS Vital Signs, $n = 2$, and Web Neuro, $n = 1$), a conventional neuropsychological test battery at UCLA ($n = 5$) and mental status examinations that documented cognitive decline or impairment ($n = 2$). They were not included for analysis with the brain regions due to the heterogeneity of these methods. Subject demographics, including mechanism of TBI are detailed in Table 1 (average age in Men: 59.5 ± 16.5; Women 68 ± 11.9; $p = 0.05$).

MRI methods

Each subject had an MRI of the brain including a 3D volumetric MPRAGE sequence on a 3.0 Tesla Siemens Scanner. All subjects were then analyzed with an FDA cleared volumetric program (Neuroreader) as detailed in prior work [10]. For each scan, 45 brain structures were quantified including the hippocampus, lobar structures, subcortical regions (thalamus, caudate, putamen, etc.), ventral diencephalon, midbrain, ventricular, and white matter volumes with an atlas-based segmentation. Total gray matter (GM), white matter (WM), and cerebrospinal fluid (CSF) volumes were also computed. Total intracranial volume (TIV) was calculated by summing GM, WM, and CSF volumes, and separate computations were done correcting individual brain structural volumes for this total head size metric. For

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Table 1
Subject information

Variable ($n = 40$)	Mean $\pm \sigma$ or Percentage
Age	67.7 ± 14.5 (Range: 25–85 y)
Gender	Women (57%), Men (43%)
Mechanism of TBI	Fall (48%), MVC (30%), Contact Sports (10%), Direct Blow (7%), Blast Injury (5%)
Time from Injury	Less than 6 months (10%), 6 months to less than 1 year (17.5%), 1 year or longer but less than 5 years (35%), 5 or more years (37.5%)
Vascular disease(HTN, T2DM, CAD)*	70%
MMSE	24.1 ± 5.9 (Median: 27, Range: 12–30)
MoCA	21.5 ± 5.9 (Median: 23, Range: 6–29)

TBI, traumatic brain injury, HTN, hypertension; T2DM, type 2 diabetes mellitus; CA, coronary artery disease; MMSE, Mini-Mental State Examination; MoCA, Montreal Cognitive Assessment; MVC, motor vehicle collision.

each brain region, the following metrics were calculated: 1) region of interest volume in ml; 2) the ratio of a region of interest volume to TIV; 3) the number of standard deviations from the normative database (NR index); 4) the number for standard deviations from the mean scaled between –2 and +2 (Z-score); and 5) percentile of comparison to the normative database. The normative database was drawn from control subjects from the Alzheimer's Disease Neuroimaging Initiative ($n = 231$) with an age range of 60–90 and 53% women, 47% men. All normative database comparisons for computing Z-scores and percentiles were adjusted for age, gender, and TIV in a multiple regression model. A typical example of a Neuroreader segmented brain is noted in Fig. 1.

A full list of structures obtained is detailed in Fig. 2.

Data analysis

All statistical analyses were conducted using SPSS (Version 25, IBM, Armonk NY). For the purposes of this evaluation, regions of two or more unscaled standard deviations below the control mean (NR index of ≥ -2) were considered abnormal. These standard deviations were averaged across all subjects. Because these averages consisted of single subject comparisons to the normative database, multiple comparison corrections were not invoked for this analysis. Interaction terms were calculated between TBI and vascular disease status and TBI and age to determine if the volume loss in relation to TBI varied as a function of age or vascular disease in multiple regression models. Separate correlations between TBI mechanism and abnormally low volume regions were also applied. Additionally, correlations were modeled between MoCA and MMSE scores and computed volumes as a proportion of TIV. Finally, we correlated time from injury to brain volumes, adjusting for age, gender, mechanism of injury, and vascular disease.

RESULTS

Table 2 displays a ranked table of the average negative NR indices. Other metrics, including the percentile, are also included. Regions with positive NR regions are not included in the table but included the parietal and occipital lobes as well as cerebellum.

Figure 3 highlights the disparity between the most severe regional volume loss in the ventral diencephalon in contrast with the relatively preserved volume in the hippocampus. The black bars show the lower ventral diencephalon NR indices compared to the hippocampus. The gray bars show the highlight the lower percentile compared to normative database in the ventral diencephalon compared to the higher percentile in the hippocampus.

There were no statistically significant interactions between TBI status and vascular disease. There were statistically significant interactions between age and TBI related atrophy such as the level of volume loss increased in older persons for total gray matter volume ($t = –3.7$, $p = 001$), ventral diencephalon ($t = –7.2$, $p < 0.001$), and putamen ($t = –5.7$, $p < 0.001$). There was a statistically significant interaction between gender and TBI status in total gray matter ($t = –2.1$, $p = 0.04$), ventral diencephalon ($t = –2.1$, $p = 0.04$), putamen ($t = –2.9$, $p = 0.006$), and left thalamus ($t = –3.1$, $p = 0.003$). These findings suggest that in these regions the findings vary as a function of gender with men with TBI having a higher degree of volume loss than women with TBI. It was noted that women experienced TBI more often than men in falls and motor vehicle collisions ($\chi = 9.56$, $p = 0.05$), but men accounted for all of the blast injuries and direct blows. There were no statistically significant differences between brain volumes and mechanism of TBI nor was there an interaction effect.

Table 3 shows statistically significant correlations between the MMSE and MoCA scores and Neuroreader quantified regions. We did not correct for TIV as it reduced the variance. Overall, the average

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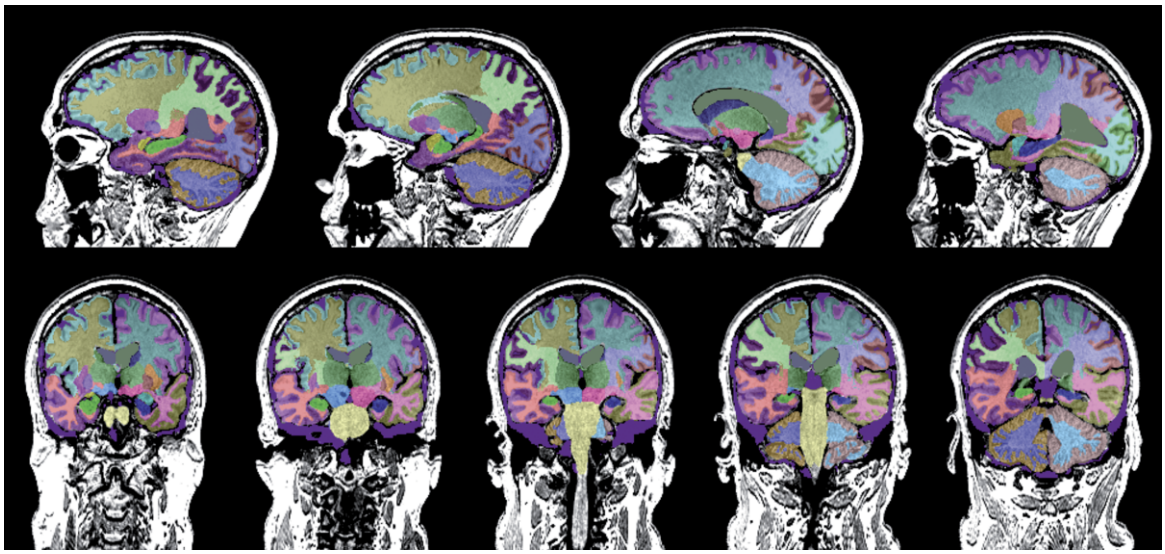

Fig. 1. Sagittal and coronal MRI images segmented by Neuroreader for one of the subjects in this study are displayed. Different colors correspond to different regions on different sides of the brain.

Whole Brain Matter	Ventral Diencephalon	Left Parietal Lobe
Gray Matter	Right Ventral Diencephalon	Occipital Lobe
White Matter	Left Ventral Diencephalon	Right Occipital Lobe
Hippocampus	Pallidum	Left Occipital Lobe
Right Hippocampus	Right Pallidum	Temporal Lobe
Left Hippocampus	Left Pallidum	Right Temporal Lobe
Amygdala	Caudate	Left Temporal Lobe
Right Amygdala	Right Caudate	Cerebellum
Left Amygdala	Left Caudate	Right Cerebellum
Putamen	Brain Stem	Left Cerebellum
Right Putamen	Frontal Lobe	CSF (+ dura)
Left Putamen	Right Frontal Lobe	Lateral Ventricle
Thalamus	Left Frontal Lobe	Right Lateral Ventricle
Right Thalamus	Parietal Lobe	Left Lateral Ventricle
Left Thalamus	Right Parietal Lobe	measured Total Intracranial Volume (mTIV)

Fig. 2. List of structures volumetrically measured by Neuroreader.

MMSE and MoCA scores met diagnostic criteria for cognitive impairment (average MMSE = 24.1 ± 5.9, average MoCA = 21.5 ± 5.9). The MMSE and MoCA scores were highly correlated ($r = –0.79$, $p < 0.001$). There were no statistically significant correlations between trauma mechanism and MMSE or MoCA scores nor was there an interaction effect. There were no statistically significantly correlations between time from injury and brain volumes.

DISCUSSION

TBI may be related to brain atrophy and subsequent cognitive decline [14]. Affected regions shown in prior studies include total gray matter volume [15], putamen [16], and ventral diencephalon [13, 17]. Prior work has suggested that the presence of TBI can accelerate age-related volume loss [18]. Other investigators have found a gender-based interaction

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Table 2
Abnormally low regional brain volumes in TBI group

Structure	NR Index	Volume (mL)	Volume/TIV (%)	Z-score	Percentile
Total Gray Matter	–12.9	492.3	26.7	–0.9	27.6
Ventral Diencephalon	–4.6	9.8	0.5	–0.3	38.4
Right Ventral Diencephalon	–4.7	4.9	0.2	–0.3	37.9
Left Ventral Diencephalon	–4.2	4.9	0.2	–0.3	39.2
Putamen	–4.3	8.3	0.4	–0.3	39.2
Right Putamen	–3.8	4.1	0.2	–0.3	40.5
Left Putamen	–4.6	4.2	0.2	–0.3	38.6
Pallidum	–3.7	3	0.1	–0.2	40.5
Left Thalamus	–2.6	8.9	0.5	–0.1	43.3
Temporal Lobes	–1.7	253.9	11.1	–0.1	45.9
Right Caudate	–1.4	3.6	0.2	–0.1	46
Brainstem	–0.7	19.5	1.1	0.8	48
Frontal Lobe	–0.7	493	20.2	0.1	48.8
Amygdala	–0.5	3.93	0.2	–0.03	49
Hippocampus	–0.4	9.6	0.4	–0.02	49.2

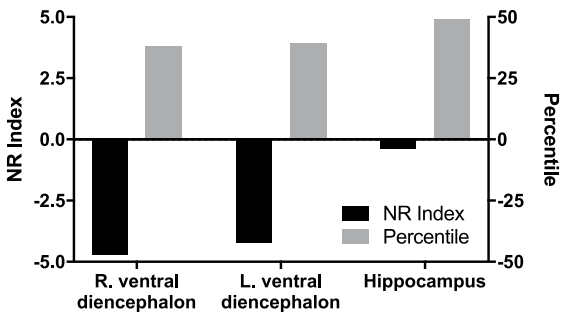

Fig. 3. Neuroreader (NR) index and percentiles of notable regions. See Table 2 for full list of regions.

with brain volumes after TBI with greater decreases in women as compared to men [19]. In this finding, our results differ as we found higher atrophy in men than in women despite the greater number and older age of the women. While women have been shown to have worse outcomes after TBI than men [20], it has also been suggested that men may have an intrinsically higher vulnerability to TBI than women [21]. In this study, we noted a trend toward statistical significance for women experiencing TBI from falls and motor vehicle collisions more often than men. By contrast, men accounted for all of the blast injuries and direct blows. These differences in mechanism of TBI may thus explain why men experienced more volume loss. Critically, the lack of interaction between vascular disease and TBI volume loss suggests that the influence of TBI on brain volumes does not vary as a function of co-morbid vascular disease.

We also found statistically significant correlations between MMSE and MoCA scores and Neuroreader-computed brain regional volumes important for cognition including the frontal, temporal, and parietal lobes and weaker correlations in the hippocampus. Previous studies have suggested that brain volumes can correlate with tests of even global cognitive function [22, 23], but evidence of this in persons with TBI is lacking. Thus, our study adds to previously understood relationships in a patient group that experiences impaired cognition. The fact that the most abnormal regions were those not correlated with MMSE or MoCA, such as the ventral diencephalon, suggests that domain specific neuropsychological tests may be needed to detect abnormalities already apparent on quantified volumetric MR imaging. Thus, both quantified volumetric MRI and neuropsychological tests are best used in conjunction in evaluating TBI as both types of data are complementary.

