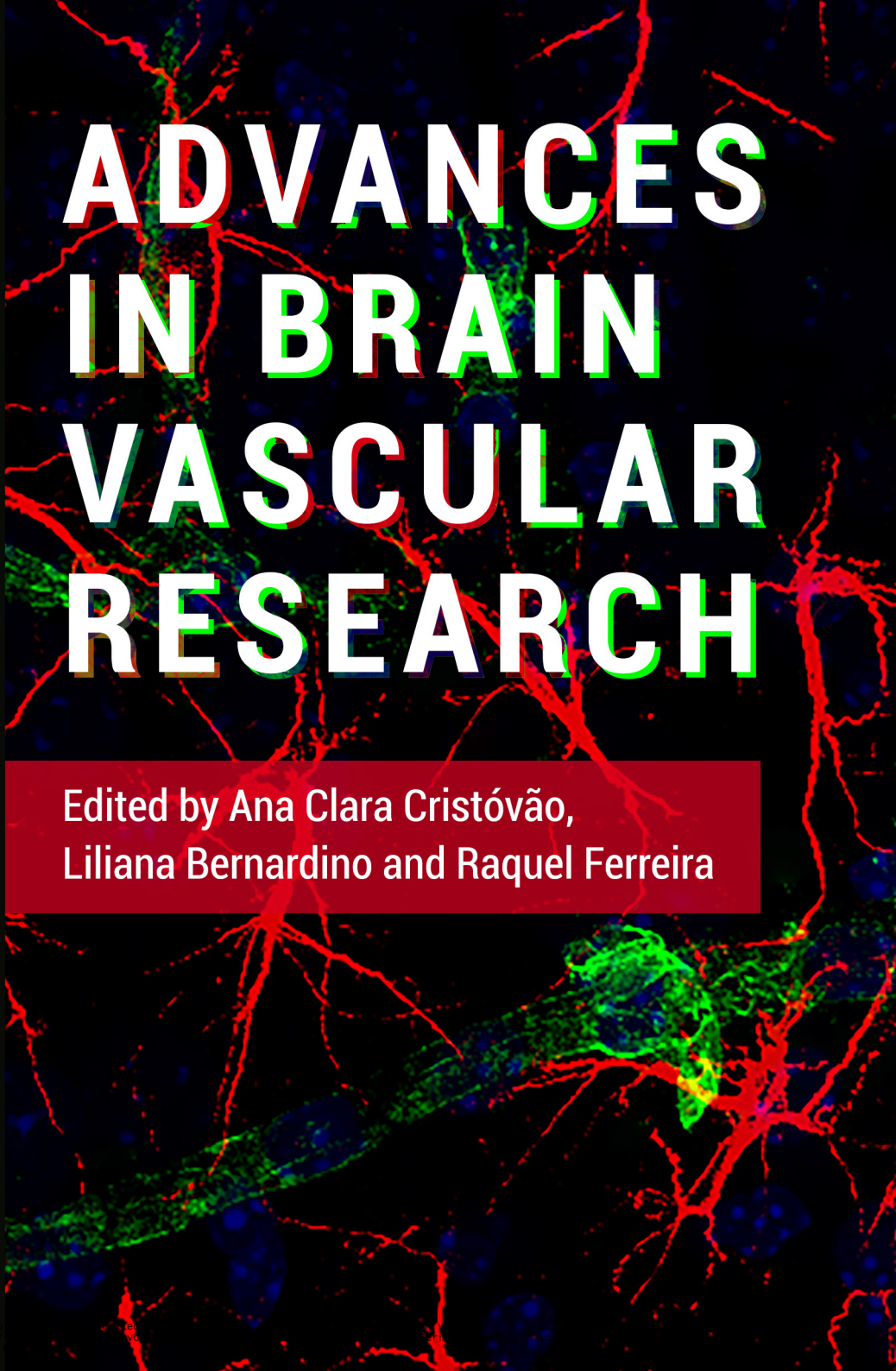
ADVANCES IN BRAIN VASCULAR RESEARCH
Edited by Ana Clara Cristóvão, Liliana Bernardino and Raquel Ferreira

Advances in Brain Vascular Research

Advances in Brain Vascular Research

Edited by

Ana Clara Cristóvão, Liliana Bernardino and Raquel Ferreira

Cambridge Scholars Publishing

Advances in Brain Vascular Research

Edited by Ana Clara Cristóvão, Liliana Bernardino and Raquel Ferreira

This book first published 2020

Cambridge Scholars Publishing

Lady Stephenson Library, Newcastle upon Tyne, NE6 2PA, UK

British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library

ISBN (10): 1-5275-5067-2
ISBN (13): 978-1-5275-5067-4

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

The editors would like to acknowledge Thomas Williams for the linguistic revision of the book. This work was funded by the Foundation for Science and Technology of Portugal (FCT), which includes funds from the State Budget and the European Regional Development Fund (FEDER), through Portugal2020 (UIDB/00709/2020) and the ICON project, Interdisciplinary Challenges On Neurodegeneration (CENTRO-01-0145-FEDER-000013); and co-financed by the Research Coordinating Institute (ICI) of the University of Beira Interior.

CHAPTER ONE

ENDOTHELIAL CYTOARCHITECTURE AND HETEROGENEITY IN THE BRAIN

MARIA ALEXANDRA BRITO

The vascular endothelium

The term endothelium was first used by Swiss anatomist Wilhelm His in 1865 to differentiate the epithelium from the inner lining of the body cavities, which included blood vessels, lymphatics, and mesothelial-lined cavities, a definition that was later narrowed down to include only the inner cell layer of blood vessels and lymphatics (Aird 2007). Within the developing vertebrate embryo, a rudimentary vascular meshwork of endothelial cells is lumenized into epithelial tubes that are differentiated into specialized subtypes, including arteries, veins, and capillaries, which become stabilized by mural cells (Fish and Wythe 2015). Arteries give rise to the microvasculature, which sequentially comprises arterioles (10-100 μm), capillaries (4-10 μm), and venules (10-100 μm) (Ge, Song, and Pachter 2005), which in turn end up in veins. Arterial and venous blood vessels are formed by an inner endothelial layer (tunica intima), a middle smooth muscle layer (tunica media) and an outer connective tissue layer (tunica adventitia). In contrast, capillaries lack a tunica media, so endothelial cells are ensheathed by cells with contractile properties, known as pericytes (Sá-Pereira, Brites, and Brito 2012).

Brain endothelial cells

The central nervous system (CNS) is protected by a complex interface, known as the blood-brain barrier (BBB). The BBB prevents a wide range of molecules present in the plasma from entering that otherwise could enter the brain and induce cellular activation and neural tissue damage; moreover, it strictly regulates the concentration of ions in the brain

parenchyma, which is essential for normal brain function (Cardoso, Brites, and Brito 2010). The anatomic basis of the BBB is formed by brain microvascular endothelial cells (BMEC), which possess unique features that limit paracellular and transcellular permeability, rendering the brain endothelium a selective barrier. Despite the fact that important interactions are established with the remaining components of the neurovascular unit, particularly the basement membrane, pericytes and astrocytes (Sá-Pereira, Brites, and Brito 2012), this chapter is focused on the main cellular and molecular characteristics of BMEC. As it is currently accepted that endothelial cells display genotypic and phenotypic heterogeneity, BMEC's variable properties are also addressed.

Endothelial cell cytoarchitecture

BMEC are characterized by elaborate junctional complexes that restrict permeability across the endothelium, by cell adhesion molecules that are involved in the crosstalk between the endothelium and immune cells, and also by specific transport systems that ensure the supply of necessary molecules to the brain and the elimination of potentially harmful molecules, the main features of which are presented below.

Junctional complexes and cell adhesion molecules

The connection between barrier integrity and function and the presence of endothelial intercellular junctions was an important hallmark in studying the BBB. Junctional complexes are formed by tight junctions (TJ), adherens junctions (AJ) and gap junctions (GJ). While in epithelial cells the distribution of TJ and AJ is more organized, with apical TJ and basolateral AJ, in endothelial cells the junctional architecture is less defined, and AJ are intermingled with TJ (Bazzoni and Dejana 2004). GJ are involved in intercellular communication (Stamatovic et al. 2016), though this type of junctions at the BBB has been far less described than TJ and AJ, or than GJ in epithelia.

Tight junctions

TJ proteins form strains along the intercellular junction region, establishing a connection with the opposing membrane and obliterating the intercellular space. TJ are formed by a set of transmembrane proteins that are linked to cytosolic proteins, which in turn establish the connection with the cytoskeleton. There are two major types of transmembrane proteins,

classified according to the number of membrane-spanning domains they contain: four-pass transmembrane proteins such as claudins, occludin, and tricellulin, and single-span proteins, including junctional adhesion molecules (JAM) (Mariano et al. 2011). Claudins form dimers and bind homotypically to claudins on adjacent cells to constitute the primary seal of the TJ. The claudin family has 27 elements that have been identified until now and are differentially expressed in epithelia and endothelia (Mineta et al. 2011). However, claudin-5 has been the family member most researched in BBB studies so far, both in vitro and in vivo (Cardoso et al. 2012, Liu et al. 2018). Interestingly, the expression of several family members has recently been demonstrated, namely claudins-1, -3, -4, -5, -11, -20 and -25, in BBB microvessels, and claudin-5 is the dominant protein in vitro (Berndt et al. 2019), explaining why it has been the most extensively studied.

Occludin was the first transmembrane TJ protein to be identified, but its requirement for TJ formation, regulation or both is still controversial. On the one hand, occludin-deficient mice did not show alterations in the barrier properties of the intestinal epithelium (Saitou et al. 2000). On the other hand, occludin's role in TJ sealing has been demonstrated (Lacaz-Vieira et al. 1999), with a direct influence on the permeability of epithelial and endothelial barriers, including the BBB (Argaw et al. 2009, Jiao et al. 2011). The recently identified tricellulin belongs to the same family as occludin and is concentrated at the intersection between three epithelial cells. Further studies on tricellulin have demonstrated that this TJ protein plays an essential role in the regulation of permeability to macromolecules (Krug et al. 2009). Tricellulin was later identified in the brain microvasculature in situ, as well as in cultured BMEC (Mariano et al. 2013), suggesting a role in BBB integrity and properties. Although the potential interactions of tricellulin with other TJ proteins and the mechanisms that regulate the expression and function of this protein are still unclear, they have recently started to be unraveled (Mariano et al. 2011).

JAM are other transmembrane proteins that mediate protein interactions in the TJ region. The JAM family is divided into two subgroups based on different binding-domain motives. Class I members interact with TJ accessory proteins and have been identified in endothelial and epithelial cells, and they have an active role in controlling endothelial permeability and leukocyte transmigration (Martin-Padura et al. 1998). Members of class II include the endothelial cell-selective adhesion molecule, a JAM protein found exclusively in endothelial cells (Nasdala et

al. 2002), loss of which was shown to enhance vascular endothelial growth factor (VEGF)-initiated permeability (Wegmann et al. 2006).

The cytoplasmic proteins of the TJ are responsible for the connection of junction complexes to the actin cytoskeleton. The most studied TJ accessory components are zonula occludens (ZO) proteins. In contrast to ZO-3, ZO-1 and -2 are both present in endothelial cells (Balda and Anderson 1993, Jesaitis and Goodenough 1994). Alterations in ZO-1 levels in BMEC have been associated with TJ disruption and loss of BBB integrity in pathological conditions (Zhong et al. 2012, Jiao et al. 2011, Palmela et al. 2012). All ZO proteins share common domains that are essential for signal transduction and enable the anchoring of transmembrane TJ proteins and actin filaments (Furuse et al. 1994, Van Itallie et al. 2009). Actin filaments serve both structural and dynamic roles in the cell, and alterations of the structural organization of this cytoskeleton protein have a direct influence on TJ integrity, as suggested in hypoxia conditions (Brown and Davis 2005).

Adherens junctions

AJ not only provide a second site for cell adhesion but are also involved in cell polarization and tissue morphogenesis (Nishimura and Takeichi 2009), as well as in receptor signaling and transendothelial migration of leukocytes (Sweeney et al. 2019). Similarly to TJ, different classes of proteins are required for AJ assembly: transmembrane and the cytoplasmic proteins, which serve as a link between the transmembrane components and the cytoskeleton (Niessen and Gottardi 2008). The most widely characterized transmembrane and cytosolic proteins in endothelial cells are vascular endothelial (VE)-cadherin and catenins, respectively.

Classical cadherins are single-pass transmembrane glycoproteins that function as homophilic receptors to mediate Ca^{2+}-dependent intercellular adhesion. Cadherin clustering is crucial for maintaining AJ integrity and is sustained by interactions between cadherin molecules of the same cell and on the opposite cell (Gumbiner 2005). The role of VE-cadherin in vascular integrity has been shown through experiments targeting the extracellular domain of this transmembrane protein, with a consequent increase in endothelial permeability (Corada et al. 2001). Studies with BMEC reinforced the role of VE-cadherin in BBB function since protein loss or redistribution by the action of cytokines led to an impairment in barrier integrity (Shen et al. 2011).

Cytosolic catenins include α-catenin and β-catenin. The majority of β-catenin in the cell is associated with AJ, where it interacts with the

cytoplasmic region of cadherin (Aberle et al. 1994). There is also a small and dynamic pool of β-catenin in the cytoplasm and nucleus that is responsible for the transduction of Wnt signals. The reduction of β-catenin levels has been shown to cause AJ fragility and increased BMEC permeability in injurious situations (Wylezinski and Hawiger 2016). Besides binding to cadherins, β-catenin contains binding information in a different domain for α-catenin (Pokutta and Weis 2000), involved in the connection of the AJ complex to actin. Despite the shortage of studies on the function of α-catenin, interference with its binding to β-catenin has shown to affect endothelial integrity and impair leukocyte transmigration (van Buul, van Alphen, and Hordijk 2009). Although alterations in TJ proteins have been associated with compromised barrier function, a growing number of studies have highlighted the crucial role of accurate AJ assembly in such endothelial function. In particular, both VE-cadherin and β-catenin have been shown to mediate the expression of BMEC claudins (Taddei et al. 2008, Liebner et al. 2008), validating their role in BBB integrity.

Platelet endothelial cell adhesion molecule-1 (PECAM-1), also known as cluster of differentiation 31 (CD31), is a transmembrane protein highly enriched at interendothelial junctions of vascular endothelial cells, suggested to contribute to the regulation of vascular integrity and remodeling. Although it has been considered by some authors to be an AJ protein (Sweeney et al. 2019), others consider it to be a transmembrane protein located outside junctional complexes (Wimmer et al. 2019). Considering that its role as a cell adhesion molecule (CAM) is widely accepted, in this chapter it is described in further detail in the section about such molecules.

Gap junctions

GJ are formed by members of the connexin (Cx) family (Eugenin et al. 2012), which is composed of several transmembrane isomers with tissue-specific expression. Canonically, connexins function as homo- or hetero-hexamers at the plasma membrane and can exist as hemichannels (HC) or GJ, the latter being characterized by the alignment of two neighboring cell surface hexamers to oppose each other. GJ mediate intercellular communication by connecting the cytoplasm of adjacent cells and allowing the passage of ions and small molecules, thus leading to signal transduction between neighboring cells (Stamatovic et al. 2016). In the brain, GJ complexes mediate the important signaling between BMEC and both astrocytes and pericytes (De Bock et al. 2014). Interestingly, in

BMEC, Cx have been shown to contribute to BBB permeability control and suggested to be associated with occludin and claudin-5, as well as to stabilize brain endothelial junctions, while nonspecific Cx channel blockers inhibited the barrier function of the TJ. The brain microvascular endothelium expresses Cx37 and Cx40, whereas Cx43 was observed in freshly isolated capillary and cultured BMEC, but not in brain slices (De Bock et al. 2014). These apparently inconsistent findings may be explained by the fact that Cx43 is expressed in the growing microvessels of the 18 week-old human telencephalon but disappears as their differentiation progresses (Virgintino et al. 2001). Cx43 redistribution in endothelial cells with increased BBB permeability has been observed with ageing, suggesting that a loss of endothelial cell communication may underpin ageing-associated barrier defects (De Bock et al. 2011).