The major implication of this study is the ability of volumetric quantification to detect brain atrophy following TBI among older patients who present with cognitive complaints [25, 26]. Additionally non-atrophy related findings of TBI such as microbleeds or significant white matter sheer injury are seen only 34% of the time on conventional MR sequences [27]. Furthermore, the fact that we did not find predominant hippocampal or temporal-parietal atrophy in this group compared to other regions suggests that TBI related brain damage is distinct, and that the mechanisms of TBI-related cognitive decline are potentially distinguishable from those for AD and other dementias. The abnormally low ventral diencephalon volumes, for example, do correspond to prior work suggesting that CTE neuropathology deposits in this area [17]. Thus, while volumetric quantification is not currently recommended in standard clinical practice guidelines for even mild TBI

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Table 3
Correlations between MMSE, MoCA, and quantified regions

Structure	Correlation with MMSE p ($n=17$)	Correlation with MoCA p ($n=27$)
Whole Brain Matter	0.585, 0.014	0.459, 0.016
Grey Matter	0.266, 0.302	0.306, 0.121
White Matter	0.441, 0.077	0.237, 0.234
Hippocampus	0.481, 0.051	0.365, 0.061
Right Hippocampus	0.433, 0.083	0.351, 0.073
Left Hippocampus	0.495, 0.043	0.369, 0.058
Amygdala	0.515, 0.035	0.453, 0.018
Right Amygdala	0.424, 0.089	0.392, 0.043
Left Amygdala	0.559, 0.020	0.485, 0.010
Putamen	0.547, 0.023	0.341, 0.082
Right Putamen	0.559, 0.020	0.361, 0.064
Left Putamen	0.515, 0.034	0.307, 0.119
Thalamus	0.733, 0.001	0.614, 0.001
Right Thalamus	0.728, 0.001	0.624, 0.001
Left Thalamus	0.715, 0.001	0.584, 0.001
Ventral Diencephalon	0.603, 0.010	0.332, 0.090
Right Ventral Diencephalon	0.598, 0.011	0.359, 0.066
Left Ventral Diencephalon	0.592, 0.012	0.299, 0.129
Pallidum	0.413, 0.099	0.231, 0.245
Right Pallidum	0.401, 0.111	0.209, 0.296
Left Pallidum	0.415, 0.098	0.240, 0.229
Caudate	0.206, 0.428	0.038, 0.851
Right Caudate	0.277, 0.282	0.073, 0.717
Left Caudate	0.127, 0.628	0.002, 0.991
Brainstem	0.553, 0.021	0.185, 0.356
Frontal Lobe	0.518, 0.033	0.334, 0.089
Right Frontal Lobe	0.526, 0.030	0.358, 0.067
Left Frontal Lobe	0.506, 0.038	0.307, 0.119
Parietal Lobe	0.584, 0.014	0.512, 0.006
Right Parietal Lobe	0.583, 0.014	0.519, 0.006
Left Parietal Lobe	0.573, 0.016	0.496, 0.009
Occipital Lobe	0.364, 0.150	0.465, 0.014
Right Occipital Lobe	0.359, 0.157	0.506, 0.007
Left Occipital Lobe	0.357, 0.159	0.405, 0.036
Temporal Lobe	0.658, 0.004	0.547, 0.003
Right Temporal Lobe	0.653, 0.005	0.578, 0.002
Left Temporal Lobe	0.628, 0.007	0.489, 0.010
Cerebellum	0.483, 0.050	0.229, 0.250
Right Cerebellum	0.489, 0.046	0.213, 0.286
Left Cerebellum	0.470, 0.057	0.242, 0.224
Cerebrospinal fluid	–0.046, 0.861	–0.361, 0.064
Lateral Ventricle	–0.211, 0.416	–0.278, 0.161
Right Lateral Ventricle	–0.177, 0.496	–0.282, 0.154
Left Lateral Ventricle	–0.237, 0.360	–0.269, 0.175

[28], it does hold potential future applications for this population.

There are several potential limitations of this study. First, we do not establish causation between the low brain volumes and cognitive impairment or the mechanism of TBI of these patients. Second, the assessment of TBI was retrospective and, consequently, fraught with problems with the recall of specific details of TBIs, as is usual for this type of study. Third, our study is additionally limited in its generalization to the overall population by the relatively small sample size. Larger samples sizes for various mechanisms may demonstrate specific differences as suggested in prior work [29]. The lack of TBI severity grading is another limitation. It is possible that the study findings may only apply to moderate or severe TBI; however, atrophy can be seen even in mild TBI with one study showing increasing atrophy with loss of consciousness [30]. Additionally, post-traumatic impairment of memory function also predicts increased atrophy [31]. Different 3T magnets were also used in this study. While this could be a potential limitation, Neuroreader has produced reproducible high quality regional brain segmentation regardless of vendor or field strength [12, 32]. The younger ages in our cohort compared to the normal database raises the possibility that atrophy may have been underestimated. Another limitation of this study is the differing sensitivities of MMSE and MoCA, with one study showing greater sensitivity of MoCA compared to MMSE in detecting cognitive impairment in relation to TBI [24]. Finally, we do not evaluate distinct white matter tracts in this study and did not find abnormally low white matter volumes in the TBI group. However prior work has shown the importance of white matter analyses in capturing the cognitive deficits in TBI [33]. Thus, future studies should incorporate combined longitudinal volumetric and white matter diffusion MR imaging analyses for maximal sensitivity in detecting TBI related brain abnormalities.

This study demonstrates total reduction of gray matter volume in TBI compared to controls with focal areas showing greater areas of volume loss. This work may form the basis for future studies that not only utilize these regions for improved accuracy of TBI related brain damage but may also serve as biomarkers for treatment response for cognitive rehabilitation programs [34]. Future studies with clinical trials will be necessary for such additional validation.

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FDDNP-PET Tau Brain Protein Binding Patterns in Military Personnel with Suspected Chronic Traumatic Encephalopathy[1]

Stephen T. Chen[a,2], Prabha Siddarth[a,b,2], David A. Merrill[a,b], Jacqueline Martinez[a,b], Natacha D. Emerson[a,b], Jie Liu[c], Koon-Pong Wong[c], Nagichettiar Satyamurthy[c], Christopher C. Giza[d], Sung-Cheng Huang[c], Robert P. Fitzsimmons[e], Julian Bailes[f], Bennet Omalu[g], Jorge R. Barrio[c] and Gary W. Small[a,b,*]

[a]*Department of Psychiatry and Biobehavioral Sciences, University of California, Los Angeles, Los Angeles, CA, USA*
[b]*UCLA Longevity Center at the Semel Institute for Neuroscience and Human Behavior, University of California, Los Angeles, Los Angeles, CA, USA*
[c]*Department of Medical and Molecular Pharmacology, University of California, Los Angeles, Los Angeles, CA, USA*
[d]*Department of Neurosurgery at the David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA, USA*
[e]*Fitzsimmons Law Offices, Wheeling, WV, USA*
[f]*Department of Neurosurgery, North Shore University Health System and University of Chicago Pritzker School of Medicine, Evanston, IL, USA*
[g]*Department of Medical Pathology and Laboratory Medicine, School of Medicine, University of California, Davis, Sacramento, CA, USA*

Abstract.
Background: Our group has shown that *in vivo* tau brain binding patterns from FDDNP-PET scans in retired professional football players with suspected chronic traumatic encephalopathy differ from those of tau and amyloid aggregate binding observed in Alzheimer's disease (AD) patients and cognitively-intact controls.
Objective: To compare these findings with those from military personnel with histories of mild traumatic brain injury (mTBI).
Methods: FDDNP-PET brain scans were compared among 7 military personnel and 15 retired players with mTBI histories and cognitive and/or mood symptoms, 24 AD patients, and 28 cognitively-intact controls. Nonparametric ANCOVAs with Tukey-Kramer adjusted *post-hoc* comparisons were used to test for significant differences in regional FDDNP binding among subject groups.

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[2]These authors contributed equally to this work.

*Correspondence to: Gary W. Small, MD, Semel Institute, 760 Westwood Plaza, Los Angeles, CA 90024, USA. E-mail: gsmall@mednet.ucla.edu.

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Results: FDDNP brain binding was higher in military personnel compared to controls in the amygdala, midbrain, thalamus, pons, frontal and anterior and posterior cingulate regions ($p < 0.01$–0.0001). Binding patterns in the military personnel were similar to those of the players except for the amygdala and striatum (binding higher in players; $p = 0.02$–0.003). Compared with the AD group, the military personnel showed higher binding in the midbrain ($p = 0.0008$) and pons ($p = 0.002$) and lower binding in the medial temporal, lateral temporal, and parietal regions (all $p = 0.02$).

Conclusion: This first study of *in vivo* tau and amyloid brain signals in military personnel with histories of mTBI shows binding patterns similar to those of retired football players and distinct from the binding patterns in AD and normal aging, suggesting the potential value of FDDNP-PET for early detection and treatment monitoring in varied at-risk populations.

Keywords: Alzheimer's disease, brain tau and amyloid, chronic traumatic encephalopathy, FDDNP-PET, mild traumatic brain injury, military personnel, retired professional football players

INTRODUCTION

Chronic traumatic encephalopathy (CTE) is a progressive neurodegenerative disease associated with prior brain trauma. Clinically, the disease is marked by cognitive, behavioral, and motor disturbances, and eventually progressive dementia. Neuropathological changes of CTE include widespread accumulation of phosphorylated tau protein as neurofibrillary tangles (similar to those observed in Alzheimer's disease, AD), astrocytic tangles, neurites, diffuse axonal injury, white matter abnormalities, inflammation, and immune pro-inflammatory cytokine responses in traumatized brain regions. Immunoreactive deposits are found in neocortical, subcortical (e.g., thalamus, caudate, putamen, midbrain, and cerebellar white matter), and medial temporal (hippocampus, entorhinal cortex, and amygdala) regions, where neuronal loss may be observed [1]. TAR DNA-binding protein 43 (TDP-43, transactive response DNA binding protein 43 kDa), is a protein encoded by the TARDBP gene that may accompany tauopathy in CTE cases, predominantly in subcortical areas, and is more prominent in motor neuron disease cases [2]. Amyloid deposition has been reported—typically after age 60—in approximately 40% to 50% of CTE cases and generally consists of sparse diffuse plaques with relatively few neuritic plaques in cortical areas, and rarely in subcortical structures. NINDS criteria for the diagnosis of CTE require p-tau aggregates around small vessels in an irregular pattern at the depths of the cortical sulci and are supported by p-tau-related pathologies in the hippocampus, mammillary bodies and other hypothalamic nuclei, amygdala, nucleus accumbens, thalamus, midbrain tegmentum, and isodendritic core [3].

In vivo imaging of neuropathological, insoluble protein aggregates with positron emission tomography (PET) can aid in the early diagnosis of neurodegenerative diseases before extensive neuronal loss and clinical symptoms become evident [4]. Investigators have studied numerous *in vivo* imaging probes with purported specificity for amyloid-β plaques, including [C-11]PiB, [F-18]3-fluoro-PiB ([F-18]flutemetamol), [C-11]SB-13, [F-18]BAY94-9172 ([F-18]florbetaben), [F-18]AV45 ([F-18]florbetapir), [C-11]BF-227, among others [5]; however, only FDDNP has been shown to also detect tau protein deposits in the living human brain. Most recently developed tau-specific ligands used with PET (e.g., [C-11]PBB3, [F-18]THK-523, [F-18]THK-5105, [F-18]THK-5117, [F-18]T808, [F-18]T807) have shown significant non-specific, "off-target" binding (e.g., T-807 or AV-1451 and others) to MAO-A, MAO-B, neuromelanin-containing neurons, and other tissue targets in AD subjects [6, 7]. In subjects with suspected CTE, with the exception of one publication using T-807 in one National Football League (NFL) subject [7], no other reports have become available [8].

FDDNP is specific for the β-pleated sheet conformation present in amyloidogenic fibrils, both *in vitro* [9] and *in vivo*, in AD and mild cognitive impairment (MCI) [10, 11] and in Gerstmann-Sträussler-Scheinker disease and other prion diseases [12, 13], as well as in tau neuropathology in frontotemporal dementia [14] and progressive supranuclear palsy [15]. Previously, our group used FDDNP-PET to detect brain patterns of tau neuropathology distribution in retired NFL players with suspected CTE consistent with models of concussion and with paired helical filament-tau distribution observed at autopsy in subjects with a history of mild traumatic brain injury (mTBI) and CTE [16, 17].

As indicated above, FDDNP is specific for protein motifs having β-sheet pleated sheets, which are present in tau and amyloid neuroaggregates and explain its successful utilization in various

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neurodegenerative diseases having tau and amyloid-containing neuroaggregates.

McKee and associates have reported that CTE is a predominant tauopathy and that amyloid, as sparse diffuse amyloid, is only observed in brain cortices of some CTE patients, typically after age 60 [18]. Thus, in the absence of amyloid aggregates, FDDNP appears to serve as a tau-imaging probe in CTE or other tauopathies like progressive supranuclear palsy [15]. Our previous work indicated that FDDNP-PET scans of retired professional football players could be differentiated from those of patients with AD [16]. This tau binding in CTE has recently been confirmed by Omalu and associates, who performed an autopsy on a 63-year-old football player previously scanned with FDDNP, which showed that FDDNP-PET revealed brain binding levels closely correlated with paired helical filament tau (PHF-tau) deposition (Spearman rank-order correlation (rs); $rs = 0.59$, $p = 0.02$) [19]. Regardless of whether FDDNP is binding to tau or amyloid, which may not be certain at the time of imaging, it is the regional pattern of FDDNP binding that distinguishes these various neurodegenerative diseases.

Brain trauma in military service members is well documented. Approximately 15% to 23% of returning military service members suffer from mTBI, [20–23] which includes concussion, sub-concussion, and mostly exposures to explosive blasts from improvised explosive devices. Military-related mTBI may be different from mTBI related to sports participation in terms of the manner of causation, including frequency or repetitiveness of impact (i.e., blast versus machine gun) and the type and angle of impact (i.e., massive blasts versus landmines). Unlike the relatively circumscribed types of trauma that occur in sports, military-related mTBI is acquired in a broad variety of ways, including sports and other recreational activities, physical training, falls, and motor vehicle accidents, though, in one study, explosive blast injury accounted for 56% of military-related TBI, of which 80% were mTBI [22]. Injuries from blasts vary depending on the magnitude of the explosive, proximity to the explosive, and the space in which the explosive detonates. McKee and Robinson [24] have commented on the variability of mTBI among military personnel, who may experience single or multiple blast injuries. Blast injury is the result of the rapid transmission of an acoustic wave through the brain tissue and accompanying blast winds that can produce forces similar to multiple, severe concussive impacts occurring over microseconds [25].

There is no consensus at this time on how to grade the severity of military-related mTBI. Military-related mTBI may be further distinguished from sports-related mTBI by its high co-occurrence with posttraumatic stress disorder (PTSD), which occurs in 44% of military personnel who have suffered mTBI and subsequent loss of consciousness [21]. PTSD can be difficult to distinguish from other sequelae of mTBI, post-concussive syndrome, and CTE. All are characterized by executive dysfunction, impulsivity, emotional lability, disinhibition, sleep disturbance, and changes in personality [26, 27]. These common neuropsychiatric features may reflect the increasingly convergent neuropathological findings in the study of post-concussive syndrome, PTSD, and CTE. A tool that could distinguish PTSD due to psychological stress from other trauma-induced conditions such as CTE would be important for developing specific treatments. To address some of these knowledge gaps, we report here the first FDDNP-PET scan findings of brain tau pathology in military personnel with suspected CTE and compare the results to those of retired NFL players, patients with AD and cognitively healthy individuals.

MATERIALS AND METHODS

Neuropsychiatric evaluations were performed on seven military personnel who were recruited for this study because of a history of head trauma and cognitive or mood symptoms. Recruitment of military personnel occurred through word of mouth and individuals who contacted our group after hearing about our previous research through media. We compared results from these subjects with those of 15 retired NFL players, 24 patients with AD, and 28 cognitively intact individuals reported previously [16]. In the current study, their FDDNP-PET scans were reanalyzed using a more complex set of regions of interest (ROIs) as defined below. Subjects in the AD group met the standard diagnostic criteria for AD and dementia [28, 29]. The control group had normal cognitive functioning for their age and did not meet criteria for mild cognitive impairment or AD [30].

Subjects had physical examinations and assessments, screening laboratory tests, and structural imaging scans (computed tomography [CT] or magnetic resonance imaging [MRI]) to rule out major medical problems and other causes of mental symptoms (e.g., stroke, tumor) and for co-registration with PET scans for ROI analyses. Four football

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players and one veteran underwent CT scans because they could not tolerate MRI (claustrophobia, body metal, body size).

The Mini-Mental State Examination (MMSE) and Hamilton Rating Scales for Depression (HAM-D) and Anxiety (HAM-A) were administered to quantitate degree of cognitive and mood changes as well as to confirm diagnoses [11, 31]. In addition, a neuropsychological test battery was administered to assess memory, language, attention, information-processing speed, executive functioning, and visuospatial ability. We used standard diagnostic criteria for MCI, as previously described to measure memory, executive functioning, attention, and language abilities [11]. Clinical assessments were performed within four weeks of scanning, and clinicians were blinded to scan results. Informed consent was obtained in accordance with UCLA Human Subjects Protection Committee procedures. Cumulative radiation dosimetry was below the mandated maximum annual dose and in compliance with state and federal regulations.

All scans (Siemens Biograph TruePoint PET/CT scanner) were performed with participants in a supine position and with the imaging plane parallel to the orbitomeatal line as detailed previously [11, 17, 32]. In brief, subjects were injected with 10 mCi of FDDNP. FDDNP binding data were quantified using Logan graphical analysis: The slope of the linear portion of the Logan plot is the relative distribution volume (DVR) of the tracer in an ROI divided by that in the reference region (cerebellum) [33]. ROIs were traced on co-registered MRI or CT scans for subcortical, limbic (amygdala, striatum, thalamus, subthalamus, midbrain, pons), and cortical (frontal, parietal, occipital, anterior cingulate, posterior cingulate, and medial and lateral temporal) regions [11, 17, 32]. Each DVR or binding value was expressed as an average of left and right regions. All scans were read and ROIs drawn by individuals blinded to clinical assessments.

Statistical analyses

Demographic and clinical measures were compared between groups using Kruskal-Wallis tests for continuous measures (followed by Mann-Whitney tests for pair-wise comparisons) and Fishers exact tests for categorical measures. Nonparametric ANCOVAs (using ranked FDDNP binding levels rather than the raw DVR values; controlling for age) with Tukey-Kramer adjusted *post-hoc* comparisons were used to test for statistically significant differences in regional FDDNP binding (DVR values) among the four subject groups. We also examined whether inclusion of sex, APOE4 status, and educational level as covariates changed any of the findings. A significance level of $p < 0.05$ (two-tailed) was used for all inferences.

RESULTS

Demographic and clinical data are shown in Table 1. Military personnel and football players were exclusively men, whereas the AD and cognitively healthy control groups had a substantial proportion of women. Military personnel were significantly younger than the other three groups. Four of the seven military personnel were cognitively normal, and three had MCI (Supplementary Table 1). All but two football players had a diagnosis of MCI; one player was cognitively normal and one had dementia (Supplementary Table 2). While the difference in education levels among subject groups did not reach statistical significance, pairwise comparisons indicated that military personnel had significantly lower education levels than both players and cognitively healthy individuals. Military personnel and players had significantly higher levels of depression and anxiety than did controls and AD subjects but military personnel and players did not differ significantly. Three military personnel and five players had moderate to severe depression, while one military and two players had moderate to severe anxiety. One military serviceman had also played football and suffered concussions.

The four groups differed significantly in all of their regional FDDNP binding levels, controlling for age ($F(3,69) = 4.4–44.0$; all p-values <0.0001 except for occipital, $p = 0.007$) (Table 2). FDDNP binding levels were significantly higher in military personnel compared to cognitively intact individuals in the following ROIs: amygdala, midbrain, thalamus, pons, frontal, anterior cingulate gyrus, and posterior cingulate gyrus ($p < 0.01–0.0001$) (Table 2, Figs. 1 and 2). Binding patterns in the military personnel were similar to those of the football players except in the amygdala and striatum, where the players had higher binding than the military personnel ($p = 0.02–0.003$). Compared with the AD group, the military personnel showed higher binding in the midbrain ($p = 0.0008$) and pons ($p = 0.002$) and

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Table 1
Demographic and clinical characteristics of the study groups

Characteristic*	Military (N = 7)	Players (N = 15)	AD AD (N = 24)	Cognitively Intact (N = 28)	Statistics**, *p*-value
Age (y)	45 (33–57)	54 (40–86)	77 (50–86)	62.5 (30–84)	24.1, <0.0001
Education (y)	14 (12–16)	16 (14–28)	16 (6–22)	18 (12–22)	7.5, 0.06
Females	0 (0)	0 (0)	12 (50)	10 (36)	0.0009
Family History of Dementia	1 (14)	5 (33)	10 (43)	14 (50)	0.4
APOE4 carriers[$]	3 (43)	2 (13)	11 (58)	12 (44)	0.06
MMSE score	29 (24–29)	27 (17–30)	22 (4–27)	30 (26–30)	49.6, <0.0001
HAM-D score	17 (1–39)	15 (0–33)	1 (0–15)	2 (0–9)	29.3, <0.0001
HAM-A score	16 (2–32)	13 (0–31)	2 (0–17)	3 (0–9)	21.1, <0.0001

*Median (range) or number (%). **Kruskal-Wallis $\chi^2(3)$ (continuous measures) or Fisher's exact test *p*-value (categorical measures). Mann-Whitney tests for pair-wise comparisons: Subjects with Alzheimer's disease (AD) were significantly older than controls (U = 758, $p = 0.03$), players (U = 179, $p = 0.001$) and military personnel (U = 30.5, $p = 0.0006$). Military personnel were younger than players (U = 45.5, $p = 0.02$) and controls (U = 52, $p = 0.005$). Military personnel had lower educational level than players (U = 44, $p = 0.02$) and controls (U = 65.5, $p = 0.02$). The AD group had lower Mini-Mental State Examination (MMSE) scores than all other groups (U = 174–411, $p = 0.002$–0.0001); controls had significantly higher MMSE scores compared to players (U = 178, $p = 0.0002$) and military (U = 59, $p = 0.005$). The players and military personnel had higher scores on the 1) Hamilton Depression Rating Scale (HAM-D) compared to AD (players: U = 371, $p = 0.0006$; military: U = 148, $p = 0.005$) and controls (players: U = 500.5, $p < 0.0001$; military: U = 207.5, $p = 0.002$); 2) Hamilton Anxiety Rating Scale (HAM-A) compared to AD (players: U = 379, $p = 0.0025$; military: U = 155.5, $p = 0.008$) and controls (players: U = 455, $p = 0.001$; military: U = 193.5, $p = 0.005$). There were no other pair-wise difference between groups. [$]Missing for 5 AD and 1 cognitively intact subject.

Table 2
Mean group 2-(1-{6-[(2-[F-18]fluoroethyl) (methyl)amino]-2-naphthyl}ethylidene)malononitrile relative distribution volumes (standard deviation values given in parentheses) and comparisons between groups

	Amygd	Midb	HypoTh	Thal	Pons	Str	MTL	LTL	F	P	ACG	PCG	O
Military Personnel	1.30	1.26	1.32	1.37	1.27	1.33	1.14	1.11	1.12	1.08	1.17	1.13	1.03
n = 7	(0.09)	(0.14)	(0.13)	(0.14)	(0.14)	(0.10)	(0.09)	(0.07)	(0.05)	(0.05)	(0.07)	(0.06)	(0.03)
Players	1.39	1.34	1.42	1.51	1.31	1.53	1.18	1.12	1.14	1.09	1.22	1.16	1.00
n = 15	(0.10)	(0.07)	(0.08)	(0.10)	(0.07)	(0.10)	(0.04)	(0.05)	(0.05)	(0.05)	(0.07)	(0.08)	(0.05)
Alzheimer's disease	1.24	1.15	1.23	1.32	1.16	1.35	1.19	1.16	1.11	1.15	1.14	1.18	1.06
n = 24	(0.06)	(0.06)	(0.06)	(0.11)	(0.06)	(0.09)	(0.02)	(0.02)	(0.03)	(0.03)	(0.07)	(0.02)	(0.06)
Cognitively Intact	1.16	1.14	1.23	1.25	1.15	1.32	1.11	1.06	1.04	1.05	1.09	1.08	1.02
n = 28	(0.04)	(0.05)	(0.05)	(0.09)	(0.08)	(0.07)	(0.02)	(0.03)	(0.03)	(0.03)	(0.04)	(0.04)	(0.05)
Nonparametric ANCOVA*	36.25	26.51	16.42	19.2	20.13	21.35	28	29.34	43.97	23.88	16.54	25.71	4.39
Tukey-Kramer adjusted pairwise *t*-test *p*-values													
Players versus Military	0.02	0.5	0.1	0.2	0.8	0.003	0.07	0.9	0.8	0.9	0.6	0.9	0.4
Cognitively intact versus Military	0.001	0.0003	0.1	0.01	0.0007	0.5	0.3	0.07	<0.0001	0.2	0.004	0.007	0.9
Alzheimer's disease versus Military	0.9	0.0008	0.1	0.3	0.002	0.5	0.02	0.02	0.7	0.02	0.3	0.4	0.8
Cognitively intact versus Players	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.006	<0.0001	<0.0001	0.5

Amygd, amygdala; Midb, midbrain; HypoTh, hypothalamus; Thal, Thalamus; Str, striatum (caudate nucleus and putamen); MTL, medial temporal lobe; LTL, lateral temporal lobe; F, frontal; P, parietal; PCG, posterior cingulate gyrus; ACG, anterior cingulate gyrus; O, occipital. *F(3,69) statistics; all *p*-values<0.0001 except for O ($p = 0.007$), controlling for age.

lower binding in the medial temporal, lateral temporal, and parietal regions (all $p = 0.02$) (Table 2). All the above findings were similar with inclusion of sex, APOE4 status, or educational level as a covariate.

DISCUSSION

This study is the first to report *in vivo* FDDNP binding patterns of brain tau and amyloid protein deposits in military personnel with suspected CTE

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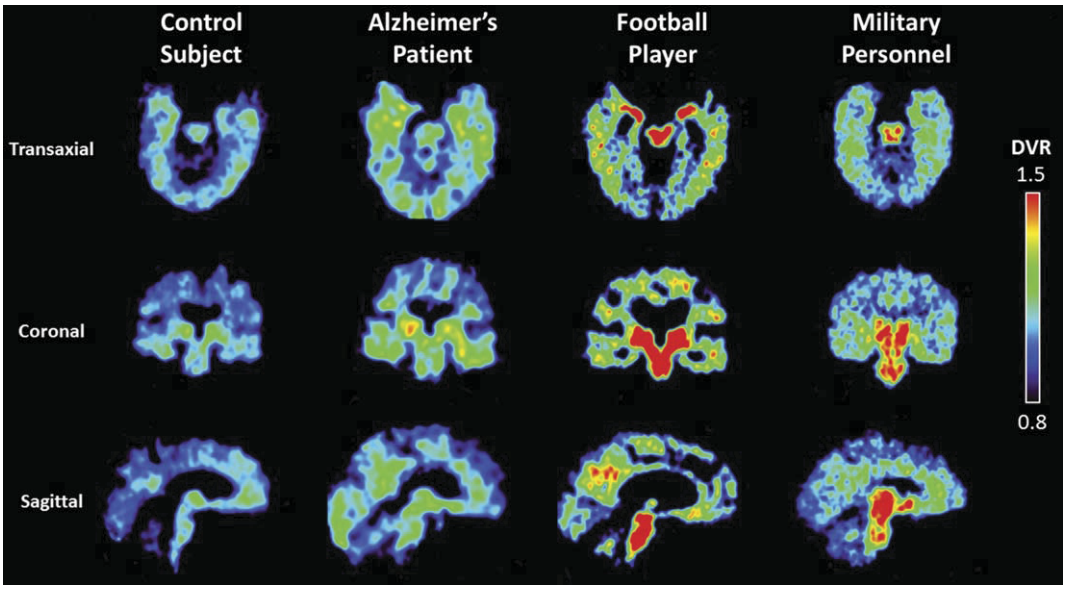

Fig. 1. Examples of FDDNP-PET DVR transaxial, coronal, and sagittal images of a cognitively healthy individual, Alzheimer's disease patient, football player, and a military subject. The Alzheimer's disease patient shows higher DVR signals in parietal, temporal and frontal regions compared to the healthy individual, who may have neuropathology deposition given the age of the subject (80 years) [11]. The football player and military subject show higher amygdala, midbrain, and other subcortical binding compared with the healthy individual and Alzheimer's disease patient. The healthy individual showed some mild cortical binding typical of other healthy individuals age 70 and older (or darker shades in greyscale) [10]. Warmer colors (or darker shades in greyscale) indicate higher FDDNP binding.

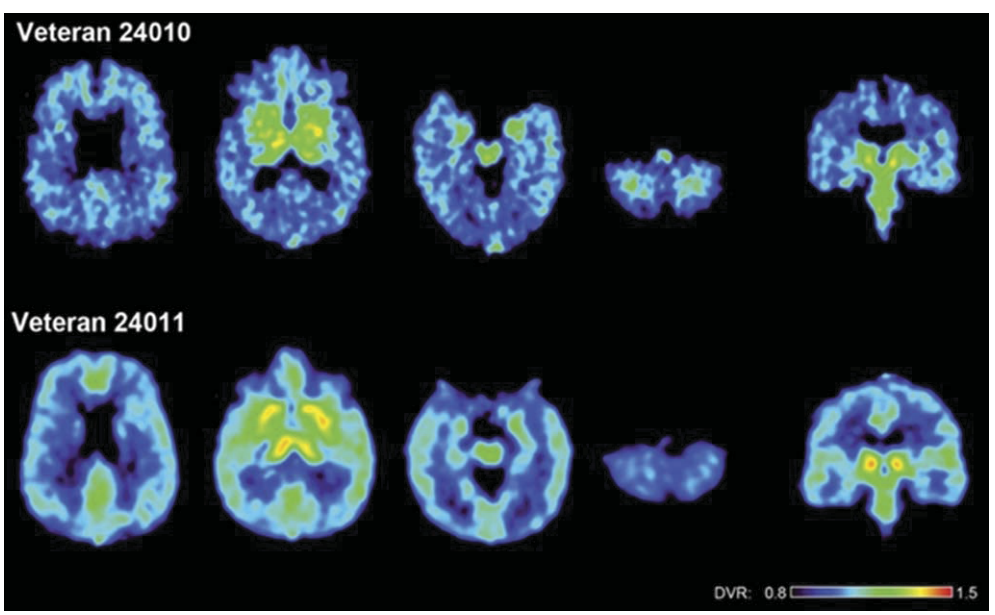

Fig. 2. FDDNP DVR parametric images of the brains of two war veterans with histories of multiple blast concussions (mTBIs) during their war zone deployment. The upper row shows a 48-year-old man (veteran 24010) and the lower row a 36-year-old man (veteran 24011). Left four images in each row show transaxial brain images from top of the brain to the bottom. The right image shows a coronal cut through the midbrain. (This figure was originally published in Barrio et al., 2015 [16].)