Cell adhesion molecules

CAM, which are involved in immune responses, have been classified into different families of proteins according to their structure: integrins, selectins and proteins from the immunoglobulin superfamily. Integrins are cell-surface transmembrane αβ heterodimers that recognize specific extracellular matrix (ECM) ligands. The expression of integrin subunits α1 and α6 is associated with the presence of β1-ECM ligands, like laminin, collagen IV and fibronectin (Paulus et al. 1993). Importantly, the diversity of roles attributed to integrins appears to be dependent on the specific combination of proteins. For instance, the function of integrin αvβ3 seems to include the induction of endothelial migration and angiogenesis, being greater after the onset of focal ischemia and influencing vascular hyperpermeability (Abumiya et al. 1999). On the other hand, loss of β1 integrin in ischemic injury correlates with maximal neuronal injury and loss of microvessel integrity (Tagaya et al. 2001). Moreover, β1 integrin block has been linked to a reduction in claudin-5 expression and an increase in microvessel permeability (Osada et al. 2011). These observations point to a novel regulatory mechanism with β1 integrin/ECM adhesion and BMEC TJ, promoting BBB integrity.

The selectin family consists of three Ca^{2+}-dependent CAM: leukocyte (L-), endothelial (E-), and platelet (P-) selectins, which vary depending on the number of extracellular consensus repeats with homology to complement regulatory proteins: two, six or nine, respectively (Barthel et al. 2007). In endothelial cells, both P- and E-selectins are expressed (Yoshida et al. 1996), and their levels can be increased by several stimuli, like cytokines and pathogens (Huang, Gonzalez, and Eniola-Adefeso

2013, Wong and Dorovini-Zis 1996). The selectin family plays a key role in mediation of early neutrophil rolling and adherence to BMEC, through interaction with L-selectin on the leukocyte surface (Engelhardt 2008). Although the upregulation of selectins is particularly important to recruit immune cells to resolve the injury, it may also play a detrimental role if the aberrant homing of leukocytes aggravates the symptoms. In fact, in brain ischemic injury, selectins appear to play an important role in exacerbating immune cell recruitment and consequent injury (Zhang et al. 1998), and blocking these adhesion molecules has been shown to constitute a promising therapeutic approach (Yilmaz et al. 2011). In contrast, studies in an animal model of multiple sclerosis have demonstrated that the expression of endothelial selectins is not involved in immune cell recruitment through the BBB (Doring et al. 2007). Therefore, the role of selectins in the recruitment of immune cells in pathological conditions appears to be complex and might depend on the expression of other molecules.

Proteins from the immunoglobulin superfamily share the presence of several extracellular immunoglobulin domains (Wang and Springer 1998). The most studied proteins from this family in endothelial cells are intercellular CAM-1 (ICAM-1), PECAM-1 and vascular CAM-1 (VCAM-1), which typically have 5, 6 and 7 immunoglobulin domains, respectively. In BMEC, these three CAM are crucial for the recruitment of immune cells to the injured brain area, especially in pathological conditions such as multiple sclerosis (Engelhardt et al. 1997, Washington et al. 1994).

Transport across the BBB

Due to elaborate junctional complexes, the brain endothelium can be seen as a continuous cell membrane across which lipid molecules can diffuse and enter the brain passively, whereas polar and high molecular weight molecules are not able to freely cross the endothelium (Abbott et al. 2010). These properties allow the movement of blood gases oxygen and carbon dioxide across the BBB but prevent many essential molecules from entering the brain, including nutrients, hormones and vitamins, and restrict the elimination of unwanted compounds (Abbott et al. 2010, Sweeney et al. 2019). To ensure the metabolic demands of the brain and its homeostasis, endothelial cells of brain capillaries are equipped with a number of transport systems that can be categorized as efflux transporters, solute carrier-mediated transporters, and transcytosis.

Efflux transporters

Some molecules that have a high lipid solubility have a lower brain penetrance than might be expected based on their physicochemical characteristics. This fact results from the existence of efflux transporters in BMEC that promote the efflux from endothelial cells into the blood and/or extrusion out of the brain of both endogenous molecules and xenobiotics, thus preventing their accumulation in the brain. Despite the relevance of efflux transporters for CNS homeostasis, they represent an enormous obstacle for the treatment of brain pathologies.

The most studied efflux transporters are the ATP-binding cassette (ABC) family, which mediates the exporting of substrates from cells coupled with the hydrolysis of ATP (Shen and Zhang 2010). There are 49 genes encoding the transporters in this family, arranged into seven subfamilies (termed A–G), among which the subfamilies B, C and G, which encode for P-glycoprotein (ABCB1), MRP (or multidrug resistance-associated protein) (ABCC1-9) and BCRP (or breast cancer resistance protein) (ABCG2) (Videira, Reis, and Brito 2014). P-glycoprotein and BCRP are expressed at BBB level (Gomez-Zepeda et al. 2019) in the same order of magnitude (Morris, Rodriguez-Cruz, and Felmlee 2017). Both BCRP and P-glycoprotein are expressed at the luminal surface (Roberts et al. 2008), although a fraction of P-glycoprotein in the abluminal membrane has also been detected (Tai et al. 2009). A clear expression of MRP4 has been demonstrated in the luminal membrane of rat BBB endothelial cells (Roberts et al. 2008). As far as other MRP are concerned, and particularly for MRP1, conflicting results can be found in the literature. In fact, some researchers have described a weak expression at BBB capillaries (Roberts et al. 2008), while others have stated that it is present in choroid plexus capillaries but not in those of the BBB (Gazzin et al. 2011), which overall indicate a low expression of this efflux transporter at the BBB. Differences among species in the expression of the efflux transporters have been described, with a higher expression of BCRP and a lower expression of P-glycoprotein and of MRP4 in humans than in rodents (Morris, Rodriguez-Cruz, and Felmlee 2017).

BCRP and P-glycoprotein are important elements of barrier function. These transporters have substrate overlap, as shown by the fact that inhibition of one of the two transporters is not sufficient to deliver anticancer drugs into the brain because of compensation by the other transporter, which led to the paradigm that P-glycoprotein and BCRP work as a “cooperative team of gatekeepers” at the BBB (Agarwal et al. 2011). This does not exclude some substrate specificity, as recently shown for

benzylpenicillin that is transported by BCRP but not by P-glycoprotein (Li et al. 2016).

To overcome the action of efflux transporters in therapeutics, their inhibition has been used in the treatment of brain pathologies like amyotrophic lateral sclerosis (Jablonski et al. 2014) and brain cancer (On and Miller 2014). The importance of P-glycoprotein and BCRP to BBB function and brain homeostasis has been highlighted and their deregulation in neurodegenerative diseases has been demonstrated. In fact, an upregulation of both P-glycoprotein and BCRP has been shown in amyotrophic lateral sclerosis, epilepsy, as well as in stroke and ischemic brain injury, whereas a selective decrease of P-glycoprotein, with no changes in BCRP, has been observed in Alzheimer's disease (AD) and multiple sclerosis (Qosa et al. 2015).

Solute carrier-mediated transport

Endothelial cells forming the BBB express a large number of solute carriers that provide transport of a wide variety of solutes and nutrients, mediating their flux into and out of the brain. These transporters have a specific and polarized distribution in endothelial cells and are expressed in either the luminal or abluminal membrane, or both, depending on the direction of transport into or out of the brain, or can be bidirectional (Abbott et al. 2010). These transporters are grouped based on the type of molecule transported, which include carbohydrates, amino acids, nucleotides, and organic anions and cations (Ohtsuki and Terasaki 2007, Sweeney et al. 2019). Energy transporters are one of the most important systems, and energy sources include glucose, mannose, and creatine, among others. One of the most studied groups of transporters in BMEC is the glucose transporter (GLUT) family with 14 isoforms (GLUT 1-14) (Patching 2017), responsible for mannose and glucose transport. GLUT-1 is highly enriched in BMEC, with higher expression on the luminal side than at the abluminal membrane, as a mechanism to generate a glucose transport gradient (Moura et al. 2019). Due to the relevance of this transporter at the BBB, glucose and other sugar derivatives have been considered as promising shuttles to achieve drug delivery to the brain via GLUT-1 (Patching 2017). Evidence highlights the importance of GLUT-1 expression for proper brain function, as this transporter is reduced in disorders that affect the brain, like AD (Hooijmans et al. 2007).

Transcytosis

Transcytotic mechanisms allow the transporting of a variety of large molecules and complexes across the BBB that otherwise would be physically prevented from entering the brain parenchyma (Abbott et al. 2010). This type of transport involves endocytic mechanisms, in which extracellular molecules are internalized at one pole of the plasma membrane and subsequently are transported via caveolae or clathrin-coated vesicles to the opposite side, where they are exocytosed. Caveolae are vesicles rich in sphingolipids and cholesterol, with caveolins as the main protein component (Stan 2005), and particularly caveolin-1 (Cardoso, Brites, and Brito 2010). The expression of caveolin-2 alone does not lead to caveolae formation and requires caveolin-1 for transportation (Parolini et al. 1999), whereas experiments in mice lacking caveolin-1 highlighted its importance in the formation of caveolae (Park et al. 2002). In the brain, caveolin-1 has been identified in BMEC (Ikezu et al. 1998, Bernas et al. 2010) and suggested to play an active role in the regulation of BBB vesicular permeability (Predescu, Predescu, and Malik 2007), partially because of its influence on junction proteins (Nag, Venugopalan, and Stewart 2007, Zhong et al. 2008, Kronstein et al. 2012). Clathrin-coated vesicles are negatively charged and, thus, repel anionic molecules. So, only a small fraction of the plasma proteins, characterized by a positive charge, can be transcytosed by clathrin-coated vesicles (Herve, Ghinea, and Scherrmann 2008).

Soluble plasma molecules can be randomly taken up by caveolae with a bulk of blood plasma and then be transported across brain endothelial cells. This process, known as bulk-phase or fluid-phase transcytosis (FMT), is independent of any interaction between the transported molecules and the caveolar vesicle membrane. Due to the negative charge of clathrin-coated vesicles, only a very small portion of the plasma proteins can be transcytosed randomly within the fluid phase of transport by these vesicles (Herve, Ghinea, and Scherrmann 2008). In contrast to the FMT, transcytosis can involve the interaction of a ligand with moieties expressed at the luminal surface of endothelial cells. This type of transcytosis may occur via specific binding to a receptor (receptor-mediated transcytosis, RMT) or by a nonspecific process (adsorptive-mediated transcytosis, AMT). Both in RMT and AMT, the lysosomal compartment needs to be avoided to prevent degradation of the protein or peptide and achieve transcytosis (Abbott et al. 2010).

RMT is characterized by the binding of a macromolecular ligand to the corresponding receptor in the endothelial cell surface that triggers an endocytic process with formation of a caveola. The caveola is then

released and the ligand-receptor cluster is internalized into the endothelial cell and routed across the cytoplasm towards the opposite pole of the cell where the vesicle content is exocytosed, with dissociation of the complex occurring during cellular transit or the exocytotic event (Abbott et al. 2010). Among the several receptors involved in RMT at the BBB are the insulin receptor, the transferrin receptor, the lipoprotein receptor-related protein receptor-1 (LRP-1), and the receptor for advanced glycation end products (RAGE) (Abbott et al. 2010, Sweeney et al. 2019). Insulin and transferrin receptors allow insulin and iron-loaded transferrin to enter the brain to supply it with the hormone and iron respectively (Paterson and Webster 2016, Lajoie and Shusta 2015). Importantly, these receptors have been exploited as BBB shuttles for the delivery of drugs to the brain, in a strategy known as Trojan horses (Pardridge 2006). LRP-1 is mainly localized in the abluminal membrane of endothelial cells, while RAGE is mainly expressed at the luminal membrane (Sweeney et al. 2019). LRP-1 and RAGE transport amyloid-β (Aβ) peptide, one of the hallmarks of AD across the BBB. LRP-1 transports Aβ out of the brain by transcytosis, mediating its efflux from the brain and representing the major efflux transporter for Aβ across the microvasculature (Shibata et al. 2000). LRP-1 is decreased in the BBB of AD patients (Silverberg, Messier, et al. 2010), which contributes to Aβ accumulation in the brain parenchyma. In contrast to LRP-1, RAGE constitutes an entrance gate for peripheral Aβ to enter the brain parenchyma and is upregulated in AD (Silverberg, Miller, et al. 2010), thus promoting Aβ accumulation in the brain. Targeting of these transport systems to promote Aβ elimination and/or to prevent peripheral Aβ entrance into the brain parenchyma has emerged as a promising strategy for AD treatment (Deane et al. 2012, Kook et al. 2012, Sagare et al. 2013).

In AMT, a compound is internalized through a direct electrostatic interaction with the endothelial cell surface, which triggers the endocytic process (Moura et al. 2019). Thus, positively charged molecules like albumin interact with the negatively charged cell luminal membrane of BBB endothelial cells to be internalized (Pulgar 2018). Interestingly, AMT was recently suggested to be the transcytosis mechanism by which α-synuclein-containing erythrocyte-derived extracellular vesicles cross the BBB towards the brain and further points to AMT as a mechanism involved in the communication between the periphery and the brain, as well as in α-synuclein-related pathology initiation and spread (Matsumoto et al. 2017). The AMT mechanism has been used for drug delivery to the brain of large therapeutic molecules such as neuropeptides and proteins or even drug-encapsulated vectors like liposomes and nanoparticles using

cationic proteins and basic oligopeptides, such as cell-penetrating peptides, as targets (Lu 2012).

Endothelial cell heterogeneity

Endothelial cells have the ability to sense and respond to their local environment and meet the physiological requirements of the underlying tissue (Aird 2007). Therefore, in contrast to the long naively considered layer of functionally invariant cells, endothelial heterogeneity is now acknowledged at several levels, namely between different organs, between large and small vessels within the same organ, and between adjacent vascular segments, which reflect functional diversity (Ge, Song, and Pachter 2005). Accordingly, gene expression analysis has revealed significant variation in gene expression patterns between endothelial cells from microvessels of different tissues, from large vessels and microvessels, and from arteries and veins, as well as in pathological conditions, as reviewed by Ge et al. (Ge, Song, and Pachter 2005). These authors have also pointed out the differences between arterioles, capillaries and venules in the organization of junctional complexes, rate of transcytosis, and the expression of some enzymes. Furthermore, segmental characterization has revealed that as the vessels' size increases, there is a progressive decrease in the expression of transporters enriched at the BBB, P-glycoprotein and GLUT-1, together with a progressive increase in the expression of α-smooth muscle actin. It has also been demonstrated that P-glycoprotein expression is homogeneous in the whole cerebral capillary bed, reflecting the well-known protective role of the efflux transporter (Saubamea et al. 2012). Expression of the key marker of rat BBB, endothelial barrier antigen (EBA), is not uniform in the cerebral vasculature, being undetectable in arterioles and strongly expressed in venules, while an uneven expression has been observed in capillaries, with some vascular segments strongly labelled and others only faintly stained. This unexpected pattern for a protein associated with the integrity of the BBB suggests that it may be dynamically regulated at single-cell level in healthy brains (Saubamea et al. 2012). Variability in the endothelial surface charge has also been shown by the less intense anionic sites in venules than in capillaries or arterioles (Vorbrodt 1987), in line with the negative surface charge of the BBB mentioned above. Leukocyte extravasation occurs solely at the level of post-capillary venules (Engelhardt and Ransohoff 2005), which have leaky junctions and are loosely covered by a layer of pericytes; moreover, local changes in hemodynamics result in greatly reduced blood flow rates that increase the

chances of leukocyte contact with the vessel's endothelial lining (Muller 2013).

Along the wall of pre-capillary arterioles, capillaries, and post-capillary venules, there are variations in the abundance of pericytes, which rise at post-capillary venules and tend to disappear and be replaced by smooth muscle cells as the vein caliber increases (Sá-Pereira, Brites, and Brito 2012). Moreover, the degree of vascular coverage by pericytes varies with tissue type, appearing to be correlated with the degree of tightness of the interendothelial junctions. Although it is recognized that CNS pericytes are numerous surrounding brain capillaries, the precise extent of the vascular surface covered by pericytes is still unclear. In fact, Frank et al. (Frank, Dutta, and Mancini 1987) mention that pericytes cover 22–30% of the cerebral capillary surface, whereas Dalkara and colleagues (Dalkara, Gursoy-Ozdemir, and Yemisci 2011) state 30–70%, and Engelhardt and Sorokin (Engelhardt and Sorokin 2009) claim that they cover 99% of the abluminal surface of the capillary basement membrane in the brain. In any case, the pericyte-to-endothelia ratio in the brain is higher than in other organs (1:3 compared with 1:100 in striated muscles) (Dalkara, Gursoy-Ozdemir, and Yemisci 2011), and pericyte coverage in retina is even higher than in brain capillaries (Frank, Dutta, and Mancini 1987), in accordance with the suggested role of pericytes in maintaining the BBB and the blood-retinal barrier. There are also variations among species, as the average ratio of pericytes to ECs in the rat capillary is 1:5, whereas it is 1:4 in the mouse, and 1:3-4 in humans (Dore-Duffy and Cleary 2011).