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and compare them to those of retired football players with suspected CTE, as well as FDDNP tau and amyloid binding patterns in patients with AD and cognitively intact individuals. As predicted, FDDNP binding patterns in military personnel with suspected CTE were similar to those of retired football players with suspected CTE, but different in certain brain regions from those with AD, and distinct from those with normal cognition.

The FDDNP binding patterns in these military personnel show high levels of binding in brain regions demonstrating tau accumulation found in postmortem studies of individuals with CTE [19, 34]. While athletes have been the focus of similar studies, the present investigation points to the potential utility of this technology in other at-risk populations. Only one subject (football player) had dementia, suggesting that FDDNP may potentially detect CTE-related neuropathology well before individuals suffer from major cognitive impairment.

Efforts toward developing tau-specific PET ligands have not thus far yielded useful results, as the developed probes have shown a lack of *in vivo* specificity and a lack of correlation with *in vitro* tau deposition due to binding to other tissue targets leading to significant 'off-target' binding [35]. FDDNP is the only available, well-studied tau-PET ligand that, thus far, has been used in the PET imaging of any tauopathy, including progressive supranuclear palsy or CTE, and has been shown to differentiate CTE from AD and normal cognition [15, 16]. We have previously shown that FDDNP binds to both fibrillary tau and amyloid, abnormal protein deposits that accumulate in AD [10, 11]. Since neuropathological determinations indicate that CTE is a predominant tauopathy [34] and amyloid cortical deposition—as diffuse amyloid—is observed as a function of age later in the development of the disease (typically in subjects older than 60 years of age) [1], our imaging results indicate that the FDDNP signal in our cases of suspected CTE likely represent fibrillary tau deposition, as confirmed in our recent work. Further, our results in the present study confirm our group's findings that the pattern of abnormal brain tau deposition is the key feature of FDDNP-PET that offers a potentially useful biomarker for differentiating these and other important forms of neurodegeneration [16].

The military and retired football player groups showed similar FDDNP tau binding patterns although there were some regional distinctions, specifically in the amygdala and striatum. The distinctions in FDDNP binding values between these two groups with suspected CTE may reflect the differences of the concussion-based CTE development versus the blast-based mechanism of CTE (Blast-Variant CTE) [16]. The subjects in the military group are typically subjected to impacts that may be fairly powerful but less frequent than the concussive and sub-concussive injuries occurring while playing football. Among athletes with concussion-based CTE, our prior research [16] suggests a progression of neuropathology over four stages of severity (T1-T4) [24], whereas in blast-mediated CTE experienced by military personnel, such a progressive pattern of neuropathological change has not yet been described.

Limitations to interpretation of our findings include the small number of military personnel studied, the absence of women in the two groups with mTBI, the lack of specificity of injury to military personnel, and lack of comparison with individuals with mTBI but no symptoms or signs of CTE. Previous studies have not reported any gender based differences observed in FFDNP binding. One subject from the military personnel group also had an athletic background and may have suffered mTBI related to sports and/or military experience. Several subjects in the military and player groups suffered from moderate to severe depression and anxiety. In a previous study of individuals with MCI and without a history of mTBI, our group found that depression and trait anxiety correlated with cortical FDDNP binding [36], suggesting that current findings may also be attributable to psychopathology unrelated to mTBI. Other investigators posit that inflammation, which has been implicated along with tau and amyloid in the cascade of causative events in neurodegenerative diseases, could also play a role in depression and post-traumatic stress disorder (PTSD) associated with TBI [37, 38]. We did not screen for PTSD, which is highly prevalent among military personnel with mTBI and may in itself be correlated with neuropathology [39, 40]. Other factors that could influence the results would be differences in cerebrovascular health, substance use, and genetic risk among subjects with suspected CTE and normal cognition. Given these limitations, our findings warrant further study before they can be applied to clinical settings. Despite such limitations, our findings are consistent with our a priori hypothesis and the binding patterns in subjects with suspected CTE—both the players and the military personnel—are consistent with known neuropathological deposition patterns in multiple cases of CTE confirmed through neuropathological examination at autopsy [1, 2].

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Further study of CTE biomarkers in at-risk individuals, both symptomatic and asymptomatic, is warranted to better characterize disease progression and develop effective strategies to treat CTE in military personnel, athletes, and other at-risk groups. It is also critically important to avoid premature assumptions of CTE based on nonspecific symptomatology, and it is here that objective biomarkers may prove very useful clinically. Longitudinal studies of FDDNP and other CTE biomarkers can objectively quantify injury severity, predict patient outcome and risk for future neurodegeneration, identify injury mechanisms and therapeutic targets, aid in assessment of therapeutic efficacy, and monitor recovery or decline, though the relationship between neuropathological burden and symptom severity has not yet been established. The current results suggest that FDDNP-PET may have the potential to become a valuable biomarker for early detection, treatment monitoring, and prevention of CTE in varied at-risk populations. Given the large number of people at risk, the public health impact of such a biomarker would be considerable.

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The University of California, Los Angeles, owns a U.S. patent (6,274,119) entitled "Methods for Labeling β-Amyloid Plaques and Neurofibrillary Tangles;" which has been licensed to TauMark, LLC. Drs. Satyamurthy, Huang, Omalu, Barrio and Small are among the inventors.

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/17-1152r3).

SUPPLEMENTARY MATERIAL

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Inflammation in Traumatic Brain Injury

Teodor T. Postolache[a,b,c,d,*], Abhishek Wadhawan[a,e], Adem Can[f], Christopher A. Lowry[b,c,g,h], Margaret Woodbury[a,i], Hina Makkar[a], Andrew J. Hoisington[b,j], Alison J. Scott[k], Eileen Potocki[i], Michael E. Benros[l] and John W. Stiller[a,m,n]

[a]*Mood and Anxiety Program, Department of Psychiatry, University of Maryland School of Medicine, Baltimore, MD, USA*
[b]*Veterans Health Administration, Rocky Mountain Mental Illness Research Education and Clinical Center (MIRECC), Veterans Integrated Service Network (VISN) 19, Aurora, CO, USA*
[c]*Military and Veteran Microbiome: Consortium for Research and Education (MVM-CoRE), Aurora, CO, USA*
[d]*Mental Illness Research, Education and Clinical Center (MIRECC), Veterans Integrated Service Network (VISN) 5, VA Capitol Health Care Network, Baltimore, MD, USA*
[e]*Saint Elizabeths Hospital, Department of Psychiatry, Washington, DC, USA*
[f]*School of Medicine, University of Maryland Baltimore, Baltimore, MD, USA*
[g]*Department of Integrative Physiology and Center for Neuroscience, University of Colorado Boulder, Boulder, CO, USA*
[h]*Department of Physical Medicine and Rehabilitation and Center for Neuroscience, University of Colorado Anschutz Medical Campus, Aurora, CO, USA*
[i]*VA Maryland Healthcare System, Baltimore VA Medical Center, Baltimore, MD, USA*
[j]*Systems Engineering and Management, Air Force Institute of Technology, Wright-Patterson AFB, OH, USA*
[k]*Department of Microbial Pathogenesis, University of Maryland School of Dentistry, Baltimore, MD, USA*
[l]*Copenhagen Research Center for Mental Health-CORE, Mental Health Centre Copenhagen, Copenhagen University Hospital, Copenhagen, Denmark*
[m]*Maryland State Athletic Commission, Baltimore, MD, USA*
[n]*Saint Elizabeths Hospital, Neurology Consultation Services, Washington, DC, USA*

Abstract. There is an increasing evidence that inflammation contributes to clinical and functional outcomes in traumatic brain injury (TBI). Many successful target-engaging, lesion-reducing, symptom-alleviating, and function-improving interventions in animal models of TBI have failed to show efficacy in clinical trials. Timing and immunological context are paramount for the direction, quality, and intensity of immune responses to TBI and the resulting neuroanatomical, clinical, and functional course. We present components of the immune system implicated in TBI, potential immune targets, and target-engaging interventions. The main objective of our article is to point toward modifiable molecular and cellular mechanisms that may modify the outcomes in TBI, and contribute to increasing the translational value of interventions that have been identified in animal models of TBI.

Keywords: Depression, glia, immune challenge, immunomodulation, inflammation, priming, probiotic, traumatic brain injury

*Correspondence to: Teodor T. Postolache, MD, 685 W. Baltimore Street, MSTF Building, Suite # 930, Mood and Anxiety Program, Department of Psychiatry, University of Maryland School of Medicine, Baltimore, MD 21201, USA. Tel.: +1 410 706 2323; E-mail: tpostola@som.umaryland.edu.

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Traumatic brain injury (TBI) is an all-encompassing term to describe an intracranial injury after an external mechanical force results in damage to the brain structure or alteration in its functioning [1]. Both the causes and courses of TBI are heterogenous. Mechanisms of injury resulting in TBI are varied, including blunt forces (from bumps to blows), inertial loading, penetrating wounds, and blast injuries [1]. Just as the causes of TBI vary, the extent of damage also varies. Immediate injury after TBI can result in complications ranging from an unnoticed bruise with transient deficits to bleeding, or brain dysfunction resulting in permanent disability, or even death [2]. In evaluation of potential TBI patients, it is important to gain an accurate history of injury and complications during injury (including loss of consciousness), evaluate and treat symptoms [2], and to perform serial assessments in the post-injury period [3].

Etiological and pathophysiological heterogeneity of TBI are two of the major factors that produce inconsistent results between animal research and randomized clinical trials. It is essential to identify precise mechanisms for the primary [1] and secondary injuries associated with TBI to adequately target specific subgroups that are characterized by shared pathophysiology. After TBI, the cellular cascade of inflammation could have potentially beneficial effects [4–7]. However, if inflammation is too intense, prolonged, and unremitting, it may be harmful. Secondary injury has a slower progression ranging from minutes to years following the mechanical insult and is driven by several pathways, including: 1) free radical generation causing damage to molecular structures of cellular membranes [8], 2) excitotoxicity secondary to excess of glutamate [9, 10], and 3) systemic and central nervous system (CNS) immune activation [11]. It has been hypothesized that immune activation and modulation can significantly change the clinical and functional course in TBI [12]. Within minutes of impact, glia and recruited immune cells generate signals that initiate an inflammatory cascade [13]. The activation of the immune system has a vital role in cleaning the areas of the primary injury and preventing subsequent infections. However, to this day, targeting immune activation has been unsuccessful in improving clinical outcome in patients with TBI [14, 15], perhaps because of the greater heterogeneity of the causes of inflammation and their interactions in clinical TBI as compared to animal models, as well as genetic, environmental, pharmacological, and cultural differences between participants. The complex immunological mechanisms, including the influence of interacting immune-modifying factors involved in human TBI, presents challenges in developing an adequate animal model.

Interventional paradigms may be designed to:

1) Target early inflammatory processes that stimulate beneficial effects and inhibit detrimental ones.
2) Modulate overly prolonged or intense immune activation.
3) Clarify how immune challenges before or following TBI (e.g., reactivating infections, allergen exposure in atopic individuals, or exacerbation of autoimmune disorders) may affect it's course and outcomes.

A better understanding of the complex molecular cascade of inflammation and immune function post-TBI is vital for both patient-specific diagnostic interventions and development of novel treatments, as well as in developing coupling between animal models and clinical interventional studies.

SEVERITY OF TBI

The methods of assessing the severity of TBIs in a variety of clinical settings include the mechanism [closed versus (vs.) penetrating], the clinical severity, and the type and degree of structural damage [16]. The most common tool used to evaluate TBI severity in the clinical setting is the Glasgow Coma Scale (GCS). The GCS includes assessment of best verbal response, best eye-opening response, and best motor response, with an overall score range of 3–15, with 3 being the most severe and 15 the least severe [1].

The three major categories of TBI severity are mild, moderate, and severe, often dichotomized into mild vs. moderate/severe.

EPIDEMIOLOGY OF TRAUMATIC BRAIN INJURY

TBI is a significant public health problem. It has been referred to as the "silent epidemic". Yet, even if underestimated, the epidemiological characteristics of TBI are staggering. The lifetime prevalence of TBI is up to 40% of adults [17]. The global estimate of TBI indicates an incidence of 64 million and 74 million new cases yearly worldwide, with the highest incidence in USA, Canada, and Europe [18]. Overall, the most common causes of TBI are falls and

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motor vehicle accidents [19]. TBI incidence peaks three times during the lifespan: childhood, adolescence, and older adulthood [17]. TBI is the leading cause of death in children over 1 year old worldwide [20]. The estimated incidence of TBI in children aged 0–14 years old is about 475,000 in the United States [21]. The geriatric population incurs the most TBI-related hospital visits and deaths. Given the aging population, the number of older adults afflicted by TBI is likely to continue to increase [17]. Military personnel are also at particularly high risk due to combat, accidents, and activities during training [22].

ANIMAL MODELS OF TRAUMATIC BRAIN INJURY

Presenting the immune changes induced by TBI, common for all models and specific to each animal model in detail, is beyond the scope of the current article. Yet, we will briefly describe specific models with some of the inflammation findings uncovered using those models. For a presentation in depth, the reader is directed to a recent review of the topic [23].

Given the complexity of TBI, it is not surprising to see a variety of approaches that aim at modeling it in animals [24–26]. Animal models of TBI are commonly designed and classified by how the head injury is induced in the animal subject, rather than by its severity [25, 26]. Severity of injury in a particular animal model depends on the specific mechanical impact parameters that are used in the experiment. Therefore, in most cases, the same animal model can be used to represent both severe and milder forms of TBI, depending on the particular parameters used in the study [27]. At minimum, animal models of TBI can be classified by a single distinguishing factor, namely, closed vs. open head models. In the closed-head injury models, the animal's skull is left intact, while in open-head models, craniotomy or craniectomy is performed to open a window on the skull to reach the brain for induction of TBI. Neuroinflammation is a key feature of all animal TBI models. Proliferation of reactive astrocytes and microglia, which is a hallmark cellular response to TBI and is often evidenced by upregulation of certain proteins, such as glial fibrillary acidic protein (GFAP) and ionized calcium-binding adaptor molecule 1 (Iba1), along with increased levels of proinflammatory cytokines and chemokines, has been consistently observed across different types of animal models of TBI [23, 28, 29]. Another common finding is that repeated injuries exacerbate the neuroinflammatory response across different animal models [1, 30].

Closed-head models

Most closed-head animal TBI models require a blunt impact to the head for the induction of TBI. Some models only mimic the rotational forces that the brain is subjected to during TBI but not the mechanical impact [31–35]. Blast-injury models can be considered impactless since brain injury is achieved by the shock wave from the blast rather than a mechanical blow to the head [36]. The critical factor in interpreting the results of these animal models of TBI is the possibility of contusion, hematoma, and skull fractures. Presence of hematoma or contusion can change the course of the immune response and should be taken into account when the results are interpreted [37, 38].

In a weight drop model of TBI, a solid object falls freely in a guiding tube from a predetermined height onto a subject's head, which is usually anesthetized during the procedure to induce TBI [39–42]. Enhanced reactive gliosis and astrogliosis have been shown with several variations of the weight drop model in both mouse and rat subjects [43–46], in addition to increased proinflammatory cytokine [e.g., interleukin (IL)-1, tumor necrosis factor-alpha (TNF-α)] levels and their gene expression [41, 47]. These neuroinflammatory responses are enhanced with repeated injuries [1, 44]. Another method to induce closed-head TBI is the utilization of pneumatically or electronically controlled piston as the impactor instead of a free-falling weight [48–52]. Similar to the findings in the weight drop models, drastically increased GFAP and Iba1 levels have been reported in this TBI model [53–55]. However, elevation of proinflammatory cytokines has not been consistently observed with this model [53, 54, 56].

An alternative to the weight drop- and piston-based animal models of TBI are the projectile impactor models. These models use accelerated projectiles as impactors and have the ability to model the head impacts from high-speed small mass objects [57–61]. Using this approach, it has been demonstrated that repeated impacts to the head causes extensive increases in neuroinflammatory markers and microgliosis, including increased M1-type microglia [61, 62].

The blast-induced TBI model is another example of a closed-head TBI model. In blast-induced TBI mod-

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els, the animal is secured in a blast tube where a shock wave travels and reaches the animal's head to create TBI [36]. An acute neuroinflammatory response occurs acutely in blast-induced TBI models and levels of various chemokines and cytokines [e.g., IL-1α, IL-6, IL-10, interferon-gamma (IFN-γ), monocyte chemoattractant protein-1] increase in a short time frame (2-4 h after blast TBI) [63, 64].

Open-head models

The controlled cortical impact (CCI) model is one of the most utilized animal models of TBI that was originally developed in ferrets and then applied predominantly to rodents [65, 66]. In CCI, anesthetized animals undergo a craniectomy or craniotomy procedure, depending on the experimental design, and the dura is exposed [65, 66]. During the injury, a piston impactor moving at a predetermined speed hits the exposed dura to create TBI. Modified versions of CCI in which the skull is kept intact and the piston impacts the skull directly should be considered a separate model [67]. CCI was originally thought to produce focal injury around the impact site, but later studies showed that it also produces more diffuse injury and neurodegeneration throughout the brain distant from the cortical impact site [68]. CCI has been shown to induce acute increases in chemokines (i.e., $CXCL_1$) and cytokines (i.e., IFN-γ, TNF-α) and also certain anti-inflammatory cytokines (IL-4, IL-13) along with activated microglia [69, 70]. Fluid percussion injury (FPI) is another commonly used open-head TBI animal model [71–74]. FPI and CCI are similar in terms of their application of craniectomy to open a window on the skull. Through this opening, however, instead of blunt force impact from a piston, rapid and transient hydraulic pressure is applied through the opening in the skull to create TBI [75]. FPI induces both focal and diffuse injury as well [25, 75]. FPI increases activated microglia and macrophages in the brain [76]. In a more recent study, FPI has been shown to initiate a neuroinflammatory response acutely and increase levels of various chemokines and cytokines in the brain [77].

Even though relatively uncommon among civilian TBI patients, penetrating injury to the brain from a ballistic high-speed object (i.e., a bullet) is a particular concern for the military. The penetrating ballistic-like brain injury (PBBI) model was developed to address this particular type of injury [78]. In PBBI, similar to CCI and FPI, a skull window is opened through craniectomy. After that, a probe with an inflatable tip is inserted into the brain. To create injury, the probe tip is inflated very rapidly. This mimics effects of a bullet wound in the brain [78]. PBBI has been shown to induce loss of brain tissue, extensive intracranial hemorrhages and edema [78–80]. Neuroinflammation after PBBI is widespread and occurs rapidly, and given the major disruption of the blood-brain barrier (BBB), the central events are rapidly mirrored in the periphery. Levels of proinflammatory cytokines (IL-1β, IL-6, IFN-γ, TNF-α) and markers of microglial reactivity increase acutely after PBBI [81, 82].

THE IMMUNE SYSTEM: A BRIEF SUMMARY

The immune system includes structures, cells, and molecules that together are charged with defending the self against pathogens, malignant cells, transplanted tissue and, most commonly, microorganisms, as well as with eliminating damaged tissue that consumes resources and represents a risk of systemic toxicity, and ultimately sepsis. The innate immune system is characterized by rapid and nonspecific responses and limited capacity to cascade the response based on the memory of previous encounters with antigens. It plays an essential role in preventing or limiting the invasion by microorganisms, and in the removal of debris, wound healing, and tissue remodeling. The innate immune system includes anatomical barriers (e.g., skin, mucosa) and physiological gradient barriers (e.g., pH, temperature), antimicrobial peptides, cellular effectors such as innate lymphoid cells (e.g., natural killer cells), phagocytes (e.g., neutrophils, macrophages, microglia, and dendritic cells), and granulocytes (e.g., eosinophils, basophils, mast cells, and neutrophils) that mainly release immune molecular mediators from their granules [83, 84]. To sense danger signals or pathogens and to trigger immune responses, these martial cells use "pattern recognition receptors", including nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), Toll-like receptors (TLRs), and scavenger receptors [83, 85]. Chemokines and cytokines are released to amplify and initiate a cascade of immune responses and recruit other immune cells. Distal signaling via the vascular system and neural input to the brain may affect behavior, ultimately centralizing behavior towards the bare essentials necessary to survive the threat (i.e., "sickness behavior", involving an overall retreat from the environment, a decrease in energy and purposeful

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activity, an increase in sleepiness and propensity to sleep, and decrease in appetite and food intake).

Adaptive immunity involves the cascading activation and expansion of T and/or B lymphocytes targeting specific antigens, and a memory of previous antigen exposure allowing a targeted, intense, and rapid response to future antigen challenges [86, 87]. T lymphocytes ($CD4^+$ and $CD8^+$ T cells) have cytotoxic, helper, or regulatory functions, and are activated and reactivated by recognizing antigens displayed in major histocompatibility complexes. B lymphocytes produce immunoglobulins and have reciprocal interactions with T cells and cells of the innate immunity [88]. Adaptive immunity is also involved in autoimmune responses, as well as atopy.

SPECIFIC COMPONENTS OF THE IMMUNE RESPONSE TO TBI

The timeline of immune response to TBI, reflecting the elevation of various components of the immune system, is summarized in Fig. 1. Several methods, including pharmacological probing, targeting with antibodies, and utilizing genetically modified animals, have been used to infer the role of molecular signaling pathways and cellular immunity implicated in TBI (described in Tables 1A and 1B). Molecular pathways and cell types involved in the inflammatory response to TBI are well-described in other mechanism-oriented reviews [11, 30, 89, 90]. Below, we list some of the components of this immune response.

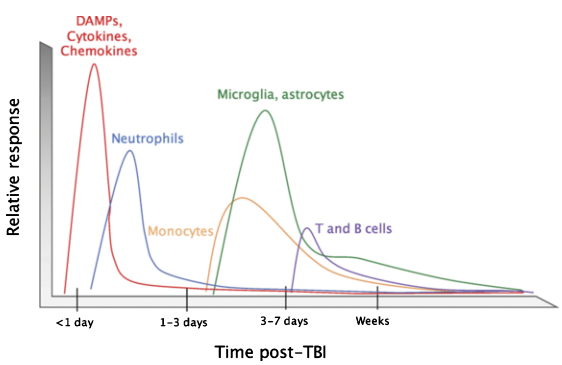

Fig. 1. Timeline of the immune response to TBI. After impact, rapid tissue release of damage-associated molecular patterns (DAMPs) prompts resident cells to secrete chemokines and cytokines. These molecules attract neutrophils, which contribute to circumscribing the injury site and promoting the removal of damaged and dead cells and debris. Infiltrating monocytes and activated glia begin to predominate 3-5 days post-injury to defend against infection and perform reparative functions. T and B cells can also be recruited to sites of brain pathology at later time points in the response (3–7 days post-injury). The timeline is modeled to human TBI across modalities, and is likely to correspond to the great majority of animal models of TBI (Reproduced from McKee CA, Lukens JR (2016) Emerging roles for the immune system in traumatic brain injury. *Front Immunol* **7**, 556).

Early immune "danger" signaling in TBI

In the damaged CNS, the initiation of immune signaling is started by recognition of damage-associated molecular patterns (DAMPS), while microorganism invasion in the CNS is signaled by pathogen-associated molecular patterns (PAMPS) [91]. These molecules interact with receptors, such as TLRs, NLRs, and scavenger receptors that serve as "danger" sensors and help to initiate the inflammatory cascade [85, 92–94].

The DAMPS' recognition leads to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB)-inducing kinase [94] and elevation of proinflammatory cytokines, such as TNF-α and IL-6 [95]. A reduced expression of inflammatory markers and smaller brain lesions after impact were observed in TLR4-deficient mice relative to wild-type controls [96], and less edema was observed after TBI after administration of VGX-1027, a selective TLR4 blocker [97]. Brain injury in humans and experimental animals induces expression of High mobility group box 1 protein (HMGB1) [98], a protein binding to TLR4 and initiating inflammatory cascades [99–101], and its inhibition diminishes both inflammation as well as BBB breakdown [102]. Other proteins triggering inflammation after TBI are the *protein chaperones* involved in tissue reorganization and wound healing [103, 104], and have immunomodulatory and neuroprotective effects [105–108]. Other inflammation triggering and perpetuating mechanisms include the activation of the purinergic receptors by adenosine tri-phosphate (ATP) released in the extracellular space secondary to tissue damage, leading to activation of inflammatory cascades [109, 110] and promotion of neutrophil recruitment [111, 112], as well as the formation of inflammasomes in the cytosol [113, 114]. Multiple cells in the CNS—such as neurons, astrocytes, microglia, macrophages, and astrocytes—can contain assembled inflammasomes [115, 116], and levels of inflammasome proteins in the cerebrospinal fluid (CSF) generally predict a poorer outcome in TBI [117].

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Table 1
Rodent models used to characterize the role of signaling pathways and immune cell types in TBI. Table 1A describes molecular signaling pathways and Table 1B describes the cellular components of the immune system in TBI, illustrating approaches based on antibody targeting, as well as genetically modified mice (Reproduced from McKee CA, Lukens JR (2016) Emerging roles for the immune system in traumatic brain injury. Front Immunol 7, 556)

Table 1A. Molecular signaling pathways

Inflammatory mediator	Animal line/model	Purpose	Major findings in animals exposed to TBI	Reference
IL-1	Anti-IL-1β antibody	Blockade of IL-1β signaling	Reductions in macrophages/microglia, neutrophils, and T cell numbers in the brain, improvement in learning tasks, and decreased tissue loss	[289, 290]
	IL-1 R antagonist	Neutralize IL-1	Higher expression of proinflammatory cytokines in macrophages	[291]
ASC	Anti-ASC	Limit inflammasome assembly	Reduced caspase-1 activation and IL-1β production, decreased lesion volume	[292]
	ASC knockout	Abrogate inflammasome assembly	No improvements in lesion volume, histopathology, cell death, or motor function	[293]
NLRP1	NLRP1 knockout	Prevent NLRP1 inflammasome assembly	No improvements in lesion volume, histopathology, cell death, or motor function	[293]
IL-6	IL-6 knockout	Ablation of IL-6 signaling	Fewer reactive astrocytes and macrophages, increased neuronal death	[294]
	IL-6 knockout	Ablation of IL-6 signaling	Poor behavioral performance, higher IL-1β levels in the cortex	[295]
	GFAP-IL-6 overexpression	Increase IL-6 expression in astrocytes	Greater recruitment of glia and immune cells to the lesion, decreased oxidative stress and neuronal death	[296]
	Anti-IL-6 antibody	Neutralize IL-6	Reduced some inflammatory and behavioral effects of post-injury hypoxia	[297]
TNF-α	TNF-α inhibitor post-TBI	Inhibit TNF-α signaling	Early administration improved cognitive performance, and decreased neuronal apoptosis and astrogliosis	[298]
	TNFR1 knockout	Disrupt TNF-α signaling through TNFR1	Improved neurological function and neuronal survival/lesion volume, decreased numbers of CD11b+cells in the brain	[299]
	TNFR2 knockout	Reduce TNFR2 signaling	Worsened neurological function and no protection from tissue loss	[299]
	TNFR2/Fas knockout	Abrogate TNF-α signaling through TNFR2	Impaired motor and cognitive performance	[142]
G-CSF	G-CSF injection post-TBI	Enhance G-CSF signaling	Improved cognitive performance and increased hippocampal neurogenesis, higher glial activation and production of BDNF and GDNF	[300]
GM-CSF	GM-CSF knockout	Disrupt GM-CSF signaling	Worsened cognitive deficits as well as cell and tissue loss, reduced astrogliosis	[301]
Type 1 IFN	IFNAR knockout or IFNAR blocking antibody	Block type 1 IFN signaling	Reduced lesion volume, more anti-inflammatory cytokine signaling, increased glial activation, these effects were hematopoietic cell-dependent	[302]
IL-10	IL-10 knockout, IL-10 injection	Modulate IL-10 signaling	Diminished protective effects of hyperbaric oxygen treatment, including lesion volume, edema, cognitive improvement, and decreased cytokine production in IL-10 knockout mice, while IL-10 injection improved these outcomes	[303]