Brain endothelial diversity

In line with the organ-specific heterogeneity in both genotypic and phenotypic characteristics and signature gene regulatory networks, brain endothelial cells present the most prominent upregulation of the "Wnt signaling" and "adherens junction" pathways. Moreover, analysis of regulatory transcription factors that could maintain brain endothelial cell-specific upregulation of the Wnt signaling pathway have revealed the upregulation of lymphoid enhancer-binding factor 1, known to interact with β-catenin and regulate differentiation of the BBB in vivo (Jambusaria et al. 2018).

Besides organ-specific endothelial heterogeneity, there is also variability in endothelial cell populations within the brain. In fact, there are regional differences in BBB function that reflect functional diversity within the CNS' anatomically distinct regions (Wilhelm et al. 2016) and there are certain brain regions devoid of BBB, as at the level of

circumventricular organs (CVO) (Cardoso, Brites, and Brito 2010). CVO include important elements of the neuroendocrine system and secretory organs (e.g. posterior pituitary, pineal gland, median eminence, subcommissural organ and the subfornical organ) where an exchange of circulating substances takes place. In these CVO, capillaries are characterized by thinner endothelial cells that contain fenestrations and discontinuous TJ, together with a lower expression of TJ proteins, which result in enhanced permeability (Wilhelm et al. 2016). It should also be highlighted that there are even differences among CVO microvessels, which display higher permeability in central regions, where TJ proteins like occludin, claudin-5 and ZO-1 are undetectable, whereas a low level of staining was detectable in distal subdivisions of secretory CVO (Morita et al. 2016).

It has been shown that BBB permeability for specific molecules is heterogeneous throughout the brain. For example, brain uptake of insulin in rats is higher in the hippocampus than in the cortex, changes that could not be attributed to differences in insulin receptor expression levels (Banks and Kastin 1998). On the other hand, the chemokine C-C motif ligand 11 (CCL11) crosses the BBB with transport rates that vary between brain regions, with the fastest transport occurring in the striatum (Erickson et al. 2014). Regarding interleukin (IL)-1 transport in SAMP8 mice, the fastest transport was observed in the pons-medulla (Moinuddin, Morley, and Banks 2000). Studies by Villasenor and colleagues (Villasenor et al. 2017) have shown that transcytosis significantly increased in a region-specific manner, and suggested that the regional variations in BBB permeability could be determined by different molecular pathways regulating transcellular permeability in BMEC.

Interestingly, even in the same brain region and tissue section, endothelial variability has been observed in the expression of a widely expanded set of endothelial markers, and it appears that this molecular heterogeneity is a property generalized to many markers (Lee et al. 2017). To explain this observation, the authors propose that the coordinate patterns of expression could define several different endothelial cell types within the brain or that cells exhibit distinct molecular patterns due to environmental cues. Importantly, analysis of such markers may make it possible to target populations for alterations in endothelial cells in specific conditions that could be used in personalized medicines.

In line with regional heterogeneity, pathological processes of specific brain regions are usually associated with BBB impairment in those regions, as exemplified by the fact that after status epilepticus induced in rats, BBB leakage was observed in limbic regions, while cortical brain

regions were not affected (van Vliet et al. 2014). There are also regional differences in BBB permeability associated with ageing as revealed by magnetic resonance imaging (MRI) analysis of humans showing that the hippocampus and the caudate nucleus, but not other brain regions, present BBB hyperpermeability in people with no cognitive impairment, an effect that was accelerated in cognitively impaired individuals (Montagne et al. 2015).

Conclusion

BMEC have the common characteristic of forming a single cell layer that lines all brain blood vessels and regulates exchanges between the bloodstream and the surrounding tissue. The most relevant knowledge about BMEC cytoarchitecture was brought together here, essential for understanding the unique barrier properties of the complex structure that they form, the BBB. Moreover, alterations occurring in BBB endothelial cells in brain disorders were pinpointed, in line with the new paradigm according to which vascular dysfunction is a key player in the onset and progression of brain diseases. Moreover, overcoming the BBB for therapeutic purposes based on the shuttling of drugs relying on BMEC transport systems was addressed. Despite the common and specific properties of brain endothelial cells, heterogeneity within brain microvasculature has increasingly been recognized. Such diversity among different brain regions and even within the same one was also addressed. Importantly, understanding brain microvascular dissimilarities may pave the way to cell-specific targeting approaches in personalized medicine.

References

Abbott, N. J., A. A. Patabendige, D. E. Dolman, S. R. Yusof, and D. J. Begley. 2010. "Structure and function of the blood-brain barrier." *Neurobiol Dis* no. 37 (1):13-25. doi: S0969-9961(09)00208-3 [pii] 10.1016/j.nbd.2009.07.030.

Aberle, H., S. Butz, J. Stappert, H. Weissig, R. Kemler, and H. Hoschuetzky. 1994. "Assembly of the cadherin-catenin complex in vitro with recombinant proteins." *J Cell Sci* no. 107 (Pt 12):3655-63.

Abumiya, T., J. Lucero, J. H. Heo, M. Tagaya, J. A. Koziol, B. R. Copeland, and G. J. del Zoppo. 1999. "Activated microvessels express vascular endothelial growth factor and integrin alpha(v)beta3 during focal cerebral ischemia." *J Cereb Blood Flow Metab* no. 19 (9):1038-50. doi: 10.1097/00004647-199909000-00012.

Agarwal, S., A. M. Hartz, W. F. Elmquist, and B. Bauer. 2011. "Breast cancer resistance protein and P-glycoprotein in brain cancer: two gatekeepers team up." *Curr Pharm Des* no. 17 (26):2793-802.

Aird, W. C. 2007. "Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms." *Circ Res* no. 100 (2):158-73. doi: 10.1161/01.RES.0000255691.76142.4a.

Argaw, A. T., B. T. Gurfein, Y. Zhang, A. Zameer, and G. R. John. 2009. "VEGF-mediated disruption of endothelial CLN-5 promotes blood-brain barrier breakdown." *Proc Natl Acad Sci U S A* no. 106 (6):1977-82. doi: 10.1073/pnas.0808698106.

Balda, M. S., and J. M. Anderson. 1993. "Two classes of tight junctions are revealed by ZO-1 isoforms." *Am J Physiol* no. 264 (4 Pt 1):C918-24.

Banks, W. A., and A. J. Kastin. 1998. "Differential permeability of the blood-brain barrier to two pancreatic peptides: insulin and amylin." *Peptides* no. 19 (5):883-9.

Barthel, S. R., J. D. Gavino, L. Descheny, and C. J. Dimitroff. 2007. "Targeting selectins and selectin ligands in inflammation and cancer." *Expert Opin Ther Targets* no. 11 (11):1473-91. doi: 10.1517/14728222.11.11.1473.

Bazzoni, G., and E. Dejana. 2004. "Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis." *Physiol Rev* no. 84 (3):869-901. doi: 10.1152/physrev.00035.2003.

Bernas, M. J., F. L. Cardoso, S. K. Daley, M. E. Weinand, A. R. Campos, A. J. Ferreira, J. B. Hoying, M. H. Witte, D. Brites, Y. Persidsky, S. H. Ramirez, and M. A. Brito. 2010. "Establishment of primary cultures of human brain microvascular endothelial cells to provide an in vitro cellular model of the blood-brain barrier." *Nat Protoc* no. 5 (7):1265-72. doi: nprot.2010.76 [pii]10.1038/nprot.2010.76.

Berndt, P., L. Winkler, J. Cording, O. Breitkreuz-Korff, A. Rex, S. Dithmer, V. Rausch, R. Blasig, M. Richter, A. Sporbert, H. Wolburg, I. E. Blasig, and R. F. Haseloff. 2019. "Tight junction proteins at the blood-brain barrier: far more than claudin-5." *Cell Mol Life Sci* no. 76 (10):1987-2002. doi: 10.1007/s00018-019-03030-7.

Brown, R. C., and T. P. Davis. 2005. "Hypoxia/aglycemia alters expression of occludin and actin in brain endothelial cells." *Biochem Biophys Res Commun* no. 327 (4):1114-23. doi: 10.1016/j.bbrc.2004.12.123.

Cardoso, F. L., D. Brites, and M. A. Brito. 2010. "Looking at the blood-brain barrier: molecular anatomy and possible investigation approaches." *Brain Res Rev* no. 64 (2):328-63.

doi: 10.1016/j.brainresrev.2010.05.003S0165-0173(10)00067-6 [pii].

Cardoso, F. L., A. Kittel, S. Veszelka, I. Palmela, A. Toth, D. Brites, M. A. Deli, and M. A. Brito. 2012. "Exposure to lipopolysaccharide and/or unconjugated bilirubin impair the integrity and function of brain microvascular endothelial cells." *PLoS One* no. 7 (5):e35919. doi: 10.1371/journal.pone.0035919PONE-D-11-18048 [pii].

Corada, M., F. Liao, M. Lindgren, M. G. Lampugnani, F. Breviario, R. Frank, W. A. Muller, D. J. Hicklin, P. Bohlen, and E. Dejana. 2001. "Monoclonal antibodies directed to different regions of vascular endothelial cadherin extracellular domain affect adhesion and clustering of the protein and modulate endothelial permeability." *Blood* no. 97 (6):1679-84.

Dalkara, T., Y. Gursoy-Ozdemir, and M. Yemisci. 2011. "Brain microvascular pericytes in health and disease." *Acta Neuropathol* no. 122 (1):1-9. doi: 10.1007/s00401-011-0847-6.

De Bock, M., M. Culot, N. Wang, M. Bol, E. Decrock, E. De Vuyst, A. da Costa, I. Dauwe, M. Vinken, A. M. Simon, V. Rogiers, G. De Ley, W. H. Evans, G. Bultynck, G. Dupont, R. Cecchelli, and L. Leybaert. 2011. "Connexin channels provide a target to manipulate brain endothelial calcium dynamics and blood-brain barrier permeability." *J Cereb Blood Flow Metab* no. 31 (9):1942-57. doi: 10.1038/jcbfm.2011.86.

De Bock, M., R. E. Vandenbroucke, E. Decrock, M. Culot, R. Cecchelli, and L. Leybaert. 2014. "A new angle on blood-CNS interfaces: a role for connexins?" *FEBS Lett* no. 588 (8):1259-70. doi: 10.1016/j.febslet.2014.02.060S0014-5793(14)00191-4 [pii].

Deane, R., I. Singh, A. P. Sagare, R. D. Bell, N. T. Ross, B. LaRue, R. Love, S. Perry, N. Paquette, R. J. Deane, M. Thiyagarajan, T. Zarcone, G. Fritz, A. E. Friedman, B. L. Miller, and B. V. Zlokovic. 2012. "A multimodal RAGE-specific inhibitor reduces amyloid beta-mediated brain disorder in a mouse model of Alzheimer disease." *J Clin Invest* no. 122 (4):1377-92. doi: 10.1172/JCI5864258642 [pii].

Dore-Duffy, P., and K. Cleary. 2011. "Morphology and properties of pericytes." *Methods Mol Biol* no. 686:49-68. doi: 10.1007/978-1-60761-938-3_2.

Doring, A., M. Wild, D. Vestweber, U. Deutsch, and B. Engelhardt. 2007. "E- and P-selectin are not required for the development of experimental autoimmune encephalomyelitis in C57BL/6 and SJL mice." *J Immunol* no. 179 (12):8470-9.

Engelhardt, B. 2008. "Immune cell entry into the central nervous system: involvement of adhesion molecules and chemokines." *J Neurol Sci* no. 274 (1-2):23-6. doi: S0022-510X(08)00250-5 [pii] 10.1016/j.jns.2008.05.019.

Engelhardt, B., and R. M. Ransohoff. 2005. "The ins and outs of T-lymphocyte trafficking to the CNS: anatomical sites and molecular mechanisms." *Trends Immunol* no. 26 (9):485-95. doi: 10.1016/j.it.2005.07.004.

Engelhardt, B., and L. Sorokin. 2009. "The blood-brain and the blood-cerebrospinal fluid barriers: function and dysfunction." *Semin Immunopathol* no. 31 (4):497-511. doi: 10.1007/s00281-009-0177-0.

Engelhardt, B., D. Vestweber, R. Hallmann, and M. Schulz. 1997. "E- and P-selectin are not involved in the recruitment of inflammatory cells across the blood-brain barrier in experimental autoimmune encephalomyelitis." *Blood* no. 90 (11):4459-72.

Erickson, M. A., Y. Morofuji, J. B. Owen, and W. A. Banks. 2014. "Rapid transport of CCL11 across the blood-brain barrier: regional variation and importance of blood cells." *J Pharmacol Exp Ther* no. 349 (3):497-507. doi: 10.1124/jpet.114.213074.

Eugenin, E. A., D. Basilio, J. C. Saez, J. A. Orellana, C. S. Raine, F. Bukauskas, M. V. Bennett, and J. W. Berman. 2012. "The role of gap junction channels during physiologic and pathologic conditions of the human central nervous system." *J Neuroimmune Pharmacol* no. 7 (3):499-518. doi: 10.1007/s11481-012-9352-5.

Fish, J. E., and J. D. Wythe. 2015. "The molecular regulation of arteriovenous specification and maintenance." *Dev Dyn* no. 244 (3):391-409. doi: 10.1002/dvdy.24252.

Frank, R. N., S. Dutta, and M. A. Mancini. 1987. "Pericyte coverage is greater in the retinal than in the cerebral capillaries of the rat." *Invest Ophthalmol Vis Sci* no. 28 (7):1086-91.

Furuse, M., M. Itoh, T. Hirase, A. Nagafuchi, S. Yonemura, S. Tsukita, and S. Tsukita. 1994. "Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions." *J Cell Biol* no. 127 (6 Pt 1):1617-26.

Gazzin, S., A. L. Berengeno, N. Strazielle, F. Fazzari, A. Raseni, J. D. Ostrow, R. Wennberg, J. F. Ghersi-Egea, and C. Tiribelli. 2011. "Modulation of Mrp1 (ABCc1) and Pgp (ABCb1) by Bilirubin at the Blood-CSF and Blood-Brain Barriers in the Gunn Rat." *PLoS One* no. 6 (1):e16165. doi: 10.1371/journal.pone.0016165.

Ge, S., L. Song, and J. S. Pachter. 2005. "Where is the blood-brain barrier ... really?" *J Neurosci Res* no. 79 (4):421-7. doi: 10.1002/jnr.20313.

Gomez-Zepeda, D., M. Taghi, M. Smirnova, P. Sergent, W. Q. Liu, C. Chhuon, M. Vidal, M. Picard, E. Thioulouse, I. Broutin, I. C. Guerrera, J. M. Scherrmann, Y. Parmentier, X. Decleves, and M. C. Menet. 2019. "LC-MS/MS-based quantification of efflux transporter proteins at the BBB." *J Pharm Biomed Anal* no. 164:496-508. doi: 10.1016/j.jpba.2018.11.013.

Gumbiner, B. M. 2005. "Regulation of cadherin-mediated adhesion in morphogenesis." *Nat Rev Mol Cell Biol* no. 6 (8):622-34. doi: 10.1038/nrm1699.

Herve, F., N. Ghinea, and J. M. Scherrmann. 2008. "CNS delivery via adsorptive transcytosis." *AAPS J* no. 10 (3):455-72. doi: 10.1208/s12248-008-9055-2.

Hooijmans, C. R., C. Graven, P. J. Dederen, H. Tanila, T. van Groen, and A. J. Kiliaan. 2007. "Amyloid beta deposition is related to decreased glucose transporter-1 levels and hippocampal atrophy in brains of aged APP/PS1 mice." *Brain Res* no. 1181:93-103. doi: S0006-8993(07)02043-4 [pii] 10.1016/j.brainres.2007.08.063.

Huang, R. B., A. L. Gonzalez, and O. Eniola-Adefeso. 2013. "Laminar shear stress elicit distinct endothelial cell E-selectin expression pattern via TNFalpha and IL-1beta activation." *Biotechnol Bioeng* no. 110 (3):999-1003. doi: 10.1002/bit.24746.

Ikezu, T., H. Ueda, B. D. Trapp, K. Nishiyama, J. F. Sha, D. Volonte, F. Galbiati, A. L. Byrd, G. Bassell, H. Serizawa, W. S. Lane, M. P. Lisanti, and T. Okamoto. 1998. "Affinity-purification and characterization of caveolins from the brain: differential expression of caveolin-1, -2, and -3 in brain endothelial and astroglial cell types." *Brain Res* no. 804 (2):177-92.

Jablonski, M. R., S. S. Markandaiah, D. Jacob, N. J. Meng, K. Li, V. Gennaro, A. C. Lepore, D. Trotti, and P. Pasinelli. 2014. "Inhibiting drug efflux transporters improves efficacy of ALS therapeutics." *Ann Clin Transl Neurol* no. 1 (12):996-1005. doi: 10.1002/acn3.141.

Jambusaria, A., J. Klomp, Z. Hong, S. Rafii, Y. Dai, A. B. Malik, and J. Rehman. 2018. "A computational approach to identify cellular heterogeneity and tissue-specific gene regulatory networks." *BMC Bioinformatics* no. 19 (1):217. doi: 10.1186/s12859-018-2190-6.

Jesaitis, L. A., and D. A. Goodenough. 1994. "Molecular characterization and tissue distribution of ZO-2, a tight junction protein homologous to ZO-1 and the Drosophila discs-large tumor suppressor protein." *J Cell Biol* no. 124 (6):949-61.

Jiao, H., Z. Wang, Y. Liu, P. Wang, and Y. Xue. 2011. "Specific role of tight junction proteins claudin-5, occludin, and ZO-1 of the blood-

brain barrier in a focal cerebral ischemic insult." *J Mol Neurosci* no. 44 (2):130-9. doi: 10.1007/s12031-011-9496-4.

Kook, S. Y., H. S. Hong, M. Moon, C. M. Ha, S. Chang, and I. Mook-Jung. 2012. "Abeta(1)(-)(4)(2)-RAGE interaction disrupts tight junctions of the blood-brain barrier via Ca(2)(+)-calcineurin signaling." *J Neurosci* no. 32 (26):8845-54. doi: 10.1523/JNEUROSCI.6102-11.2012.

Kronstein, R., J. Seebach, S. Grossklaus, C. Minten, B. Engelhardt, M. Drab, S. Liebner, Y. Arsenijevic, A. A. Taha, T. Afanasieva, and H. J. Schnittler. 2012. "Caveolin-1 opens endothelial cell junctions by targeting catenins." *Cardiovasc Res* no. 93 (1):130-40. doi: cvr256 [pii] 10.1093/cvr/cvr256.

Krug, S. M., S. Amasheh, J. F. Richter, S. Milatz, D. Gunzel, J. K. Westphal, O. Huber, J. D. Schulzke, and M. Fromm. 2009. "Tricellulin forms a barrier to macromolecules in tricellular tight junctions without affecting ion permeability." *Mol Biol Cell* no. 20 (16):3713-24. doi: E09-01-0080 [pii] 10.1091/mbc.E09-01-0080.

Lacaz-Vieira, F., M. M. Jaeger, P. Farshori, and B. Kachar. 1999. "Small synthetic peptides homologous to segments of the first external loop of occludin impair tight junction resealing." *J Membr Biol* no. 168 (3):289-97.

Lajoie, J. M., and E. V. Shusta. 2015. "Targeting receptor-mediated transport for delivery of biologics across the blood-brain barrier." *Annu Rev Pharmacol Toxicol* no. 55:613-31. doi: 10.1146/annurev-pharmtox-010814-124852.

Lee, S. J., S. Kwon, J. R. Gatti, E. Korcari, T. E. Gresser, P. C. Felix, S. G. Keep, K. C. Pasquale, T. Bai, S. A. Blanchett-Anderson, N. W. Wu, C. Obeng-Nyarko, K. M. Senagbe, K. C. Young, S. Maripudi, B. C. Yalavarthi, D. Korcari, A. Y. Liu, B. C. Schaffler, R. F. Keep, and M. M. Wang. 2017. "Large-scale identification of human cerebrovascular proteins: Inter-tissue and intracerebral vascular protein diversity." *PLoS One* no. 12 (11):e0188540. doi: 10.1371/journal.pone.0188540.

Li, Y., Q. Wu, C. Li, L. Liu, K. Du, J. Shen, Y. Wu, X. Zhao, M. Zhao, L. Bao, J. Gao, R. F. Keep, and J. Xiang. 2016. "Role of Human Breast Cancer Related Protein versus P-Glycoprotein as an Efflux Transporter for Benzylpenicillin: Potential Importance at the Blood-Brain Barrier." *PLoS One* no. 11 (6):e0157576. doi: 10.1371/journal.pone.0157576.

Liebner, S., M. Corada, T. Bangsow, J. Babbage, A. Taddei, C. J. Czupalla, M. Reis, A. Felici, H. Wolburg, M. Fruttiger, M. M. Taketo, H. von Melchner, K. H. Plate, H. Gerhardt, and E. Dejana. 2008.

"Wnt/β-catenin signaling controls development of the blood-brain barrier." *J Cell Biol* no. 183 (3):409-17. doi: jcb.200806024 [pii] 10.1083/jcb.200806024.

Liu, P., R. Zhang, D. Liu, J. Wang, C. Yuan, X. Zhao, Y. Li, X. Ji, T. Chi, and L. Zou. 2018. "Time-course investigation of blood-brain barrier permeability and tight junction protein changes in a rat model of permanent focal ischemia." *J Physiol Sci* no. 68 (2):121-127. doi: 10.1007/s12576-016-0516-6.

Lu, W. 2012. "Adsorptive-mediated brain delivery systems." *Curr Pharm Biotechnol* no. 13 (12):2340-8.

Mariano, C., I. Palmela, P. Pereira, A. Fernandes, A. S. Falcao, F. L. Cardoso, A. R. Vaz, A. R. Campos, A. Goncalves-Ferreira, K. S. Kim, D. Brites, and M. A. Brito. 2013. "Tricellulin expression in brain endothelial and neural cells." *Cell Tissue Res* no. 351 (3):397-407. doi: 10.1007/s00441-012-1529-y.

Mariano, C., H. Sasaki, D. Brites, and M. A. Brito. 2011. "A look at tricellulin and its role in tight junction formation and maintenance." *Eur J Cell Biol* no. 90 (10):787-96. doi: 10.1016/j.ejcb.2011.06.005 S0171-9335(11)00127-0 [pii].

Martin-Padura, I., S. Lostaglio, M. Schneemann, L. Williams, M. Romano, P. Fruscella, C. Panzeri, A. Stoppacciaro, L. Ruco, A. Villa, D. Simmons, and E. Dejana. 1998. "Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration." *J Cell Biol* no. 142 (1):117-27.

Matsumoto, J., T. Stewart, L. Sheng, N. Li, K. Bullock, N. Song, M. Shi, W. A. Banks, and J. Zhang. 2017. "Transmission of alpha-synuclein-containing erythrocyte-derived extracellular vesicles across the blood-brain barrier via adsorptive mediated transcytosis: another mechanism for initiation and progression of Parkinson's disease?" *Acta Neuropathol Commun* no. 5 (1):71. doi: 10.1186/s40478-017-0470-4.

Mineta, K., Y. Yamamoto, Y. Yamazaki, H. Tanaka, Y. Tada, K. Saito, A. Tamura, M. Igarashi, T. Endo, K. Takeuchi, and S. Tsukita. 2011. "Predicted expansion of the claudin multigene family." *FEBS Lett* no. 585 (4):606-12. doi: S0014-5793(11)00058-5 [pii] 10.1016/j.febslet.2011.01.028.

Moinuddin, A., J. E. Morley, and W. A. Banks. 2000. "Regional variations in the transport of interleukin-1alpha across the blood-brain barrier in ICR and aging SAMP8 mice." *Neuroimmunomodulation* no. 8 (4):165-70. doi: 10.1159/000054814.

Montagne, A., S. R. Barnes, M. D. Sweeney, M. R. Halliday, A. P. Sagare, Z. Zhao, A. W. Toga, R. E. Jacobs, C. Y. Liu, L. Amezcua, M. G. Harrington, H. C. Chui, M. Law, and B. V. Zlokovic. 2015. "Blood-brain barrier breakdown in the aging human hippocampus." *Neuron* no. 85 (2):296-302. doi: 10.1016/j.neuron.2014.12.032 S0896-6273(14)01141-6 [pii].

Morita, S., E. Furube, T. Mannari, H. Okuda, K. Tatsumi, A. Wanaka, and S. Miyata. 2016. "Heterogeneous vascular permeability and alternative diffusion barrier in sensory circumventricular organs of adult mouse brain." *Cell Tissue Res* no. 363 (2):497-511. doi: 10.1007/s00441-015-2207-7.

Morris, M. E., V. Rodriguez-Cruz, and M. A. Felmlee. 2017. "SLC and ABC Transporters: Expression, Localization, and Species Differences at the Blood-Brain and the Blood-Cerebrospinal Fluid Barriers." *AAPS J* no. 19 (5):1317-1331. doi: 10.1208/s12248-017-0110-8.

Moura, R. P., C. Martins, S. Pinto, F. Sousa, and B. Sarmento. 2019. "Blood-brain barrier receptors and transporters: an insight on their function and how to exploit them through nanotechnology." *Expert Opin Drug Deliv* no. 16 (3):271-285. doi: 10.1080/17425247.2019.1583205.

Muller, W. A. 2013. "Getting leukocytes to the site of inflammation." *Vet Pathol* no. 50 (1):7-22. doi: 10.1177/0300985812469883.

Nag, S., R. Venugopalan, and D. J. Stewart. 2007. "Increased caveolin-1 expression precedes decreased expression of occludin and claudin-5 during blood-brain barrier breakdown." *Acta Neuropathol* no. 114 (5):459-69. doi: 10.1007/s00401-007-0274-x.

Nasdala, I., K. Wolburg-Buchholz, H. Wolburg, A. Kuhn, K. Ebnet, G. Brachtendorf, U. Samulowitz, B. Kuster, B. Engelhardt, D. Vestweber, and S. Butz. 2002. "A transmembrane tight junction protein selectively expressed on endothelial cells and platelets." *J Biol Chem* no. 277 (18):16294-303. doi: 10.1074/jbc.M111999200.

Niessen, C. M., and C. J. Gottardi. 2008. "Molecular components of the adherens junction." *Biochim Biophys Acta* no. 1778 (3):562-71. doi: 10.1016/j.bbamem.2007.12.015.

Nishimura, T., and M. Takeichi. 2009. "Remodeling of the adherens junctions during morphogenesis." *Curr Top Dev Biol* no. 89:33-54. doi: 10.1016/S0070-2153(09)89002-9.

Ohtsuki, S., and T. Terasaki. 2007. "Contribution of carrier-mediated transport systems to the blood-brain barrier as a supporting and protecting interface for the brain; importance for CNS drug discovery and development." *Pharm Res* no. 24 (9):1745-58.

doi: 10.1007/s11095-007-9374-5.

On, N. H., and D. W. Miller. 2014. "Transporter-based delivery of anticancer drugs to the brain: improving brain penetration by minimizing drug efflux at the blood-brain barrier." *Curr Pharm Des* no. 20 (10):1499-509.

Osada, T., Y. H. Gu, M. Kanazawa, Y. Tsubota, B. T. Hawkins, M. Spatz, R. Milner, and G. J. del Zoppo. 2011. "Interendothelial claudin-5 expression depends on cerebral endothelial cell-matrix adhesion by beta(1)-integrins." *J Cereb Blood Flow Metab* no. 31 (10):1972-85. doi: 10.1038/jcbfm.2011.99.

Palmela, I., H. Sasaki, F. L. Cardoso, M. Moutinho, K. S. Kim, D. Brites, and M. A. Brito. 2012. "Time-dependent dual effects of high levels of unconjugated bilirubin on the human blood-brain barrier lining." *Front Cell Neurosci* no. 6:22. doi: 10.3389/fncel.2012.00022.

Pardridge, W. M. 2006. "Molecular Trojan horses for blood-brain barrier drug delivery." *Curr Opin Pharmacol* no. 6 (5):494-500. doi: S1471-4892(06)00117-2 [pii] 10.1016/j.coph.2006.06.001.

Park, D. S., S. E. Woodman, W. Schubert, A. W. Cohen, P. G. Frank, M. Chandra, J. Shirani, B. Razani, B. Tang, L. A. Jelicks, S. M. Factor, L. M. Weiss, H. B. Tanowitz, and M. P. Lisanti. 2002. "Caveolin-1/3 double-knockout mice are viable, but lack both muscle and non-muscle caveolae, and develop a severe cardiomyopathic phenotype." *Am J Pathol* no. 160 (6):2207-17. doi: 10.1016/S0002-9440(10)61168-6.

Parolini, I., M. Sargiacomo, F. Galbiati, G. Rizzo, F. Grignani, J. A. Engelman, T. Okamoto, T. Ikezu, P. E. Scherer, R. Mora, E. Rodriguez-Boulan, C. Peschle, and M. P. Lisanti. 1999. "Expression of caveolin-1 is required for the transport of caveolin-2 to the plasma membrane. Retention of caveolin-2 at the level of the golgi complex." *J Biol Chem* no. 274 (36):25718-25.

Patching, S. G. 2017. "Glucose Transporters at the Blood-Brain Barrier: Function, Regulation and Gateways for Drug Delivery." *Mol Neurobiol* no. 54 (2):1046-1077. doi: 10.1007/s12035-015-9672-6.

Paterson, J., and C. I. Webster. 2016. "Exploiting transferrin receptor for delivering drugs across the blood-brain barrier." *Drug Discov Today Technol* no. 20:49-52. doi: 10.1016/j.ddtec.2016.07.009.

Paulus, W., I. Baur, D. Schuppan, and W. Roggendorf. 1993. "Characterization of integrin receptors in normal and neoplastic human brain." *Am J Pathol* no. 143 (1):154-63.

Pokutta, S., and W. I. Weis. 2000. "Structure of the dimerization and beta-catenin-binding region of alpha-catenin." *Mol Cell* no. 5 (3):533-43.

Predescu, S. A., D. N. Predescu, and A. B. Malik. 2007. "Molecular determinants of endothelial transcytosis and their role in endothelial permeability." *Am J Physiol Lung Cell Mol Physiol* no. 293 (4):L823-42. doi: 10.1152/ajplung.00436.2006.

Pulgar, V. M. 2018. "Transcytosis to Cross the Blood Brain Barrier, New Advancements and Challenges." *Front Neurosci* no. 12:1019. doi: 10.3389/fnins.2018.01019.

Qosa, H., D. S. Miller, P. Pasinelli, and D. Trotti. 2015. "Regulation of ABC efflux transporters at blood-brain barrier in health and neurological disorders." *Brain Res* no. 1628 (Pt B):298-316. doi: 10.1016/j.brainres.2015.07.005.

Roberts, L. M., D. S. Black, C. Raman, K. Woodford, M. Zhou, J. E. Haggerty, A. T. Yan, S. E. Cwirla, and K. K. Grindstaff. 2008. "Subcellular localization of transporters along the rat blood-brain barrier and blood-cerebral-spinal fluid barrier by in vivo biotinylation." *Neuroscience* no. 155 (2):423-38. doi: 10.1016/j.neuroscience.2008.06.015.

Sá-Pereira, I., D. Brites, and M.A. Brito. 2012. "Neurovascular unit: a focus on pericytes." *Mol Neurobiol* no. 45 (2):327-47.

Sagare, A. P., R. D. Bell, A. Srivastava, J. D. Sengillo, I. Singh, Y. Nishida, N. Chow, and B. V. Zlokovic. 2013. "A lipoprotein receptor cluster IV mutant preferentially binds amyloid-beta and regulates its clearance from the mouse brain." *J Biol Chem* no. 288 (21):15154-66. doi: 10.1074/jbc.M112.439570.

Saitou, M., M. Furuse, H. Sasaki, J. D. Schulzke, M. Fromm, H. Takano, T. Noda, and S. Tsukita. 2000. "Complex phenotype of mice lacking occludin, a component of tight junction strands." *Mol Biol Cell* no. 11 (12):4131-42.