(*continued*)

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Table 1
(*continued*)

Table 1A. Molecular signaling pathways

Inflammatory mediator	Animal line/model	Purpose	Major findings in animals exposed to TBI	Reference
TGF-β	TAK1 inhibition	Disrupt signaling downstream of TGF-β	Improved neuronal survival and motor function, decreased NF-κB signaling and inflammatory cytokine production	[304]
	TGIF shRNAv knockdown	Ablation of downstream TGF-β signaling	Decreased infarct volume and microglia numbers, improved motor function	[305]
APOE	APOEϵ4 overexpression	APOEϵ4 overexpression	Worsened brain pathology, BBB breakdown, and neurological impairments	[306, 307]
TREM2	TREM2 knockout	Abrogate TREM2 signaling	Altered macrophage distribution, hippocampal neuroprotection, and fewer cognitive deficits	[308]
G-CSF	G-CSF injection post-TBI	Enhance G-CSF signaling	Improved cognitive performance and increased hippocampal neurogenesis, higher glial activation and production of BDNF and GDNF	[300]
GM-CSF	GM-CSF knockout	Disrupt GM-CSF signaling	Worsened cognitive deficits as well as cell and tissue loss, reduced astrogliosis	[301]

Table 1B. Cellular mediators

Cell type	Animal line/model	Purpose	Major findings in animals exposed to TBI	Reference
Neutrophils	IgM RP-3	Neutrophil depletion	No significant decrease in BBB permeability	[151]
	Anti-Gr1 antibody	Neutrophil depletion	Decreased edema, apoptosis, and microglia/macrophage activation, no significant changes in BBB integrity	[153]
	CXCR2 knockout	Reduce neutrophil infiltration	Decreased cell death, no significant changes in BBB permeability or behavior	[148]
	Neutrophil elastase knockout	Reduce neutrophil effector functions	Decreased edema and apoptotic neurons, but no decrease in tissue volume loss or behavioral improvement	[154]
Macrophages and microglia	CD11b-TK	Deplete CD11b-expressing cells	Reductions in microglia numbers in the brain, no improvement in axonal injury, treatment toxic at high dosage	[309]
	CD11b-DTR	Deplete CD11b-expressing cells	No change in lesion size, treatment caused inflammatory response without injury	[310]
	CCX872 (CCR2 antagonist)	Reduce CCR2 signaling functions	Reduced macrophages in the brain, altered pro- and anti-inflammatory cytokine expression, less cognitive dysfunction	[162]
	CCR2 knockout	Limit CCR2-mediated recruitment of monocytes	Reduced numbers of infiltrating monocytes, improved learning and memory	[161]
	CCR2RFP/RFP	Disrupt recruitment of monocytes	Reduced monocyte recruitment, cavity volume, and axonal pathology	[160]
	CX3CR1 knockout	Abrogate CX3CR1 signaling functions in macrophages and microglia	Short-term neuroprotection and lower inflammatory response, long-term functional impairments and elevated myeloid cell activation	[311]

(*continued*)

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Table 1
(*continued*)

Table 1B. Cellular mediators				
T cells	Rag1 knockout	Genetic ablation of B and T cells	No changes in neurological outcome, BBB integrity, pro- or anti-apoptotic mediators, hippocampal architecture, or astroglial activation	[312]
	FTY720	Sequester lymphocytes and reduce their migration to the brain	Decreased circulating lymphocytes, decreased neutrophils and macrophages/microglia in ipsilateral hemisphere	[313]

Inflammatory gene expression

After TBI, a program of immune gene expression is induced that determines the progression of the subsequent inflammatory cascade [118]. Qualitatively, genes implicated in antigen presentation [CD74, CD86, major histocompatibility complex (MHC) II], phagocytosis [C3, C4, Fc gamma receptor (FcgR) and FCGR4], astrocyte activation [Aquaporin (AQP)4 and GFAP), chemotaxis [chemokine ligand (CCL)2, CCL4, chemokine (C-X-C motif) ligand (CXCL)1 and CXCL4], cytokine signaling [IL-1β, IFN-γ, IL-6, IL-12, IL-10 and transforming growth factor-beta (TGF-β)] are, surprisingly, similarly upregulated in both mild and severe TBI [118], and thus, are unlikely to be key mediators of severity of TBI outcome.

Peripheral innate immune activation after TBI

It is known that the body responds to TBI by engineering both central and peripheral inflammatory responses [119–122], and that a single brain injury can elicit complex changes in the systemic immune system. In fact, the immunologic complex known as the systemic inflammatory response syndrome characterized by alterations in circulating leukocyte numbers, complement proteins, coagulants, and inflammatory cytokines [120, 122], seems to result from post-TBI disruption of the BBB and CNS vasculature along with consequent leakage of central inflammatory mediators into the periphery [119–122].

The complement system

Various complement components have been found in the CSF of TBI patients [123, 124] and correlate with the severity of BBB dysfunction [124], and interrupting the complement cascade in various animal models of TBI has been consistently found to be neuroprotective [125–132].

Cytokines

Cytokines are cellular messengers that are secreted by multiple cells implicated in TBI that can convey proinflammatory or anti-inflammatory signals to other immune and nonimmune cells [133]. Often produced during sterile injury immune responses, IL-1β is elevated in the CSF of patients with severe TBI for at least 24 h, with levels that are linked with a less favorable prognosis [134, 135]. This signal is not just a marker of severity, but likely a component of a mediating adaptive mechanism, as administering an IL-1β antagonist in animal models reduces neurological symptoms, improves neurological functioning, and reduces the lesion volume [136].

Similarly, IL-18 is detected early post-TBI [137] and administering IL-18 antagonists in rodents one hour post-brain injury led to improved symptomatic recovery [137]. Thus, IL-1β and IL-18 have been associated with negative outcomes post-TBI. In contrast, other cytokines have been associated with either a good or a bad prognosis. For instance, a classical proinflammatory cytokine, TNF-α, shows heterogenous links with outcome in TBI. On one hand, it is elevated in TBI patients and, moreover, neuroprotective effects can be elicited by its blockade in rodent TBI models [138, 139]. However, while less severe neurological symptoms and functional deficits occur one week after TBI in mice that are genetically deficient in TNF-α, greater deficits and motor symptoms were seen at 2–4 weeks after injury with larger lesions at later time points [140]. It also appears that the experimental model of TBI plays a strong moderating effect on the functional outcome of TNF-α inhibition. In particular, concomitant Fas receptor and TNF-α inhibition appears to lead to worse functional outcomes in a mouse concussion model with no structural brain damage based on impact and head acceleration, while in models of cerebral contusion, the Fas receptor and TNF-α inhibition improved functional outcome and reduced tissue damage [141,

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142]. This suggests that TNF-α has a dual role in contributing to both tissue repair and recovery, as well as to neuroinflammation and excitotoxicity.

Anti-inflammatory cytokines, such as IL-10 and TGF-β, counteract the effects of proinflammatory cytokines and reduce neuroinflammation, and overall, improve the functional and symptomatic outcomes and lesion dimension. For instance, the cytokine IL-10 appears to be neuroprotective if administered early and peripherally (but not intrathecally) in a fluid percussion concussion [143], suggesting that its anti-inflammatory effect has to act, at least initially, on the peripheral immune system for benefic effects to occur. Similarly, TGF-β1 appears to be neuroprotective in a weight drop model in rats, where the cytokine is expressed in perilesional neurons and astrocytes, and consistently, knockout of the TGF-β1 results in increased neuronal death, larger lesion size, and worse neurological deficits [16].

Phagocytes/glial cells

Post-TBI, myeloid cells in the CNS are first to be activated followed by neutrophils, which are usually among the first peripheral immune cells to be mobilized towards the site of injury within just a few hours (h) [111, 144–146]. There is a significant surge in the number of peripheral neutrophils in the early hours after TBI that lasts until 48 h post-injury [147]. Perivascular spaces and meninges seem particularly vulnerable to alignment of neutrophils after cortical injury [37, 111]. Promoting complexity, there are data to define neutrophils as neuroprotective [111, 148], actively involved in healing after neurological injury [149, 150], neutral [151, 152], or alternatively, neurotoxic [121, 151–154]. Similarly, monocytes and macrophages are directed by temporal and biological contextual factors towards either tissue damage or repair [155, 156].

The perivascular spaces and brain parenchyma become hosts for monocytes within the first two days after injury. These monocytes then differentiate into macrophages and remain in the same location for weeks after the original injury [157]. The chemokine CCL2 signals the deployment of monocytes [158, 159] after TBI and elicits CCR2 + monocyte recruitment. Some studies suggest that interfering with this pathway decreases the lesion size and may lead towards improved neurological outcomes in animal models of TBI [158, 160–162]. Yet, other studies demonstrate the repair capacity of macrophages after CNS injury [163–165]. For microglial priming, the reader is directed to discussion later in the article at the section on "Age effects/microglial priming."

Astrocytes

Astrocytes respond directly and indirectly to TBI via HMGB1/Receptor for advanced glycation end products (RAGE) signaling and activation of the NF-kB pathway [98], leading to secretion of chemokines/cytokines and enhanced astrocyte phagocytic activity. Yet, post-TBI reactive astrogliosis limits the spread of damage and inflammatory response to the unaffected CNS area, with astrocytes also providing metabolic support for neurons and their synapses [166, 167]. Astrocytes interact with other immune cells, such as microglia, in releasing various growth factors like insulin like growth factor 1 (IGF1) and nerve growth factor (NGF) that promote post-TBI healing [4].

T cells

Brain injury results in a cell-mediated immune activation [168, 169] with recruitment of T cells in the brain [170] independent of their antigenic specificity. Detailing the adaptive immunity activation in detail is beyond the intended scope of this review, However, it is important to mention that while overreactive adaptive immunity has been thought to represent a negative prognostic factor, the $CD4^+$ T cells autoreactive to CNS antigens appear to be mainly neuroprotective, in particular for injured axons [5–7].

LIPIDS, IMMUNE MODULATION, AND TBI

Polyunsaturated fatty acids (PUFA)

Lipids are a diverse molecular class, important for signaling and immunomodulation. A number of studies reported increases in levels of free fatty acids (FFA), including polyunsaturated fatty acids (PUFA), in tissues, CSF, and serum in both human TBI and experimental models of TBI [171, 172]. Notably, two independent mass spectroscopy imaging studies of TBI reported PUFA-containing lipid signatures within the injury site in rat models, including multiple phosphatidylcholines [173], diacylglycerols, and sphingolipids [174].

PUFAs are important substrates for the cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) pathways, ultimately yielding a

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range of immunomodulatory molecules, such as prostaglandins, thromboxanes, leukotrienes, and resolvins that mediate signaling through G-protein coupled receptors [175, 176]. Two classes of PUFAs, ω-3 (i.e., docosahexaenoic acid, DHA; eicosapentaenoic acid, EPA) and ω-6 (arachidonic acid) are generally implicated in opposing anti- and proinflammatory functions, respectively, that have been extensively studied [177–179].

Multiple regimens for supplementation with ω-3 s have been implemented to limit putative immune-mediated pathology in various rodent models of TBI [180–183]. Furthermore, in rodent models of mild to severe TBI, treatment with DHA or fish oil (containing DHA and other ω-3 s) showed significant improvements in cognitive function and mitigation of neuronal damage [181–184]. In humans, there are no adequate data to make a determination about efficacy and safety of ω-3 s treatment in TBI, and despite anecdotal evidence, pathophysiological targeting, as well as advocacy [185], well-designed human clinical trials are necessary. Additionally, a theoretical possibility of impaired initiation of clotting, and thus, increased risk of bleeding due to decreased eicosanoid synthesis exists [186], even if this is disputed based on analogical reasoning [185], and to our knowledge, is unsupported by large-scale safety data.

Statins

Cholesterol-lowering drugs, in principal statins, have also been implicated in reduction of TBI pathogenesis, as well as in anti-inflammatory effects. The primary effect of statins is inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase (a rate-limiting enzyme in the mevalonate pathway), but there are well-recognized pleiotropic effects of statins, including direct immunomodulation [187].

The positive significant immune effects of statins in animal models are reductions of IL-6, TNF-α, and intracellular adhesion molecule 1 (ICAM-1), attenuation of cerebral edema and glial cell activation, and reduction of the permeability of the BBB [188, 189]. The upstream immunomodulatory mechanism of statins is the inhibition of TLR4 and NF-κB [188]. It has been shown that statins have an anti-apoptotic effect via downregulating the caspase pathways, which can be overly activated after TBI [190]. In animal models of TBI, statins have resulted in reduction of parenchymal hemorrhage [191], improved cerebral blood flow [192], decreased cerebral edema [193], improved neuronal survival and regenerative angiogenesis and synaptogenesis [194], improved neurological outcome [195], reduced intravascular thrombosis, improved spatial learning [196], and reduced cognitive deficits [197], including in severe forms of TBI [198]. In a small double-blind randomized trial in patients with moderate-severe TBI, 72 h after TBI, rosuvastatin significantly decreased the level of TNF-α but not of IL-1β, IL-6, or IL-10, and led to a reduction in the disability score [199]. Preinjury atorvastatin use leads to a reduction in mortality and improved functional outcomes in elderly exposed to TBI [200].

Consistent with the findings described above, a retrospective analysis found a significant reduction of hospital mortality in statin-using patients admitted for TBI, as compared to nonusers [201]. In contrast, a recent observational study did not confirm any benefit of statins in TBI [202]. Meanwhile, another study in Asia [203] did not find any benefit of a statin pretreated status in patients with moderate-severe TBI—a difference that could be attributed to: 1) using multiple statins rather than purely atorvastatin; 2) a predominantly Asian population; 3) potential pharmacokinetic differences; or, finally, 4) withholding the statin medications during the inpatient hospitalizations. In a randomized clinical trial of atorvastatin 20 mg as compared to placebo in patients after moderate-severe TBI (32 participants in each group), no statin–placebo difference in the volume of the contusion and its expansion was found at any time point, and yet, significantly superior scores on the Glasgow Outcome Scale and the Disability Rating Scale were observed in the statin group at 3-month follow-up, with no differences at baseline [204]. An important area of research is to evaluate the differential effects of statins that more easily penetrate the BBB (lipophilic ones) vs. those that act mainly at the periphery on neuronal cholesterol levels, neuroprotection, neuroproliferation, neuroinflammation, and oxidative stress. This will guide the selection of specific statins for specific molecular pathways implicated in TBI.