Saubamea, B., V. Cochois-Guegan, S. Cisternino, and J. M. Scherrmann. 2012. "Heterogeneity in the rat brain vasculature revealed by quantitative confocal analysis of endothelial barrier antigen and P-glycoprotein expression." *J Cereb Blood Flow Metab* no. 32 (1):81-92. doi: 10.1038/jcbfm.2011.109.

Shen, S., and W. Zhang. 2010. "ABC transporters and drug efflux at the blood-brain barrier." *Rev Neurosci* no. 21 (1):29-53.

Shen, W., S. Li, S. H. Chung, L. Zhu, J. Stayt, T. Su, P. O. Couraud, I. A. Romero, B. Weksler, and M. C. Gillies. 2011. "Tyrosine phosphorylation of VE-cadherin and claudin-5 is associated with TGF-beta1-induced permeability of centrally derived vascular endothelium." *Eur J Cell Biol* no. 90 (4):323-32. doi: 10.1016/j.ejcb.2010.10.013.

Shibata, M., S. Yamada, S. R. Kumar, M. Calero, J. Bading, B. Frangione, D. M. Holtzman, C. A. Miller, D. K. Strickland, J. Ghiso, and B. V. Zlokovic. 2000. "Clearance of Alzheimer's amyloid-ss(1-40) peptide from brain by LDL receptor-related protein-1 at the blood-brain barrier." *J Clin Invest* no. 106 (12):1489-99. doi: 10.1172/JCI10498.

Silverberg, G. D., A. A. Messier, M. C. Miller, J. T. Machan, S. S. Majmudar, E. G. Stopa, J. E. Donahue, and C. E. Johanson. 2010. "Amyloid efflux transporter expression at the blood-brain barrier declines in normal aging." *J Neuropathol Exp Neurol* no. 69 (10):1034-43. doi: 10.1097/NEN.0b013e3181f46e25.

Silverberg, G. D., M. C. Miller, A. A. Messier, S. Majmudar, J. T. Machan, J. E. Donahue, E. G. Stopa, and C. E. Johanson. 2010. "Amyloid deposition and influx transporter expression at the blood-brain barrier increase in normal aging." *J Neuropathol Exp Neurol* no. 69 (1):98-108. doi: 10.1097/NEN.0b013e3181c8ad2f.

Stamatovic, S. M., A. M. Johnson, R. F. Keep, and A. V. Andjelkovic. 2016. "Junctional proteins of the blood-brain barrier: New insights into function and dysfunction." *Tissue Barriers* no. 4 (1):e1154641. doi: 10.1080/21688370.2016.1154641.

Stan, R. V. 2005. "Structure of caveolae." *Biochim Biophys Acta* no. 1746 (3):334-48. doi: 10.1016/j.bbamcr.2005.08.008.

Sweeney, M. D., Z. Zhao, A. Montagne, A. R. Nelson, and B. V. Zlokovic. 2019. "Blood-Brain Barrier: From Physiology to Disease and Back." *Physiol Rev* no. 99 (1):21-78. doi: 10.1152/physrev.00050.2017.

Taddei, A., C. Giampietro, A. Conti, F. Orsenigo, F. Breviario, V. Pirazzoli, M. Potente, C. Daly, S. Dimmeler, and E. Dejana. 2008. "Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5." *Nat Cell Biol* no. 10 (8):923-34. doi: ncb1752 [pii] 10.1038/ncb1752.

Tagaya, M., H. P. Haring, I. Stuiver, S. Wagner, T. Abumiya, J. Lucero, P. Lee, B. R. Copeland, D. Seiffert, and G. J. del Zoppo. 2001. "Rapid loss of microvascular integrin expression during focal brain ischemia reflects neuron injury." *J Cereb Blood Flow Metab* no. 21 (7):835-46. doi: 10.1097/00004647-200107000-00009.

Tai, L. M., P. S. Reddy, M. A. Lopez-Ramirez, H. A. Davies, D. K. Male, A. J. Loughlin, and I. A. Romero. 2009. "Polarized P-glycoprotein expression by the immortalised human brain endothelial cell line, hCMEC/D3, restricts apical-to-basolateral permeability to rhodamine 123." *Brain Res* no. 1292:14-24. doi: 10.1016/j.brainres.2009.07.039.

van Buul, J. D., F. P. van Alphen, and P. L. Hordijk. 2009. "The presence of alpha-catenin in the VE-cadherin complex is required for efficient transendothelial migration of leukocytes." *Int J Biol Sci* no. 5 (7):695-705.

Van Itallie, C. M., A. S. Fanning, A. Bridges, and J. M. Anderson. 2009. "ZO-1 stabilizes the tight junction solute barrier through coupling to the perijunctional cytoskeleton." *Mol Biol Cell* no. 20 (17):3930-40. doi: 10.1091/mbc.E09-04-0320.

van Vliet, E. A., W. M. Otte, J. A. Gorter, R. M. Dijkhuizen, and W. J. Wadman. 2014. "Longitudinal assessment of blood-brain barrier leakage during epileptogenesis in rats. A quantitative MRI study." *Neurobiol Dis* no. 63:74-84. doi: 10.1016/j.nbd.2013.11.019.

Videira, M., R. L. Reis, and M. A. Brito. 2014. "Deconstructing breast cancer cell biology and the mechanisms of multidrug resistance." *Biochim Biophys Acta* no. 1846 (2):312-325. doi: S0304-419X(14)00064-X [pii] 10.1016/j.bbcan.2014.07.011.

Villasenor, R., B. Kuennecke, L. Ozmen, M. Ammann, C. Kugler, F. Gruninger, H. Loetscher, P. O. Freskgard, and L. Collin. 2017. "Region-specific permeability of the blood-brain barrier upon pericyte loss." *J Cereb Blood Flow Metab* no. 37 (12):3683-3694. doi: 10.1177/0271678X17697340.

Virgintino, D., D. Robertson, M. Errede, V. Benagiano, M. Bertossi, G. Ambrosi, and L. Roncali. 2001. "Expression of the gap junction protein connexin43 in human telencephalon microvessels." *Microvasc Res* no. 62 (3):435-9. doi: 10.1006/mvre.2001.2345.

Vorbrodt, A. W. 1987. "Demonstration of anionic sites on the luminal and abluminal fronts of endothelial cells with poly-L-lysine-gold complex." *J Histochem Cytochem* no. 35 (11):1261-6. doi: 10.1177/35.11.3655325.

Wang, J., and T. A. Springer. 1998. "Structural specializations of immunoglobulin superfamily members for adhesion to integrins and viruses." *Immunol Rev* no. 163:197-215.

Washington, R., J. Burton, R. F. Todd, 3rd, W. Newman, L. Dragovic, and P. Dore-Duffy. 1994. "Expression of immunologically relevant endothelial cell activation antigens on isolated central nervous system microvessels from patients with multiple sclerosis." *Ann Neurol* no. 35 (1):89-97. doi: 10.1002/ana.410350114.

Wegmann, F., B. Petri, A. G. Khandoga, C. Moser, A. Khandoga, S. Volkery, H. Li, I. Nasdala, O. Brandau, R. Fassler, S. Butz, F. Krombach, and D. Vestweber. 2006. "ESAM supports neutrophil

extravasation, activation of Rho, and VEGF-induced vascular permeability." *J Exp Med* no. 203 (7):1671-7. doi: 10.1084/jem.20060565.

Wilhelm, I., A. Nyul-Toth, M. Suciu, A. Hermenean, and I. A. Krizbai. 2016. "Heterogeneity of the blood-brain barrier." *Tissue Barriers* no. 4 (1):e1143544. doi: 10.1080/21688370.2016.1143544.

Wimmer, I., S. Tietz, H. Nishihara, U. Deutsch, F. Sallusto, F. Gosselet, R. Lyck, W. A. Muller, H. Lassmann, and B. Engelhardt. 2019. "PECAM-1 Stabilizes Blood-Brain Barrier Integrity and Favors Paracellular T-Cell Diapedesis Across the Blood-Brain Barrier During Neuroinflammation." *Front Immunol* no. 10:711. doi: 10.3389/fimmu.2019.00711.

Wong, D., and K. Dorovini-Zis. 1996. "Regualtion by cytokines and lipopolysaccharide of E-selectin expression by human brain microvessel endothelial cells in primary culture." *J Neuropathol Exp Neurol* no. 55 (2):225-35.

Wylezinski, L. S., and J. Hawiger. 2016. "Interleukin 2 Activates Brain Microvascular Endothelial Cells Resulting in Destabilization of Adherens Junctions." *J Biol Chem* no. 291 (44):22913-22923. doi: 10.1074/jbc.M116.729038.

Yilmaz, G., S. Vital, C. E. Yilmaz, K. Y. Stokes, J. S. Alexander, and D. N. Granger. 2011. "Selectin-mediated recruitment of bone marrow stromal cells in the postischemic cerebral microvasculature." *Stroke* no. 42 (3):806-11. doi: 10.1161/STROKEAHA.110.597088.

Yoshida, M., W. F. Westlin, N. Wang, D. E. Ingber, A. Rosenzweig, N. Resnick, and M. A. Gimbrone, Jr. 1996. "Leukocyte adhesion to vascular endothelium induces E-selectin linkage to the actin cytoskeleton." *J Cell Biol* no. 133 (2):445-55.

Zhang, R., M. Chopp, Z. Zhang, N. Jiang, and C. Powers. 1998. "The expression of P- and E-selectins in three models of middle cerebral artery occlusion." *Brain Res* no. 785 (2):207-14.

Zhong, Y., E. J. Smart, B. Weksler, P. O. Couraud, B. Hennig, and M. Toborek. 2008. "Caveolin-1 regulates human immunodeficiency virus-1 Tat-induced alterations of tight junction protein expression via modulation of the Ras signaling." *J Neurosci* no. 28 (31):7788-96. doi: 28/31/7788 [pii] 10.1523/JNEUROSCI.0061-08.2008.

Zhong, Y., B. Zhang, S. Y. Eum, and M. Toborek. 2012. "HIV-1 Tat triggers nuclear localization of ZO-1 via Rho signaling and cAMP response element-binding protein activation." *J Neurosci* no. 32 (1):143-50. doi: 10.1523/JNEUROSCI.4266-11.2012.

CHAPTER TWO

NEUROGENESIS AND THE BRAIN VASCULATURE

LILIANA BERNARDINO
AND RAQUEL FERREIRA

Introduction

Neurogenesis, the formation of new neurons from neural stem cells (NSC), and the formation of new blood vessels are closely connected processes. Endothelial cells constitute the inner cellular lining of vessels and are highly metabolically active units required for key structural functions. In particular, brain endothelial cells display a series of traits that enable them to provide oxygen, nutrients and other cues to all surrounding cellular types present in the brain parenchyma without compromising their immune privilege (Aird 2012). Brain endothelial cells typically present complex tight junctions between them, higher resistance, a lower number of pinocytic vesicles, and higher amounts of energy-producing mitochondria than the peripheral endothelium, rendering them more impermeable and responsive (Rizzo and Leaver 2010). There are a few exceptions, considering the close connection between brain endothelial cells and NSC in the neurogenic niches. Within these niches, blood vessels display unique characteristics such as a lack of tight junctions between endothelial cells and reduced mural cell coverage of the blood vessels in proximity to NSC, allowing more direct contact with the circulation. Additionally, blood vessels can be used as migrating scaffolds by neuroblasts, i.e. immature neurons, in a process known as vasophilic migration (Bovetti et al. 2007). This migration mode occurring in a physiological and pathological context was reviewed recently (Segarra, Kirchmaier, and Acker-Palmer 2015). Evidence suggests that both endothelial cell-secreted factors and contact-dependent signaling, as well as blood-borne molecules, contribute to the ability of NSC to either

maintain their quiescent state, to proliferate or to regenerate (Rosa et al. 2016). Conversely, neural circuits regulate vessel patterning and blood vessel growth, the first event in the development of the central nervous system vasculature, termed vasculogenesis. The formation of new capillary sprouts from pre-existing blood vessels, or angiogenesis, is another important mechanism that involves endothelial cell proliferation and migration, degradation of extracellular matrix and tube formation (Distler et al. 2003). It is mainly regulated by environmental cues such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF) and angiopoietins (Ang)-1 and -2 (Rundhaug 2005, Distler et al. 2003, Saito et al. 2007).

In sum, the cerebral endothelium plays a critical role in neurogenesis during development and in the adult brain. This chapter will first review the neurogenic process and later discuss its interplay with blood vessels.

Neurogenesis

NSC are multipotent self-renewing cells from which neurons are generated, in a process that involves multiple signaling cascades. Neurogenesis occurs throughout the embryonic and adult stages of life and for diverse purposes, such as neuronal replenishment after injury.

Developmental neurogenesis is a dynamic but highly controlled process that begins approximately around the 5th gestational week with neuroblast proliferation and expansion. Neuroblasts then gradually transform into radial glial that later divide asymmetrically into radial glial cells and neural progenitor cells, which in turn give rise to post-mitotic immature neurons that will integrate local neuronal circuits (Urban and Guillemot 2014). Embryonic neurogenesis is regulated by genetic, environmental and epigenetic cues that start to decline by the early postnatal stage.

In the adult mammalian brain, neurogenesis occurs in two main neurogenic niches: the subventricular zone lining the lateral ventricles and the subgranular zone of the hippocampus, a thin cellular layer between the granule cell layer and hilus of the dentate gyrus. This endogenous regenerative process has been extensively studied in rodents and, despite recent controversies, evidence has shown that it also occurs in humans (Altman and Das 1965, Altman 1969, Eriksson et al. 1998, Spalding et al. 2013, Sorrells et al. 2018, Boldrini et al. 2018). The rodent subventricular zone contains three main cell types, in which type A cells form tangentially oriented chains surrounded by type B cells and by scattered clusters of type C cells. NSC, also called B1 cells, are quiescent cells that

can re-enter the cell cycle upon stimulation. Type B2 are considered niche astrocytes. Activated B cells proliferate and give rise to highly proliferative type C transit-amplifying progenitors. Type C cells differentiate into type A neuroblasts, i.e. immature neurons, that migrate long distances along the rostral migratory stream towards the granular cell layer and the periglomerular layer of the olfactory bulb where they then differentiate into inhibitory interneurons (Doetsch, Garcia-Verdugo, and Alvarez-Buylla 1997).

In short, the integration of adult-born neurons into the olfactory bulb circuitry of rodents contributes to neural plasticity of olfactory information processing and pattern discrimination. Instead, in the infant human brain under 18 months of age, the subventricular zone produces new neurons destined for the olfactory bulb and the prefrontal cortex, which is responsible for determining our cognitive and social behavior (Sanai et al., 2011). To date, no rostral migratory stream and no addition of new neurons has been found in the adult human olfactory bulb, a structure which is less physiologically relevant to humans than to rodents. Nevertheless, some do not rule out this possibility. In one study, the olfactory bulbs from a patient submitted to tumor resection and three olfactory bulbs from post-mortem individuals were removed for gene expression analysis. Approximately 20% of genes found in these tissues were related to nervous system development, leading authors to suggest that although these genes could be performing non-neurogenetic functions, they could more likely be supporting neuronal development in the adult olfactory bulb (Lotsch et al. 2014). In the adult, subventricular zone-derived new neurons migrate to the human striatum (Emst et al., 2014). In the subgranular zone, NSC, also called radial glia-like cells, are slowly dividing cells that give rise to rapidly dividing cells (transient amplifying progenitors or TAP). These progenitors, found predominantly in the subgranular zone and outer hilus, differentiate into newly born neurons that migrate short distances to the dentate gyrus granule cell layer. The generation and functional integration of new neurons in the dentate gyrus is essential for memory and learning processes.

Many of these processes occur in the vicinity of blood vessels within a specialized neurovascular niche. Moreover, several studies support reciprocal signaling between endothelial cells and NSC, both before and throughout their maturation. Therefore, it is of utmost importance to investigate this crosstalk, both in normal and injured brains. This knowledge is crucial to devise effective approaches for brain repair.