OTHER IMMUNE-TARGETING INTERVENTIONS IN ANIMAL MODELS OF TBI

Metabotropic glutamate receptor 5 agonists

Metabotropic glutamate receptor 5 (MGluR5) agonists, such as (RS)-2-chloro-5-hydroxyphenylglycine (CHPG), attenuate microglial activation by

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actions at MGlurR5 on microglia and neurons and inhibit caspase-dependent apoptosis. CHPG attenuates microglial activation through NADPH oxidase [205] with a potent neuroprotective effect observed early in treatment [206]. Moreover, a delayed administration reduced neurodegeneration and long-term inflammation. Specifically, if administered after 1 month, it lowered subsequent activation of microglia expressing NADPH oxidase subunits, inhibited hippocampal neuronal loss, lesion progression measured by T2-weighted MRI (at one, two, and three months), as well as white matter loss measured by diffusion tensor imaging at four months post-TBI [207].

Minocycline

Minocycline, a common antibiotic, has been explored for its ability to attenuate inflammation after brain injury and improve prognosis. Minocycline is a lipophilic, tetracycline-derivative antibiotic that is most commonly associated with treatment of acne and various infections. Its anti-inflammatory and anti-edematous properties at higher concentrations were investigated in the treatment of TBI, given its favorable safety profile and ability to cross the BBB [2, 12, 208]. The drug decreases secretion of cytokines and chemokines that promote inflammation. Minocycline results in decreased downstream nitric oxide production and decreases the activation of macrophages and microglia, and decreases expression of multiple cytokines (TNF-α, IL-1β, IL-6, and MMP-9) [2, 90, 209]. Additionally, minocycline promotes neuroprotective responses after brain injury in mice [210]. Several experimental studies have shown promise for use of minocycline as an intervention after TBI. These results indicated that minocycline decreased cerebral edema lasting up to 24 h post-injury by decreasing immediate release of inflammatory markers like IL-1β and MMP-9, but with no difference in oxidative stress as measured by glutathione levels [208]. On a macroscopic level, minocycline has been shown to decrease gray and white matter changes after TBI in mice [209]. Lesions after TBI are tempered, as evidenced by decreased focal brain lesion volume and decreased olfactory bulb atrophy in treated mice [209, 211]. Minocycline was able to attenuate memory deficits, an effect that was sustained until at least 13 weeks post-injury [212]. Other studies have indicated mice with minocycline treatment after TBI perform better on the tasks of spatial learning and memory [12].

Considering the difficulty in extrapolating the results from animal studies into practice, there are a number of guidelines for future immune targeting interventions to be developed. Studies should be conducted across models and species, to provide evidence of molecular or cellular target engagement, and should add histological (whenever possible) or imaging outcomes to the behavioral ones. Factors such as brain penetration as well as pharmacokinetic and pharmacodynamic measurements with optimal dosage should be identified [213] and the therapeutic window for any intervention intended to become a treatment should not be less than 6 h [213].

THE TIME COURSE OF TBI-INDUCED INFLAMMATION

As previously discussed, mechanical forces leading to TBI lead to direct, primary damage that results in axonal injury, contusion, or hemorrhage [16]. The secondary damage occurs hours to days after the initial insult [16] as a result of widespread neuroinflammation [11], oxidative stress [214], and excitotoxicity [9]. Within the inflammatory milieu, TBI is not an isolated event. *Subsequent*, *co-occurring*, and especially *previous* immune challenge and inflammation-mediated medical conditions interact with TBI with synergistic, antagonistic, or additive effects.

Both central and peripheral nervous systems are involved in the complex and dynamic inflammatory response induced by TBI, with genetics, location and severity of the trauma, secondary injury cascades, sex, and age as important moderators [11]. It has been proposed that modulation of the immune response could be central in identifying clinically relevant and effective therapeutic interventions for TBI, as all brain injuries trigger inflammatory responses [12].

A common immune phenomenon in the long-term recovery after TBI is the *tardive enhanced inflammatory response* that occurs post-injury. Experimental TBI results in changes that are usually coupled with prolonged elevation in glial/macrophage reactivity as a component of neuroinflammation [215–218], which is consistent with studies in humans that identified increased binding of PK-[11C](R)PK11195 (expressed by activated microglia) ligand on imaging between 11 months and 17 years post-injury [219], and higher mRNA expression of microglial markers CD68 and OX-6 at 1-year post-injury [220]. Many

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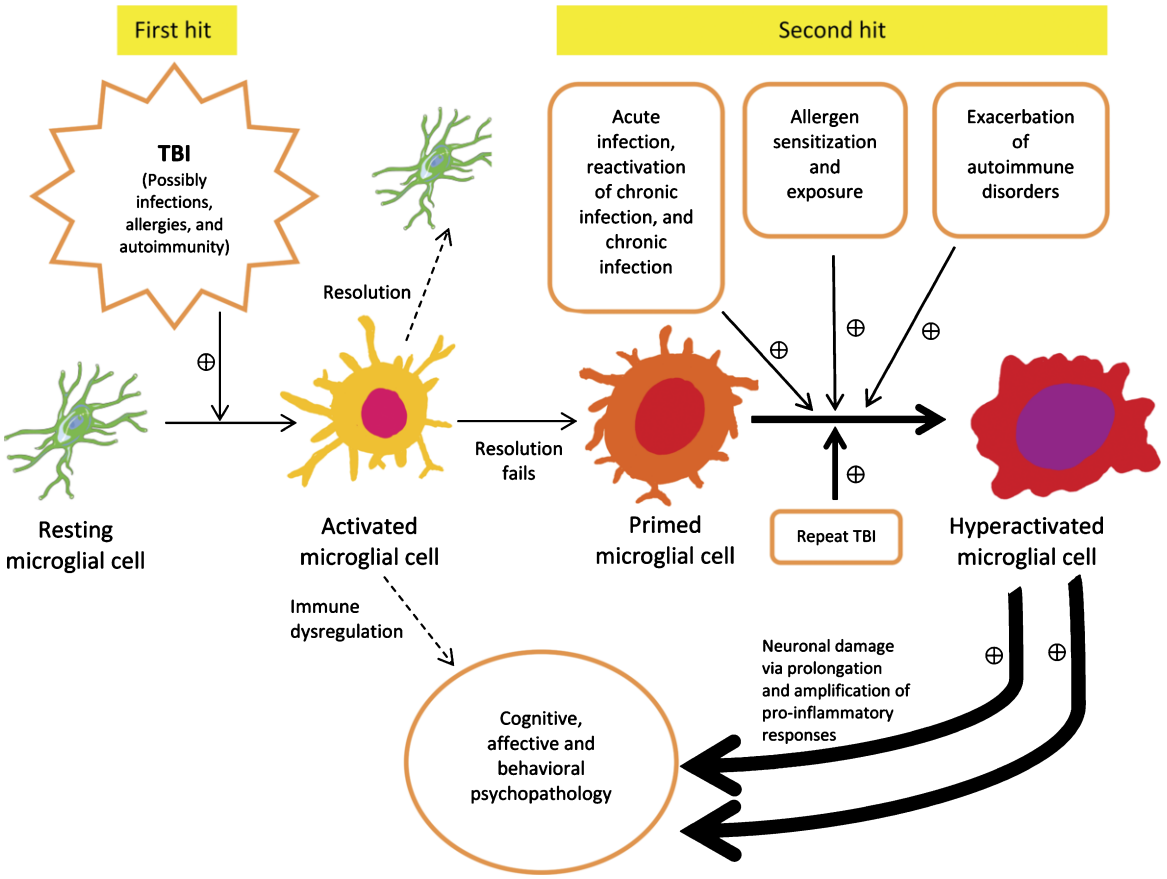

Fig. 3. Double hit model illustrates how during the first hit microglia become activated, and then potentially primed. A second hit may include any source of inflammation, such as intense stress, autoimmune disease reactivation, allergen exposure in atopic individuals, and most commonly, infections (acute, chronic, chronic reactivated) and/or repeat TBI. The primed microglia convey a risk for developing behavioral, affective, and cognitive disorders, and exposure to subsequent inflammation driven "hits" exponentially increases that probability, as well as symptomatic intensity and functional deterioration. The double hit model is expected to fully represent all TBI models in rodents, as well as its translation to clinical TBI, with the difference that in human TBI, a multitude of individual immune stressors interact with TBI in contrast to experimental animals living in conditions characterized by limited variation between individuals in exposure to various immune modifiers. This is a top-of-the-list possible explanation of why immune targeting interventions for TBI that are benefic in rodents do not translate in humans, with few exceptions (e.g., aerobic exercise).

age-related neurodegenerative disorders, including Alzheimer's disease, also have cell-mediated neuroinflammation as a prominent feature [221].

Age effects/microglial priming

Whereas microglia display an amplified proinflammatory response with aging that is referred to as "microglial priming" (first described in a model of prion disease) [222], macrophages display an age-related reduction in the proinflammatory response to an immune challenge [223]. Characteristics of a primed profile of microglia, in addition to morphological changes, include: 1) reduced activation threshold to express and release proinflammatory molecules; 2) exaggerated inflammatory response to immune challenge; and 3) increased basal expression of inflammatory markers and mediators [224]. Additionally, in older rodents, in contrast to younger rodents, although white matter demyelination injury induces a reduced macrophage/microglia response [225], a gray matter injury provokes an enhanced macrophage/microglia response [70, 226] (Fig. 2).

PERSISTENT PROINFLAMMATORY PROFILES AFTER TBI

Reactive microglia

Brain trauma induces a persistent proinflammatory profile with increased inflammatory cytokines

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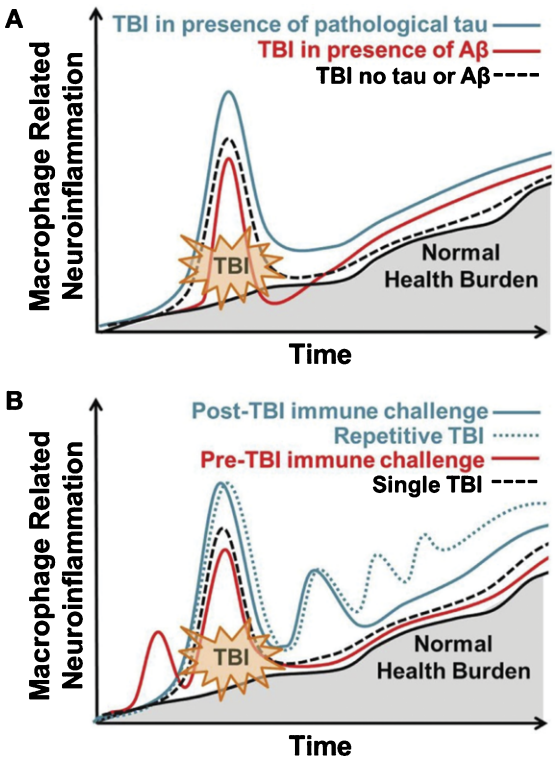

Fig. 2. Traumatic brain injury-induced macrophage response varies in reaction to immune stressors that occur before, with, and after the injury. A solid black line and gray shading depicts normal, age-related health burden. A) Traumatic brain injury (TBI) occurring in the absence of Aβ (dotted black line) or tau results in acute macrophage-related neuroinflammation that subsides over time. TBI in the presence of Aβ (solid red line) results in an acute blunted macrophage response that increases at chronic post-injury time points. TBI in the presence of pathological tau (solid blue line) results in an enhanced macrophage response to TBI that remains elevated at chronic post-injury time points. B) Over time, macrophage-related neuroinflammation increases with normal health burden. Single TBI (dotted black line) results in acute macrophage-related neuroinflammation that subsides over time. Pre-injury peripheral immune challenge at sub-threshold levels (red line) attenuates the post-injury macrophage-related inflammatory response to TBI. Post-injury peripheral immune challenge (solid blue line) causes a hyper-active macrophage response correlating with behavioral dysfunction. Repetitive post-injury immune challenge (dotted blue line), similar to what is observed in repetitive TBI, increases macrophage-related neuroinflammation and correlates with the advanced neuropathology. The figure represents a model of what occurs in human TBI, translating specific findings in rodent TBI studies. (Reproduced from Kokiko-Cochran ON, Godbout JP (2018) The inflammatory continuum of traumatic brain injury and Alzheimer's disease. *Front Immunol* **9**, 672.)

TNF-α and IL-6 (detectable in the CSF for up to 12 months and directly associated with measures of disinhibition and functional impairment [227, 228]) and priming in microglia [229–231] (Fig. 3). Increased CR3/43$^+$ (MHC-II+) and CD68$^+$ reactive microglia have been reported several months after brain injury in an immunohistochemical analysis of autopsied brains [232]. Especially, white matter in these autopsied brains had an increased immune load of microglial reactivity in the presence of traumatic axonal injury [232]. An association of CR3/43$^+$ cells with increased myelin basic protein long-term after brain trauma (2–8 years) has also been reported in other studies [220].

Multiple Immune Challenges and multi-hit models

Pre-injury peripheral mild immune challenges may improve recovery following TBI

Experimental models of TBI have a reported improved behavioral recovery and reduced lesion volume after pretreatment with low doses of LPS [233]. Additionally, attenuation of IL-1β and TNF-α overexpression and neuronal loss was found in the hippocampus when LPS treatment was administered before TBI [234]. Even a higher dose of LPS administered over shorter duration (1.0 mg/kg, single intraperitoneal dose for 4 days) prior to a lateral cryogenic brain injury has been demonstrated to decrease lesion volume and neuronal death [235]. In sum, a pre-treatment inflammatory stimulus may help protect, in part, the CNS from a secondary, generally more potent, stimulus, such as TBI, likely via pre-conditioning leading to the induction of neuroprotective responses (see Fig. 2, upper graph).

Immune challenges after TBI

A primed microglial phenotype, defined by increased expression of MHC-II and CD68 and altered morphology is consistently induced by experimental models of TBI [231, 236]. Primed microglia show some increased reactivity at baseline, but a strongly increased reactivity after an immune trigger [30].