The brain vasculature

Mesoderm precursor-derived hemangioblasts are multipotent precursor cells that can give rise to hematopoietic stem cells and endothelial progenitor cells (angioblasts), which in turn differentiate respectively into blood circulating cells and endothelial cells that constitute the building blocks of a primitive vascular network. Vasculogenesis has been detected in the mouse around embryonic day 7.5 (Risau and Flamme 1995, Sabin 2002) and, three days later, blood is already circulating in the systemic vascular bed (reviewed by (Yoder 2012)). This process is mainly regulated by VEGF and by interactions with the extracellular matrix; it was first described by Risau (Risau 1997). After the formation of the primary vascular plexus, a growing number of vessels continue to be formed and shaped into a functional circulatory system. The generation of new capillaries extending from pre-existing blood vessels is termed angiogenesis. These events are modulated by several cues such as transcription and growth factors, adhesion molecules like vascular endothelial (VE)-cadherin and platelet endothelial cell adhesion molecule 1 (PECAM-1), and mechanical forces (Risau and Flamme 1995). Both processes can occur simultaneously. Initially, vasculogenesis was thought to occur only in the embryonic period, but several reports have detected it also in adults, particularly in response to a pathological event (e.g. tumor growth, neovascularization after injury). Overall, vasculogenesis and angiogenesis are complex processes, especially when they are required to occur in the brain and to ensure the maintenance of a blood-brain barrier. The morphological and functional properties of brain endothelial cells offer the ability to provide the central nervous system with nutrients and oxygen while protecting it from blood circulating neurotoxic substances and/or microorganisms (Wolburg and Lippoldt 2002, Wilhelm et al. 2016). Hence, blood-brain barrier injury or impairment can have serious consequences and there are several clinical trials testing the correlation between neuroinflammation and vascular dysfunction in brain disorders such as Alzheimer's or Parkinson's disease (Machado-Pereira et al. 2017). Vascular injury can trigger different repair mechanisms depending on the type and extent of the lesion. For instance, denudation injury is mostly resolved by the migration and proliferation of neighboring endothelial cells (Schwartz and Benditt 1976). If the basement membrane and/or larger portions of the blood vessel wall are damaged, endothelial progenitor cells are recruited from the bone marrow to secure vascular regeneration. In animal models of injury, post-ischemia forelimb strength was significantly improved in animals receiving endothelial progenitor

cells or their conditioned media, indicating the importance of their secretome for repair (Rosell et al. 2013). These cells were first reported by Asahara and colleagues (Asahara et al. 1997) and have been the subject of some controversy regarding the most appropriate method of isolation and the use of unequivocal cell surface markers for their identification; most groups define endothelial progenitor cells as CD34+/KDR+/CD133+ cells (reviewed by (Yoder 2012)).

Crosstalk between normal angiogenesis and neurogenic niches

The cytoarchitecture and dynamics of the subventricular zone vasculature are unique when compared with other cerebral vessels, including the vasculature found in the subgranular zone. Indeed, activated type B cells and the transit-amplifying C cells are highly associated with vascular regions devoid of either glial end-feet or pericytes. These traits are consistent with a leaky blood-brain barrier. Type B1 cells have also been found to extend processes that can contact striatal vessels (Lacar et al. 2011, Mirzadeh et al. 2008). Moreover, the subventricular zone niche blood vessels lack tight junctions between endothelial cells, further reinforcing the concept of a more permissive barrier. Migrating type A neuroblasts in the rostral migratory stream run along with aligned blood vessels, but most of them are not as close to the endothelial surface as dividing type B and C cells (Tavazoie et al. 2008, Shen et al. 2008, Snapyan et al. 2009). Indeed, the vascular network runs along the rostral migratory stream in a parallel orientation, in part dictated by astrocyte-derived VEGF, which allows tangential migration of neuroblasts to the olfactory bulb (Licht and Keshet 2015, Bozoyan, Khlghatyan, and Saghatelyan 2012). The radial migration of neuroblasts occurring in the olfactory bulb is also supported by the local vasculature (Bovetti et al. 2007). Khlghatyan and Saghatelyan have detailed a protocol that enables the identification of neuronal precursors and the real-time imaging of cell migration; furthermore, they advise on how to analyze data (Khlghatyan and Saghatelyan 2012).

Blood vessels in the subventricular zone appear to be more permeable than in other regions of the brain. This enhanced leakiness in the subventricular zone supports a fast response from NSC to alterations in the composition of the blood and the cerebrospinal fluid, meaning that NSC and neuroblasts are also more exposed to blood circulating molecules of high molecular weights. Indeed, Tavazoie and colleagues showed that the subventricular zone vasculature promoted easier access to the brain

parenchyma from fluorescent tracers administered systemically (Tavazoie et al. 2008). On the other hand, the rostral migratory stream and olfactory bulb present impermeable and continuous blood vessels. These data appear to suggest that specialized vascular niches are not a requirement for neurogenesis per se to occur, but a means to ensure cell proliferation (Colin-Castelan, Ramirez-Santos, and Gutierrez-Ospina 2016). Ang-1 is a secreted glycoprotein that has been found to enhance cell proliferation in subventricular zone cell cultures via the Tie-2 receptor, which is expressed by neuronal progenitors and neurons in vivo. Moreover, the exogenous administration of Ang-1 increased the number of mature neurons and promoted neurite outgrowth (Rosa et al. 2010). The subventricular zone vessels also present fractones that are in direct contact with the lateral ventricles. Fractones are long tubular extensions of extracellular matrix (enriched in laminin and heparan-sulphate proteoglycans) devoid of cellular material (Mercier, Kitasako, and Hatton 2002). Direct contact with the cerebrospinal fluid allows the scavenging of growth factors by heparan sulphate proteoglycans and can either stimulate or inhibit subventricular zone cell proliferation (Mercier, Kitasako, and Hatton 2002) (Mercier and Douet 2014).

The hippocampal vascular niche is arranged very differently from the subventricular zone niche. In the hippocampus, capillaries stem from arterioles at the hippocampal fissure and descend into the granular cell layer until they reach the hilus, where some vessels migrate along the subgranular zone, fostering the opportunity for more physical contacts (Licht and Keshet 2015). Several studies have shown that VEGF-induced neovascularization is also accompanied by an increase in hippocampal neurogenesis (Cao et al. 2004, Jin et al. 2002, Licht et al. 2011, Udo et al. 2008). Increased microvascular density and blood flow derived from exercise have also been associated with enhanced neurogenesis (Van der Borght et al. 2009). In this work, mice exposed to the running wheel showed an increase in glucose transporter Glut-1 expression as well as a higher number of immature neurons. When mice were subsequently deprived of physical activity, Glut-1 expression returned abruptly to basal levels; the newly formed neurons were only able to survive for a few days. Other blood circulating factors are capable of inhibiting neurogenesis. Increasing the blood levels of CCL11, or eotaxin-1, was able to inhibit neurogenesis and impair learning and memory in mice, similar to what occurs in aged humans (higher CCL11 levels are linked to cognitive and neurogenic decline) (Villeda et al. 2011). The hippocampus responds to thyroidal, gonadal, and adrenal hormones, which regulate dentate gyrus volume, among other functions (McEwen 1999). Interestingly, while N-

methyl-D-aspartate (NMDA) annuls an increase in dentate gyrus cell proliferation induced by the removal of adrenal glands, NMDA receptor inhibition blocks corticosterone-stimulated reduced proliferation (Cameron, Tanapat, and Gould 1998).

The crosstalk between NSC and blood vessels can be modulated by different factors such as niche properties, contact-dependent signaling, endothelial-secreted factors, blood circulating molecules, fractone-mediated signaling, and NSC signaling (reviewed by (Licht and Keshet 2015)). The contact between NSC and endothelial cells is essential to maintain the neurovascular niche and NSC properties. Integrin α6β1 is the main receptor for vascular laminins, key elements of basement membranes (Schaff et al. 2013). Blocking α6β1 integrin inhibits and further deters subventricular zone progenitor cells from blood vessels (Shen et al. 2008). Others have shown that contact with brain endothelial cells partially supports proliferation and stemness of subventricular zone cells. Effects dependent on brain endothelial cell-derived laminin binding to α6β1 integrin are decreased in co-cultures incubated with anti-α6 integrin neutralizing antibody and in co-cultures with subventricular zone β1−/− NSC (Rosa et al. 2016). Integrin signaling is regulated by eph/ephrin signaling, which is involved in a series of processes during embryonic development as well as in adulthood (e.g. angiogenesis, stem cell differentiation) (Arvanitis and Davy 2008). B class ephrins are expressed in the subventricular zone and blocking interaction between ephrinB and its receptors leads to increased progenitor cell proliferation while endothelial ephrinB2 enforces NSC quiescence (Ottone et al. 2014). In vascular niches, endothelial cells provide soluble cues (angiocrine factors) that regulate NCS activity by enhancing self-renewal, cell proliferation or neuronal differentiation (Shen et al. 2004). Angiocrine factors include stromal cell-derived factor 1 (SDF1 or CXCL12), pigmented epithelium-derived factor (PEDF), neurotrophin-3 (NT-3), betacellulin, sphingosine-1-phosphate and prostaglandin-D2 (Kokovay et al. 2010) (Andreu-Agullo et al. 2009) (Ramirez-Castillejo et al. 2006) (Delgado et al. 2014) (Gomez-Gaviro et al. 2012). These factors may be used to achieve vascular repair and promote neurogenesis in a context of neurovascular injury. We have recently shown that ischemic endothelial cells treated with retinoic acid-loaded nanoparticles modulated the content and effect of vascular cues. Pre-treated ischemic endothelial cells were shown to release factors that enhanced neural stem cell survival and promoted differentiation (Ferreira et al. 2016). The use of nanomedicine to enhance specific targeting and delivery of a molecule with clinical potential is becoming increasingly relevant for brain regeneration strategies.

In pathological conditions, regaining or maintaining blood vessel integrity is pivotal, particularly when a neuronal injury occurs. For instance, animal models have shown that subventricular zone-derived neuroblasts migrate and concentrate along blood vessels in post-stroke striatum (Thored et al. 2007, Yamashita et al. 2006); migration and later survival of neuroblasts depend on the formation and/or repair of blood vessels. In stroke patients, greater microvessel density in the ischemic border was reported to correlate with a longer period of survival (Krupinski et al. 1994). Brain-derived neurotrophic factor (BDNF) from rostral migratory stream endothelial cells or induced by ectopic expression in striatal endothelial cells promotes the migration of neuroblasts along the rostral migratory stream in normal conditions or towards the injured striatum upon ischemic lesion respectively (Grade et al. 2013, Snapyan et al. 2009). Likewise, apoptosis of brain endothelial cells negatively affects repair mechanisms essential for neurological recovery after stroke (Saghatelyan 2009). Alzheimer's and Parkinson's diseases and other neurodegenerative conditions also show a disrupted blood-brain barrier, with decreased expression or decreased efficacy of P-glycoprotein transporters (Sweeney, Sagare, and Zlokovic 2018). Since the process of reconstructing blood vessels can be abnormal or defective, brain repair is delayed, and age-associated decline of cognitive functions can be exacerbated (Rizzo and Leaver 2010). The mechanisms and therapeutic potential underlying vascular regulation of adult neurogenesis under physiological and pathological conditions are further reviewed in (Sawada, Matsumoto, and Sawamoto 2014).

Hence, several factors can determine how effective the regeneration process can be, particularly the interaction between NSC and the vascular niches located near the subventricular zone and the hippocampus. Recently, lactate was shown to potentiate both angiogenesis and neurogenesis via the NF-κB signaling pathway in a rat model of intracerebral hemorrhage (Zhou et al. 2018). Previously considered an indicator of impaired oxidative neuronal metabolism, lactate inhibition induced through the administration of lactate dehydrogenase inhibitor oxamate after an injury halted neurogenesis and angiogenesis while the infusion of sodium L-lactate promoted both processes. In recent years, there have been increasing reports on how both neurogenesis and angiogenesis are physiologically regulated by microRNA (miR), small non-coding RNA molecules involved in gene expression. In particular, miR-126, miR-195, miR-210, miR-9, and miR-17 have also been described as regulating neurogenesis and angiogenesis in several ischemic

and hemorrhagic stroke models (Madelaine et al. 2017, Qu et al. 2019, Cheng et al. 2019, Zeng et al. 2014, Yang et al. 2017).

Ultimately, NSC can also modulate blood vessel dynamics, namely by transmitting calcium currents to capillaries, mediating vasoconstriction or vasorelaxation (Lacar et al. 2011, Lacar, Herman, Platel, et al. 2012, Lacar, Herman, Hartman, et al. 2012). NSC can also induce the expression of VEGF and BDNF in endothelial cells by releasing nitric oxide. These neurotrophic factors interact with their receptors in endothelial cells stimulating angiogenesis and further showing the importance of NSC modulation of vascular niches (Li et al. 2006).

Crosstalk between tumor angiogenesis and neurogenesis

Tumor angiogenesis is known to occur when blood vessels proliferate and/or become recruited to supply oxygen and nutrients to support cancer cell growth and expansion (Dang et al. 1999). In brief, this process is initiated with the assistance of the adjacent host vasculature (co-option) and blood circulating endothelial progenitor cells that are strongly stimulated by a pro-angiogenic imbalance generated by the tumor bed. The success of tumor angiogenesis is further ensured by pro-angiogenic cytokine-producing leukocytes and monocytes, by proliferating fibroblasts that produce collagen, by basement membrane degradation, which releases growth factors and, finally, by vessel remodeling. Nevertheless, tumor vessels are morphologically different from *normal* ones, since they present a smaller number and loosely connected pericytes as well as abnormal, discontinuous endothelial cells and basement membrane (Fukumura and Jain 2007). This disorganized layout creates leakier vessels and irregular blood flow (Hashizume et al. 2000). Moreover, vessels can display a collapsed or absent lumen (Qian et al. 2009, Yao et al. 2007), which ultimately contributes to insufficient nutrient and oxygen supply to the tumor.

Accordingly, when Judah Folkman proposed approximately fifty years ago that cancer could be reverted, or at least halted, by inhibiting angiogenesis (Folkman 1971), it paved the way to the development of anti-angiogenic chemotherapy. One remarkable agent is Bevacizumab (the active principle of Avastin®), a monoclonal antibody that specifically inhibits VEGF-A. Bevacizumab was first approved by the Food and Drug Administration to treat metastatic colorectal cancer but is now used to treat other types, such as lung, renal, or breast cancer, and recurrent glioblastoma (reviewed by (Abdalla et al. 2018)). The group has also revised other anti-angiogenic agents that have since been tested to enhance

vessel normalization and that were recently approved for anti-cancer therapy or are entering clinical investigation. Most are VEGF inhibitors or block VEGF receptor and platelet-derived growth factor via tyrosine kinases inhibitors. However, these have been associated with biodistribution issues, life-threatening side effects and drug resistance (Quesada, Medina, and Alba 2007, Ebos and Kerbel 2011, Shojaei 2012). Moreover, some dispute that the normalization of tumoral vasculature per se does not prevent the growth of solid tumors (von Baumgarten et al. 2011). Additionally, since tumor vessels allow for reduced T-cell extravasation, others have suggested combining antiangiogenic agents with immunotherapy (Fukumura et al. 2018).

In the initial stages, cancer cells co-opt existing vessels but, eventually, the tumor recruits its vascular supply although it is difficult to find the shifting point between co-option and tumor-initiated vascular growth (Leenders, Kusters, and de Waal 2002). The ability of cancer cells to insert themselves into blood vessels to establish a vascular-like network, with or without the involvement of endothelial cells, requires cancer stem cells (CSC) (Hendrix et al. 2003). CSC are cellular subpopulations that share features with the normal stem or progenitor cells (e.g. self-renewal, multipotency) and that are responsible for tumor growth, heterogeneity, metastasis and recurrence, most likely because of their high resistance to treatments (Ayob and Ramasamy 2018). CSC can differentiate into endothelial cells (Ricci-Vitiani et al. 2010) and hold a vast proteome that supports this process called vasculogenic mimicry (Cao et al. 2013) (Lirdprapamongkol et al. 2012). The closeness between blood vessels and CSC creates a symbiotic relationship that favors both parties: endothelial cells support the expansion of CSC while CSC promote angiogenesis by releasing pro-angiogenic factors, ultimately sustaining tumor growth (reviewed by (Ayob and Ramasamy 2018)).

Finally, CSC are a likely consequence of deregulated proliferation and self-renewal of stem/progenitor cells and/or dedifferentiation of mature cells (Eiriz et al. 2014). A key difference is CSC's high resistance to radio- and chemotherapies that can compromise NSC viability and/or function. Therefore, a main and current concern is to devise strategies that can distinguish these cell populations to specifically target CSC and their contribution to cancer growth and recurrence.

Conclusions and future directions

Blood vessels are an integral component of any tissue or organ, particularly the brain (also within specific areas of the brain). Additionally,

these structures modulate neurogenesis by providing points of adhesion for NSC migration and interaction. Hence, the physiological state of the vascular system will determine the success of migrating neuroblasts to injury areas and/or will support neuronal survival, ultimately impacting the success of tissue regeneration. Consequently, strategies targeting angiogenesis could be promising in the treatment of neurodegenerative or acute brain conditions. Since the bloodstream also supplies key factors that may positively or negatively affect these processes, changes in flow dynamics triggered by pathology may impact neurogenesis. However, there are still unanswered questions regarding the crosstalk between neurogenesis and brain vessels, and what factors or targets can be used to devise efficient clinical approaches for neuronal regeneration. It is particularly unclear what makes the subventricular zone and the hippocampal vascular beds suitable to support neurogenesis and, more importantly, what can be done therapy-wise to enhance blood vessel-supported neuronal repair. Despite the significant advances in our knowledge on the interaction between the neurogenic and vascular niches, we are far from fully understanding how NSC modulate vascular activity.

References

Abdalla, A. M. E., L. Xiao, M. W. Ullah, M. Yu, C. Ouyang, and G. Yang. 2018. "Current Challenges of Cancer Anti-angiogenic Therapy and the Promise of Nanotherapeutics." *Theranostics* no. 8 (2):533-548. doi: 10.7150/thno.21674.

Aird, W. C. 2012. "Endothelial cell heterogeneity." *Cold Spring Harb Perspect Med* no. 2 (1):a006429. doi: 10.1101/cshperspect.a006429.

Altman, J. 1969. "Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb." *J Comp Neurol* no. 137 (4):433-57. doi: 10.1002/cne.901370404.

Altman, J., and G. D. Das. 1965. "Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats." *J Comp Neurol* no. 124 (3):319-35.

Andreu-Agullo, C., J. M. Morante-Redolat, A. C. Delgado, and I. Farinas. 2009. "Vascular niche factor PEDF modulates Notch-dependent stemness in the adult subependymal zone." *Nat Neurosci* no. 12 (12):1514-23. doi: 10.1038/nn.2437.

Arvanitis, D., and A. Davy. 2008. "Eph/ephrin signaling: networks." *Genes Dev* no. 22 (4):416-29. doi: 10.1101/gad.1630408.

Asahara, T., T. Murohara, A. Sullivan, M. Silver, R. van der Zee, T. Li, B. Witzenbichler, G. Schatteman, and J. M. Isner. 1997. "Isolation of putative progenitor endothelial cells for angiogenesis." *Science* no. 275 (5302):964-7.

Ayob, A. Z., and T. S. Ramasamy. 2018. "Cancer stem cells as key drivers of tumour progression." *J Biomed Sci* no. 25 (1):20. doi: 10.1186/s12929-018-0426-4.

Boldrini, M., C. A. Fulmore, A. N. Tartt, L. R. Simeon, I. Pavlova, V. Poposka, G. B. Rosoklija, A. Stankov, V. Arango, A. J. Dwork, R. Hen, and J. J. Mann. 2018. "Human Hippocampal Neurogenesis Persists throughout Aging." *Cell Stem Cell* no. 22 (4):589-599 e5. doi: 10.1016/j.stem.2018.03.015.

Bovetti, S., Y. C. Hsieh, P. Bovolin, I. Perroteau, T. Kazunori, and A. C. Puche. 2007. "Blood vessels form a scaffold for neuroblast migration in the adult olfactory bulb." *J Neurosci* no. 27 (22):5976-80. doi: 10.1523/JNEUROSCI.0678-07.2007.

Bozoyan, L., J. Khlghatyan, and A. Saghatelyan. 2012. "Astrocytes control the development of the migration-promoting vasculature scaffold in the postnatal brain via VEGF signaling." *J Neurosci* no. 32 (5):1687-704. doi: 10.1523/JNEUROSCI.5531-11.2012.

Cameron, H. A., P. Tanapat, and E. Gould. 1998. "Adrenal steroids and N-methyl-D-aspartate receptor activation regulate neurogenesis in the dentate gyrus of adult rats through a common pathway." *Neuroscience* no. 82 (2):349-354.

Cao, L., X. Jiao, D. S. Zuzga, Y. Liu, D. M. Fong, D. Young, and M. J. During. 2004. "VEGF links hippocampal activity with neurogenesis, learning and memory." *Nat Genet* no. 36 (8):827-35. doi: 10.1038/ng1395.

Cao, Z., B. Shang, G. Zhang, L. Miele, F. H. Sarkar, Z. Wang, and Q. Zhou. 2013. "Tumor cell-mediated neovascularization and lymphangiogenesis contrive tumor progression and cancer metastasis." *Biochim Biophys Acta* no. 1836 (2):273-86. doi: 10.1016/j.bbcan.2013.08.001.

Cheng, H. Y., Y. S. Wang, P. Y. Hsu, C. Y. Chen, Y. C. Liao, and S. H. Juo. 2019. "miR-195 Has a Potential to Treat Ischemic and Hemorrhagic Stroke through Neurovascular Protection and Neurogenesis." *Mol Ther Methods Clin Dev* no. 13:121-132. doi: 10.1016/j.omtm.2018.11.011.

Colin-Castelan, D., J. Ramirez-Santos, and G. Gutierrez-Ospina. 2016. "Differential vascular permeability along the forebrain ventricular

neurogenic niche in the adult murine brain." *J Neurosci Res* no. 94 (2):161-9. doi: 10.1002/jnr.23682.

Dang, C. V., S. L. Gerson, M. Litwak, and M. Padarathsingh. 1999. "Gene therapy and translational cancer research." *Clin Cancer Res* no. 5 (2):471-4.

Delgado, A. C., S. R. Ferron, D. Vicente, E. Porlan, A. Perez-Villalba, C. M. Trujillo, P. D'Ocon, and I. Farinas. 2014. "Endothelial NT-3 delivered by vasculature and CSF promotes quiescence of subependymal neural stem cells through nitric oxide induction." *Neuron* no. 83 (3):572-85. doi: 10.1016/j.neuron.2014.06.015.

Distler, JHW, A Hirth, M Kurowska-Stolarska, and RE Gay. 2003. "Angiogenic and angiostatic factors in the molecular control of angiogenesis." *The Quarterly Journal of Nuclear Medicine and Molecular Imaging* no. 47 (3):149.

Doetsch, F., J. M. Garcia-Verdugo, and A. Alvarez-Buylla. 1997. "Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain." *J Neurosci* no. 17 (13):5046-61.

Ebos, J. M., and R. S. Kerbel. 2011. "Antiangiogenic therapy: impact on invasion, disease progression, and metastasis." *Nat Rev Clin Oncol* no. 8 (4):210-21. doi: 10.1038/nrclinonc.2011.21.

Eiriz, M.F., J.O. Malva, F. Agasse, and L. Bernardino. 2014. "Histamine in the Neural and Cancer Stem Cell Niches." In *Stem cells and cancer stem cells*, edited by Hayat MA, 3-17. Springer.

Eriksson, P. S., E. Perfilieva, T. Bjork-Eriksson, A. M. Alborn, C. Nordborg, D. A. Peterson, and F. H. Gage. 1998. "Neurogenesis in the adult human hippocampus." *Nat Med* no. 4 (11):1313-7. doi: 10.1038/3305.

Ferreira, R., M. C. Fonseca, T. Santos, J. Sargento-Freitas, R. Tjeng, F. Paiva, M. Castelo-Branco, L. S. Ferreira, and L. Bernardino. 2016. "Retinoic acid-loaded polymeric nanoparticles enhance vascular regulation of neural stem cell survival and differentiation after ischaemia." *Nanoscale* no. 8, :8126-8137. doi: 10.1039/c5nr09077f.

Folkman, J. 1971. "Tumor angiogenesis: therapeutic implications." *N Engl J Med* no. 285 (21):1182-6. doi: 10.1056/NEJM197111182852108.

Fukumura, D., and R. K. Jain. 2007. "Tumor microvasculature and microenvironment: targets for anti-angiogenesis and normalization." *Microvasc Res* no. 74 (2-3):72-84. doi: 10.1016/j.mvr.2007.05.003.

Fukumura, D., J. Kloepper, Z. Amoozgar, D. G. Duda, and R. K. Jain. 2018. "Enhancing cancer immunotherapy using antiangiogenics:

opportunities and challenges." *Nat Rev Clin Oncol* no. 15 (5):325-340. doi: 10.1038/nrclinonc.2018.29.

Gomez-Gaviro, M. V., C. E. Scott, A. K. Sesay, A. Matheu, S. Booth, C. Galichet, and R. Lovell-Badge. 2012. "Betacellulin promotes cell proliferation in the neural stem cell niche and stimulates neurogenesis." *Proc Natl Acad Sci U S A* no. 109 (4):1317-22. doi: 10.1073/pnas.1016199109.

Grade, S., Y. C. Weng, M. Snapyan, J. Kriz, J. O. Malva, and A. Saghatelyan. 2013. "Brain-derived neurotrophic factor promotes vasculature-associated migration of neuronal precursors toward the ischemic striatum." *PLoS One* no. 8 (1):e55039. doi: 10.1371/journal.pone.0055039.

Hashizume, H., P. Baluk, S. Morikawa, J. W. McLean, G. Thurston, S. Roberge, R. K. Jain, and D. M. McDonald. 2000. "Openings between defective endothelial cells explain tumor vessel leakiness." *Am J Pathol* no. 156 (4):1363-80. doi: 10.1016/S0002-9440(10)65006-7.

Hendrix, M. J., E. A. Seftor, A. R. Hess, and R. E. Seftor. 2003. "Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma." *Nat Rev Cancer* no. 3 (6):411-21. doi: 10.1038/nrc1092.

Jin, K., Y. Zhu, Y. Sun, X. O. Mao, L. Xie, and D. A. Greenberg. 2002. "Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo." *Proc Natl Acad Sci U S A* no. 99 (18):11946-50. doi: 10.1073/pnas.182296499.

Khlghatyan, J., and A. Saghatelyan. 2012. "Time-lapse imaging of neuroblast migration in acute slices of the adult mouse forebrain." *J Vis Exp* (67):e4061. doi: 10.3791/4061.

Kokovay, E., S. Goderie, Y. Wang, S. Lotz, G. Lin, Y. Sun, B. Roysam, Q. Shen, and S. Temple. 2010. "Adult SVZ lineage cells home to and leave the vascular niche via differential responses to SDF1/CXCR4 signaling." *Cell Stem Cell* no. 7 (2):163-73. doi: 10.1016/j.stem.2010.05.019.

Krupinski, J., J. Kaluza, P. Kumar, S. Kumar, and J. M. Wang. 1994. "Role of angiogenesis in patients with cerebral ischemic stroke." *Stroke* no. 25 (9):1794-8.

Lacar, B., P. Herman, N. W. Hartman, F. Hyder, and A. Bordey. 2012. "S phase entry of neural progenitor cells correlates with increased blood flow in the young subventricular zone." *PLoS One* no. 7 (2):e31960. doi: 10.1371/journal.pone.0031960.

Lacar, B., P. Herman, J. C. Platel, C. Kubera, F. Hyder, and A. Bordey. 2012. "Neural progenitor cells regulate capillary blood flow in the

postnatal subventricular zone." *J Neurosci* no. 32 (46):16435-48. doi: 10.1523/JNEUROSCI.1457-12.2012.

Lacar, B., S. Z. Young, J. C. Platel, and A. Bordey. 2011. "Gap junction-mediated calcium waves define communication networks among murine postnatal neural progenitor cells." *Eur J Neurosci* no. 34 (12):1895-905. doi: 10.1111/j.1460-9568.2011.07901.x.

Leenders, W. P., B. Kusters, and R. M. de Waal. 2002. "Vessel co-option: how tumors obtain blood supply in the absence of sprouting angiogenesis." *Endothelium* no. 9 (2):83-7.

Li, Q., M. C. Ford, E. B. Lavik, and J. A. Madri. 2006. "Modeling the neurovascular niche: VEGF- and BDNF-mediated cross-talk between neural stem cells and endothelial cells: an in vitro study." *J Neurosci Res* no. 84 (8):1656-68. doi: 10.1002/jnr.21087.

Licht, T., I. Goshen, A. Avital, T. Kreisel, S. Zubedat, R. Eavri, M. Segal, R. Yirmiya, and E. Keshet. 2011. "Reversible modulations of neuronal plasticity by VEGF." *Proc Natl Acad Sci U S A* no. 108 (12):5081-6. doi: 10.1073/pnas.1007640108.

Licht, T., and E. Keshet. 2015. "The vascular niche in adult neurogenesis." *Mech Dev* no. 138 Pt 1:56-62. doi: 10.1016/j.mod.2015.06.001.

Lirdprapamongkol, K., K. Chiablaem, M. Sila-Asna, R. Surarit, A. Bunyaratvej, and J. Svasti. 2012. "Exploring stemness gene expression and vasculogenic mimicry capacity in well- and poorly-differentiated hepatocellular carcinoma cell lines." *Biochem Biophys Res Commun* no. 422 (3):429-35. doi: 10.1016/j.bbrc.2012.05.009.

Lotsch, J., E. Schaeffeler, M. Mittelbronn, S. Winter, V. Gudziol, S. W. Schwarzacher, T. Hummel, A. Doehring, M. Schwab, and A. Ultsch. 2014. "Functional genomics suggest neurogenesis in the adult human olfactory bulb." *Brain Struct Funct* no. 219 (6):1991-2000. doi: 10.1007/s00429-013-0618-3.

Machado-Pereira, M., T. Santos, L. Bernardino, and R. Ferreira. 2017. "Vascular inter-regulation of inflammation: molecular and cellular targets for CNS therapy." *J Neurochem* no. 140 (5):692-702. doi: 10.1111/jnc.13914.

Madelaine, R., S. A. Sloan, N. Huber, J. H. Notwell, L. C. Leung, G. Skariah, C. Halluin, S. P. Pasca, G. Bejerano, M. A. Krasnow, B. A. Barres, and P. Mourrain. 2017. "MicroRNA-9 Couples Brain Neurogenesis and Angiogenesis." *Cell Rep* no. 20 (7):1533-1542. doi: 10.1016/j.celrep.2017.07.051.

McEwen, B. S. 1999. "Stress and hippocampal plasticity." *Annu Rev Neurosci* no. 22:105-22. doi: 10.1146/annurev.neuro.22.1.105.

Mercier, F., and V. Douet. 2014. "Bone morphogenetic protein-4 inhibits adult neurogenesis and is regulated by fractone-associated heparan sulfates in the subventricular zone." *J Chem Neuroanat* no. 57-58:54-61. doi: 10.1016/j.jchemneu.2014.03.005.

Mercier, F., J. T. Kitasako, and G. I. Hatton. 2002. "Anatomy of the brain neurogenic zones revisited: fractones and the fibroblast/macrophage network." *J Comp Neurol* no. 451 (2):170-88. doi: 10.1002/cne.10342.

Mirzadeh, Z., F. T. Merkle, M. Soriano-Navarro, J. M. Garcia-Verdugo, and A. Alvarez-Buylla. 2008. "Neural stem cells confer unique pinwheel architecture to the ventricular surface in neurogenic regions of the adult brain." *Cell Stem Cell* no. 3 (3):265-78. doi: 10.1016/j.stem.2008.07.004.

Ottone, C., B. Krusche, A. Whitby, M. Clements, G. Quadrato, M. E. Pitulescu, R. H. Adams, and S. Parrinello. 2014. "Direct cell-cell contact with the vascular niche maintains quiescent neural stem cells." *Nat Cell Biol* no. 16 (11):1045-56. doi: 10.1038/ncb3045.

Qian, C. N., D. Huang, B. Wondergem, and B. T. Teh. 2009. "Complexity of tumor vasculature in clear cell renal cell carcinoma." *Cancer* no. 115 (10 Suppl):2282-9. doi: 10.1002/cncr.24238.