In rats that sustained TBI via impact acceleration, an exaggerated inflammatory response associated with cognitive impairments and depressive-like behavior 3 months post-injury has been reported to be induced by administering LPS, as early as 5 days after the primary injury [237]. Findings from these studies substantiate the fact that post-TBI immune challenges negatively impact symptoms and functional outcomes and lead to increased deficits, as well as highlight the long period of increased vulnera-

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bility to subsequent immune challenges. However, a decreased production of inflammatory cytokines and reduced neuronal injury were reported if LPS treatment was given 1 day after repetitive mild TBI (3 TBIs, 5 days apart) [43]. In contrast, if the administration of LPS was delayed to 5 days after repetitive TBI, worsening of neuronal damage characterized by aggregation and phosphorylation of tau, impaired behavioral recovery, and elevation of inflammatory cytokines was observed [43]. These findings together emphasize the substantial temporality of the immune response to TBI and its interactions with other immune triggers.

Common clinically related immune challenges, such as allergies [238, 239], autoimmune disorders [240, 241] and/or infections [242, 243], produce a proinflammatory response and lower the threshold for priming of microglia [244, 245]. Thus, further clinical studies should investigate the role of these immune-mediated conditions and their treatment in altering the course of TBI. Clinically, there is potential benefit to be uncovered from assertively diagnosing and aggressively treating these conditions when co-occurring with TBI.

Psychological stress, a common occurrence in reaction to TBI and events that contributed to it and surrounding it, has been consistently demonstrated to upregulate immune responses acutely and chronically, with secondary loops related to hypothalamic-pituitary-adrenal (HPA) axis dysregulation (with both suppression and hyperactivity) contributing to dynamic sequential changes in immune function [246] and progression of symptomatic and functional deficits. HPA axis suppression and the diminished modulatory role of HPA axis on immune alterations post-TBI, and vulnerability to stress, sleep deprivation, and circadian dysregulation, represent important domains that may provide translational value for minimizing early symptoms and long-term consequences after TBI [246].

Immune challenges in aged- and TBI-exposed mice lead to an exaggerated immune response

Microglial priming, characterized by altered morphology and higher expression of complement receptor-3 (CD11b) and MHC-II, has been reported in aged rodents [247, 248]. As compared to young adult mice being challenged with LPS, aged animals have an increased microglial expression of IL-1β upon induction of TBI, leading to a depressive-like phenotype [249] and prolongation of sickness behavior [250], as well as greater spatial memory impairments in parallel to the higher expression of IL-6, TNF-α, and IL-1β in the hippocampus [251, 252]. As compared to TBI-exposed mice that were given saline, TBI-exposed mice that received a peripheral LPS challenge 30 days after brain injury were reported to have an exaggerated microglial response, which was depicted by higher depressive-like behaviors and increased TNF-α, MHC-II, and IL-1β expression [231]. Moreover, memory recall deficits in TBI mice are exaggerated by administration of LPS 30 days after brain injury [236].

CHRONIC NEURODEGENERATIVE CONSEQUENCES OF TBI

History of TBI is predictively associated with future onset and severity of all forms of neurodegenerative diseases, including chronic traumatic encephalopathy (CTE), Alzheimer's, Parkinson's, Huntington's, and vascular dementias [253], as well as psychiatric sequelae such as depression [254] believed to be, at least in part, consequences of microglial priming and perpetuation of neuroinflammation. However, complete replacement of primed microglia with nonreactive microglia failed to reduce astrocyte-mediated inflammation, and more importantly, markers of neuronal death (reviewed by Tapp et al. [246]). A single TBI produces long-term plastic changes in multiple aspects of the immune function (i.e., acute phase response, TLR signaling, NF-κB signaling, complement system) that take part in reciprocally activating and perpetuating immune molecular networks [255]. In repetitive TBI, the first TBI is a priming event, which creates the foundation for an elevated inflammatory response that occurs upon subsequent exposure to more brain injuries. It is important to note that TBI has persistent and profound effects on systemic immunity that show similarities to changes linked with aging [256].

Chronic traumatic encephalopathy

Development of CTE, a neurodegenerative disease characterized by abnormal tau accumulation in the sulci of the cortex, is an example of chronic cognitive impairment associated with repetitive TBI via participation in contact sports [257]. Even though some cases of CTE may have presence of amyloid-β (Aβ) plaques, their location and distribution are

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different from those of Alzheimer's disease subjects [258–260]. A common feature of CTE is glial reactivity, including microglial reactivity as well as astrocytic accumulation of abnormal tau [257, 261]. In a study utilizing PET imaging, retired National Football League players were reported to have increased translocator protein binding in the entorhinal cortex, supramarginal gyrus, hippocampus, and the parahippocampal cortex, as compared to sex- and age-matched control subjects who did not have a history of repetitive head trauma [262]. In parallel to these findings, other studies on American football players showed increased $CD68^{+}$ microglia in their brains, which could have partially mediated the deposition of abnormal tau [263].

A recent review describes the animal models of repetitive mild TBI and includes the discussion on available evidence related to the development of tau and Aβ pathology after the brain injury [264]. The number of injuries in repetitive TBI varies from 2-10 over a period of several days or weeks, but its severity is usually referred to as "mild." Recently, a highly repetitive mouse model of TBI has been described that includes 30 injuries [265]. Generally, on one injury day, administration of 1 or 2 brain injuries is done. At chronic post-injury time points, consistent presence of increased behavioral impairment, amyloid-β protein precursor, and phosphorylated tau have been reported in non-transgenic mice exposed to repetitive mild TBI [265].

Alzheimer's disease

Dementia risk later in life is elevated by history of TBI [266, 267]. Chronic inflammation induced by TBI has been suggested to be a major contributor to the development of neurodegenerative disease [220, 268]. Indeed, many studies have documented an association between TBI and vulnerability to dementia, with underlying mechanisms involving damage to white matter tracts and neuronal networks, impaired cognitive resources, and deposition of a Aβ and tau [266, 269], with neurotoxic forms of tau and amyloid leading to neuroinflammation and perpetuating a cascading vicious circle [270]. The interruption of this vicious circle by targeting persistent posttraumatic immune responses is a logical aim of future clinical trials in TBI [271]. The recent confirmation of long-term dysregulation of immunoregulatory genes provides optimism through targetable molecular networks that can be modified during a much longer therapeutic window, e.g., months after brain injury [255]. Future interventional studies in animals should consider strong interactive factors that can robustly modify the immunological consequences of TBI (e.g., dementia continuum), and the application in humans should account for immunological triggers and precipitants (by design, inclusion, and adjustment) that originate in environment, lifestyle (e.g., exercise, sleep patterns), and the temporal dynamic of immune-mediated medical conditions [272].

MICROBIOME AND PROBIOTIC INTERVENTIONS

One factor contributing to increases in chronic inflammatory disorders in Western countries is thought to be failing immunoregulation, attributable to reduced exposure to the microbial environment within which the mammalian immune system coevolved [273]. Our understanding of the "gut-brain axis" [274] is steeply increasing. The three proposed mechanisms bidirectionally linking the gut with the brain are: 1) changes in sympathetic sensorimotor function or vagal tone, 2) alterations in neuroendocrine signaling, and 3) alterations in immune signaling and gut, as well as BBB permeability [275]. The metabolites of the microbiome's colonizing bacteria can be both anti-inflammatory and neuroprotective [274] and the gut microbiome has a prominent role in maintaining a delicate homeostasis of the immune system [276], and integrity of barrier functions. Rodents with germ-free gastrointestinal systems have decreased tight junction integrity (and increased BBB permeability) likely contributing to increased microglial activation [274].

Changes in gut microbiome are associated with increased neuroinflammatory and autoimmune reactions in multiple sclerosis patients [277] and have been implicated in other autoimmune-mediated diseases mediated by T cells including rheumatoid arthritis, inflammatory bowel disease, and type 1 diabetes [274]. Krezalek and colleagues coined the term "pathobiome" to describe changes in the microbiome after TBI [276]. There is a proposed feedback loop in which TBI and subsequent induction of the "pathobiome" result in a neuroinflammatory cascade, thus, further augmenting the effects of brain injury by cell death, BBB disruption, edema, and hemorrhage [276]. Changes in the gut microbiome occur as soon as 2 h after a TBI in rodent studies [276], with subsequent increases in permeability in the intestinal wall making TBI recipients susceptible to transloca-

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tion of bacterial pathogens, resulting in exponentially increased local, systemic, and CNS inflammation [276].

Certain studies on probiotic administration have reported increases in anti-inflammatory cytokines such as IL-10 and decreases in inflammatory cytokine production in the intestine, and decreases in stress-related hormone production induced by brain injury [276]. A 2017 review by Brenner identifies three studies indicating a benefit for the treatment of TBI [278]. Two randomized clinical trials in TBI patients in the ICU showed patients who received probiotics had decreased ICU length of stay, decreased infections, infection with fewer number of pathogens, treatment with fewer types of antibiotics, and decreased duration (in days) on artificial ventilation. As initial studies are promising in the use of probiotics in TBI patients immediately following their injury during hospital stay [279], further research on long-term effects and benefits across various severities and types of TBI is much needed.

In sum, in TBI, in contrast to many other neuropsychiatric domains, there is a disquieting disconnect between bench and bedside, with successful preclinical studies failing to translate [255]. It is commonly stated that the lack of efficacy demonstrated in human clinical trials of very promising interventions developed in animal models of TBI, including molecules directed at immune targets, is due to the incomplete representation of human TBI by the specific animal models used, specifically the much higher pathogenetic heterogeneity of clinical TBI. Yet, we see two factors that are equally important. First, human TBI does not occur in isolation—concurrent stressors, levels of immunomodulatory hormones and vitamins, variability in fitness and common endocrine dysregulation and metabolic syndrome, common infections, allergies and exposure to allergens, past TBIs, including barely noticed concussive or subconcussive events, exposure to heat and cold, and photoperiodic and circadian factors, are all known to modify immune response, and some are known to modify neuroprotection and neurogenesis. Second, many immune factors are highly context-dependent, with a good number of them playing either a protective or degenerative role, dependent on the time sequence, dose, and interaction with other immune stressors or immune-mediated clinical conditions, either as a trait or state. Reciprocal cascading interactions exist—while there is long-term immune activation in many TBI patients, TBI has an immediate peripheral immunosuppressive effect that is similar to advanced aging [256], and that can lead to vulnerability to acute infections and reactivation of chronic infections that lead to immediate increased mortality [280], and long-term negative outcomes in TBI [281, 282]. Prior brain trauma, prior extracranial trauma, polytrauma, and their interactions with infections [283], in particular nosocomial infections, may result in a different course of brain injury and immune contribution to brain injury than if it would happen in isolation. Sleep abnormalities are often present in TBI patients [284] and in animal models of TBI [285], and are known to affect immune function, functional changes of HPA axis, and stress vulnerability [246]. These have not yet been studied in interventional animal studies of TBI that could mimic more closely the human experience of TBI. These processes are currently oversimplified in animal models that generate treatment candidates but are not formally tested before being transposed to humans. Ideally, these factors would be analytically studied in animals, determining the specific association of immune-modifying factors in interaction, considering simultaneity, precedence, sequence, repetitiveness, and dose-response [272]. While life in experimental animals is forcefully homogenized, with identical genetic background, similar food, and sleep-wake and rest-activity opportunities, it is not the case in humans. Sources of heterogeneity include genetic variation, exposure to activities that modify the response of the immune system to TBI, such as acute, latent, and chronic infections, atopy with allergen exposure, autoimmune conditions, exercise, degree of general as well as context specific psychological stress (e.g., realization of personal loss of physical, social and economical abilities via TBI and potential loss through demise or severe sequelae in others who have been co-exposed to the same trauma as the TBI patient) make it more complex. Additionally, regular or recent intake of common medications for common conditions that have immunomodulatory properties (such as statins, metformin, psychotropic medications) can markedly increase immune response heterogeneity in clinical samples. Additive or multiplicative interactions between these immune-modifying factors make the specific manipulation of candidate therapeutic molecules, identified in animal research and utilized in humans, to be overly simplified, and call for concentric interventions addressing individual combinations of interacting sources of immune dysregulation in animal models. Their translation to human studies requires large multicenter collabora-

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tions allowing the adequate number of participants to select specific interacting immune-modifying factors to be addressed by specific immune targeting interventions.

TAKE HOME MESSAGE

Attenuation of secondary injury has been a major goal of neuroprotective treatment, and despite strong experimental data, many trials for human TBI have failed to produce results [213]. In our evaluation, the complexity and interindividual differences of interactive factors that up or downregulate immune function prior to TBI in humans relative to the homogenous condition of experimental animals is the main cause of poor animal to human translation of significant interventional results with benefic outcomes in animal models. The failure of the human trials to show consistency have also been attributed to: 1) considering secondary mechanisms in isolation rather than in interaction; 2) inadequate attention to animal model species, strains, age, gender, and timing; 3) the major difference of anesthetics being used in animal models; 4) the relative impermeability of the BBB; 5) the definition of meaningful translational behavioral outcomes; 6) epigenetic variability in humans resulting in pharmacogenetic heterogeneity; 7) not considering predicting biomarkers that would justify physiologically the need of the intervention in a particular participant; 8) inadequate sample size [213]; and 9) less diffuse axonal injury because of much smaller brains in the rodents. Animal studies generally do not make pharmacokinetic and pharmacodynamic measurements, and do not try to optimize dosage to tissue concentration.

While we are noticing the disturbing lack of translation between targeting the immune system in animal models and clinical TBI, there is, however, one element of congruence—aerobic exercise that has shown significant benefits (cognitive performance) in patients with mild and moderate TBI [286], as it adequately translates the rodent study on pre-conditioning with exercise (daily running for four weeks) leading to reduced increases in IL-1β, TNF-α, and markers of BBB permeability and lesser decreases in IL-10 post-brain injury, as well as less motor function impairment after fluid percussion injury in rats [287]. Similar results have been found in a mouse study suggesting that there is a therapeutic window, specifically late after TBI (5 weeks after brain trauma) for reduction of longer-term inflammation and cognitive impairment [288]. This late administration of exercise is important given that there are concerns regarding aerobic exercise early after TBI, considering that, as shown previously in this article, immune activation appears to be benefic in the early phase of TBI for cleaning of the impacted tissue, as well as for protection of infections, in addition to increasing brain oxygen demand and generation of additional free radicals with exercise. Furthermore, the later administration of an immunomodulating intervention is consistent with literature suggesting that targetable molecular networks can be modified during a much longer therapeutic window than initially thought, e.g., months after brain injury [255], although a more exact translation of the therapeutic window in humans is highly desirable. Finding the temporal sweet-spot for switching from immunotherapies geared toward clearance of debris, reparative and neuroprotective effects of inflammation, and those geared toward decreasing chronic unremitting inflammation [11], should represent a central topic for future research.

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