Qu, M., J. Pan, L. Wang, P. Zhou, Y. Song, S. Wang, L. Jiang, J. Geng, Z. Zhang, Y. Wang, Y. Tang, and G. Y. Yang. 2019. "MicroRNA-126 Regulates Angiogenesis and Neurogenesis in a Mouse Model of Focal Cerebral Ischemia." *Mol Ther Nucleic Acids* no. 16:15-25. doi: 10.1016/j.omtn.2019.02.002.

Quesada, A. R., M. A. Medina, and E. Alba. 2007. "Playing only one instrument may be not enough: limitations and future of the antiangiogenic treatment of cancer." *Bioessays* no. 29 (11):1159-68. doi: 10.1002/bies.20655.

Ramirez-Castillejo, C., F. Sanchez-Sanchez, C. Andreu-Agullo, S. R. Ferron, J. D. Aroca-Aguilar, P. Sanchez, H. Mira, J. Escribano, and I. Farinas. 2006. "Pigment epithelium-derived factor is a niche signal for neural stem cell renewal." *Nat Neurosci* no. 9 (3):331-9. doi: 10.1038/nn1657.

Ricci-Vitiani, L., R. Pallini, M. Biffoni, M. Todaro, G. Invernici, T. Cenci, G. Maira, E. A. Parati, G. Stassi, L. M. Larocca, and R. De Maria. 2010. "Tumour vascularization via endothelial differentiation of glioblastoma stem-like cells." *Nature* no. 468 (7325):824-8. doi: 10.1038/nature09557.

Risau, W. 1997. "Mechanisms of angiogenesis." *Nature* no. 386 (6626):671-4. doi: 10.1038/386671a0.

Risau, W., and I. Flamme. 1995. "Vasculogenesis." *Annu Rev Cell Dev Biol* no. 11:73-91. doi: 10.1146/annurev.cb.11.110195.000445.

Rizzo, M. T., and H. A. Leaver. 2010. "Brain endothelial cell death: modes, signaling pathways, and relevance to neural development, homeostasis, and disease." *Mol Neurobiol* no. 42 (1):52-63. doi: 10.1007/s12035-010-8132-6.

Rosa, A. I., J. Goncalves, L. Cortes, L. Bernardino, J. O. Malva, and F. Agasse. 2010. "The angiogenic factor angiopoietin-1 is a proneurogenic peptide on subventricular zone stem/progenitor cells." *J Neurosci* no. 30 (13):4573-84. doi: 10.1523/JNEUROSCI.5597-09.2010.

Rosa, A. I., S. Grade, S. D. Santos, L. Bernardino, T. C. Chen, J. Relvas, F. M. Hofman, and F. Agasse. 2016. "Heterocellular Contacts with Mouse Brain Endothelial Cells Via Laminin and alpha6beta1 Integrin Sustain Subventricular Zone (SVZ) Stem/Progenitor Cells Properties." *Front Cell Neurosci* no. 10:284. doi: 10.3389/fncel.2016.00284.

Rosell, A., A. Morancho, M. Navarro-Sobrino, E. Martinez-Saez, M. Hernandez-Guillamon, S. Lope-Piedrafita, V. Barcelo, F. Borras, A. Penalba, L. Garcia-Bonilla, and J. Montaner. 2013. "Factors secreted by endothelial progenitor cells enhance neurorepair responses after cerebral ischemia in mice." *PLoS One* no. 8 (9):e73244. doi: 10.1371/journal.pone.0073244.

Rundhaug, Joyce E. 2005. "Matrix metalloproteinases and angiogenesis." *Journal of cellular and molecular medicine* no. 9 (2):267-285.

Sabin, F. R. 2002. "Preliminary note on the differentiation of angioblasts and the method by which they produce blood-vessels, blood-plasma and red blood-cells as seen in the living chick. 1917." *J Hematother Stem Cell Res* no. 11 (1):5-7. doi: 10.1089/152581602753448496.

Saghatelyan, A. 2009. "Role of blood vessels in the neuronal migration." *Semin Cell Dev Biol* no. 20 (6):744-50. doi: 10.1016/j.semcdb.2009.04.006.

Saito, Akiko, Akira Sugawara, Akira Uruno, Masataka Kudo, Hiroyuki Kagechika, Yasufumi Sato, Yuji Owada, Hisatake Kondo, Mayumi Sato, and Masahiko Kurabayashi. 2007. "All-trans retinoic acid induces in vitro angiogenesis via retinoic acid receptor: possible involvement of paracrine effects of endogenous vascular endothelial growth factor signaling." *Endocrinology* no. 148 (3):1412-1423.

Sawada, M., M. Matsumoto, and K. Sawamoto. 2014. "Vascular regulation of adult neurogenesis under physiological and pathological conditions." *Frontiers in Neuroscience* no. 8:53. doi: 10.3389/fnins.2014.00053.

Schaff, M., C. Tang, E. Maurer, C. Bourdon, N. Receveur, A. Eckly, B. Hechler, C. Arnold, A. de Arcangelis, B. Nieswandt, C. V. Denis, O. Lefebvre, E. Georges-Labouesse, C. Gachet, F. Lanza, and P. H. Mangin. 2013. "Integrin alpha6beta1 is the main receptor for vascular laminins and plays a role in platelet adhesion, activation, and arterial thrombosis." *Circulation* no. 128 (5):541-52. doi: 10.1161/CIRCULATIONAHA.112.000799.

Schwartz, S. M., and E. P. Benditt. 1976. "Clustering of replicating cells in aortic endothelium." *Proc Natl Acad Sci U S A* no. 73 (2):651-3.

Segarra, M., B. C. Kirchmaier, and A. Acker-Palmer. 2015. "A vascular perspective on neuronal migration." *Mech Dev* no. 138 Pt 1:17-25. doi: 10.1016/j.mod.2015.07.004.

Shen, Q., S. K. Goderie, L. Jin, N. Karanth, Y. Sun, N. Abramova, P. Vincent, K. Pumiglia, and S. Temple. 2004. "Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells." *Science* no. 304 (5675):1338-40. doi: 10.1126/science.1095505.

Shen, Q., Y. Wang, E. Kokovay, G. Lin, S. M. Chuang, S. K. Goderie, B. Roysam, and S. Temple. 2008. "Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions." *Cell Stem Cell* no. 3 (3):289-300. doi: 10.1016/j.stem.2008.07.026.

Shojaei, F. 2012. "Anti-angiogenesis therapy in cancer: current challenges and future perspectives." *Cancer Lett* no. 320 (2):130-7. doi: 10.1016/j.canlet.2012.03.008.

Snapyan, M., M. Lemasson, M. S. Brill, M. Blais, M. Massouh, J. Ninkovic, C. Gravel, F. Berthod, M. Gotz, P. A. Barker, A. Parent, and A. Saghatelyan. 2009. "Vasculature guides migrating neuronal precursors in the adult mammalian forebrain via brain-derived neurotrophic factor signaling." *J Neurosci* no. 29 (13):4172-88. doi: 10.1523/JNEUROSCI.4956-08.2009.

Sorrells, S. F., M. F. Paredes, A. Cebrian-Silla, K. Sandoval, D. Qi, K. W. Kelley, D. James, S. Mayer, J. Chang, K. I. Auguste, E. F. Chang, A. J. Gutierrez, A. R. Kriegstein, G. W. Mathern, M. C. Oldham, E. J. Huang, J. M. Garcia-Verdugo, Z. Yang, and A. Alvarez-Buylla. 2018. "Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults." *Nature* no. 555 (7696):377-381. doi: 10.1038/nature25975.

Spalding, K. L., O. Bergmann, K. Alkass, S. Bernard, M. Salehpour, H. B. Huttner, E. Bostrom, I. Westerlund, C. Vial, B. A. Buchholz, G. Possnert, D. C. Mash, H. Druid, and J. Frisen. 2013. "Dynamics of hippocampal neurogenesis in adult humans." *Cell* no. 153 (6):1219-1227. doi: 10.1016/j.cell.2013.05.002.

Sweeney, M. D., A. P. Sagare, and B. V. Zlokovic. 2018. "Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders." *Nat Rev Neurol* no. 14 (3):133-150. doi: 10.1038/nrneurol.2017.188.

Tavazoie, M., L. Van der Veken, V. Silva-Vargas, M. Louissaint, L. Colonna, B. Zaidi, J. M. Garcia-Verdugo, and F. Doetsch. 2008. "A specialized vascular niche for adult neural stem cells." *Cell Stem Cell* no. 3 (3):279-88. doi: 10.1016/j.stem.2008.07.025.

Thored, P., J. Wood, A. Arvidsson, J. Cammenga, Z. Kokaia, and O. Lindvall. 2007. "Long-term neuroblast migration along blood vessels in an area with transient angiogenesis and increased vascularization after stroke." *Stroke* no. 38 (11):3032-9. doi: 10.1161/STROKEAHA.107.488445.

Udo, H., Y. Yoshida, T. Kino, K. Ohnuki, W. Mizunoya, T. Mukuda, and H. Sugiyama. 2008. "Enhanced adult neurogenesis and angiogenesis and altered affective behaviors in mice overexpressing vascular endothelial growth factor 120." *J Neurosci* no. 28 (53):14522-36. doi: 10.1523/JNEUROSCI.3673-08.2008.

Urban, N., and F. Guillemot. 2014. "Neurogenesis in the embryonic and adult brain: same regulators, different roles." *Front Cell Neurosci* no. 8:396. doi: 10.3389/fncel.2014.00396.

Van der Borght, K., D. E. Kobor-Nyakas, K. Klauke, B. J. Eggen, C. Nyakas, E. A. Van der Zee, and P. Meerlo. 2009. "Physical exercise leads to rapid adaptations in hippocampal vasculature: temporal dynamics and relationship to cell proliferation and neurogenesis." *Hippocampus* no. 19 (10):928-36. doi: 10.1002/hipo.20545.

Villeda, S. A., J. Luo, K. I. Mosher, B. Zou, M. Britschgi, G. Bieri, T. M. Stan, N. Fainberg, Z. Ding, A. Eggel, K. M. Lucin, E. Czirr, J. S. Park, S. Couillard-Despres, L. Aigner, G. Li, E. R. Peskind, J. A. Kaye, J. F. Quinn, D. R. Galasko, X. S. Xie, T. A. Rando, and T. Wyss-Coray. 2011. "The ageing systemic milieu negatively regulates neurogenesis and cognitive function." *Nature* no. 477 (7362):90-4. doi: 10.1038/nature10357.

von Baumgarten, L., D. Brucker, A. Tirniceru, Y. Kienast, S. Grau, S. Burgold, J. Herms, and F. Winkler. 2011. "Bevacizumab has differential and dose-dependent effects on glioma blood vessels and tumor cells." *Clin Cancer Res* no. 17 (19):6192-205. doi: 10.1158/1078-0432.CCR-10-1868.

Wilhelm, I., A. Nyul-Toth, M. Suciu, A. Hermenean, and I. A. Krizbai. 2016. "Heterogeneity of the blood-brain barrier." *Tissue Barriers* no. 4 (1):e1143544. doi: 10.1080/21688370.2016.1143544.

Wolburg, H., and A. Lippoldt. 2002. "Tight junctions of the blood-brain barrier: development, composition and regulation." *Vascular Pharmacology* no. 38 (6):323-37.

Yamashita, T., M. Ninomiya, P. Hernandez Acosta, J. M. Garcia-Verdugo, T. Sunabori, M. Sakaguchi, K. Adachi, T. Kojima, Y. Hirota, T. Kawase, N. Araki, K. Abe, H. Okano, and K. Sawamoto. 2006. "Subventricular zone-derived neuroblasts migrate and differentiate into mature neurons in the post-stroke adult striatum." *Journal of Neuroscience* no. 26 (24):6627-36. doi: 10.1523/JNEUROSCI.0149-06.2006.

Yang, P., L. Cai, G. Zhang, Z. Bian, and G. Han. 2017. "The role of the miR-17-92 cluster in neurogenesis and angiogenesis in the central nervous system of adults." *J Neurosci Res* no. 95 (8):1574-1581. doi: 10.1002/jnr.23991.

Yao, X., C. N. Qian, Z. F. Zhang, M. H. Tan, E. J. Kort, X. J. Yang, J. H. Resau, and B. T. Teh. 2007. "Two distinct types of blood vessels in clear cell renal cell carcinoma have contrasting prognostic implications." *Clin Cancer Res* no. 13 (1):161-9. doi: 10.1158/1078-0432.CCR-06-0774.

Yoder, M. C. 2012. "Human endothelial progenitor cells." *Cold Spring Harb Perspect Med* no. 2 (7):a006692. doi: 10.1101/cshperspect.a006692.

Zeng, L., X. He, Y. Wang, Y. Tang, C. Zheng, H. Cai, J. Liu, Y. Wang, Y. Fu, and G. Y. Yang. 2014. "MicroRNA-210 overexpression induces angiogenesis and neurogenesis in the normal adult mouse brain." *Gene Ther* no. 21 (1):37-43. doi: 10.1038/gt.2013.55.

Zhou, J., T. Liu, H. Guo, H. Cui, P. Li, D. Feng, E. Hu, Q. Huang, A. Yang, J. Zhou, J. Luo, T. Tang, and Y. Wang. 2018. "Lactate potentiates angiogenesis and neurogenesis in experimental intracerebral hemorrhage." *Exp Mol Med* no. 50 (7):78. doi: 10.1038/s12276-018-0113-2.

CHAPTER THREE

THE BLOOD BRAIN BARRIER: MOLECULAR AND CELLULAR FUNCTION

NAGORE I. MARÍN-RAMOS, THOMAS C. CHEN AND FLORENCE M. HOFMAN

Introduction

Neural function within the central nervous system (CNS) requires a highly controlled microenvironment, with a constant supply of oxygen and nutrients. Neurons are very sensitive to subtle changes in the concentrations of many compounds. Hence, the existence of a barrier that regulates CNS homeostasis and prevents the penetration of certain macromolecules is critical for a proper synaptic signaling function (Abbott et al. 2010, Tajes et al. 2014). To maintain and properly regulate this neural microenvironment, the CNS is separated from the rest of the body by three barrier systems: the blood-brain barrier (BBB), the blood-cerebrospinal fluid barrier (BCSFB) at the choroid plexus, and the arachnoid barrier (AB). Among the three barrier systems, the BBB is the most critical, and all organisms with a well-developed CNS present a version of it (Wolburg and Lippoldt 2002, Abbott 2005, Wilhelm et al. 2016). The existence of the BBB was first observed over a century ago by Paul Ehrlich while performing physiological studies of the cerebrospinal fluid (CSF). Ehrlich noticed that the water-soluble aniline dyes injected in the peripheral circulation did not stain the brain and spinal cord. Although he initially hypothesized that this phenomenon was due to the CNS possessing a low affinity for the dye, Goldmann demonstrated that the injection of these dyes into the subarachnoid space colored the brain but not peripheral tissues (Goldmann 1909, 1913). These experiments were the first of many that provided evidence of the existence of a single non-fenestrated continuous endothelial cell (EC) layer, identified as the BBB (Chow and Gu 2015). This barrier provides a stable environment for

neural function, with the appropriate amount of nutrients and oxygen, and also functions as a protective shield for the CNS impeding the entrance of neurotoxic substances circulating in the bloodstream. As a result of the specific combination of ion channels and transporters, the BBB keeps an optimal ionic composition that allows the synaptic signaling (Wolburg and Lippoldt 2002, Wilhelm et al. 2016).

Composition of the BBB

The BBB is localized within the brain microvascular network, which is formed by arterioles, venules, and a large network of capillaries. To respond to the particular requirements of the CNS, the brain vessels forming the BBB have developed a highly specific structure that protects against potentially harmful external agents, while ensuring the correct supply of nutrients and oxygen. The functional BBB is composed of CNS EC tightly interconnected by junctional complexes, pericytes, astrocytes and neurons, which collectively form the neurovascular unit (NVU) (Figure 3-1). To achieve the most restrictive permeability required to protect the CNS, EC forming the BBB show specific characteristics compared to peripheral EC, such as lower numbers of endocytic vesicles to limit transcellular transport, lack of fenestrations, high transendothelial electrical resistance (TEER) due to the tight junctions which restrict paracellular transport, higher mitochondrial volume to support the active metabolism, low expression of leukocyte adhesion molecules (LAM), and specialized transport systems (Siegenthaler, Sohet, and Daneman 2013, Tajes et al. 2014). The fact that CNS EC have a very low expression of LAM such as E-selectin and ICAM-1 prevents the entry of immune cells from the blood. As a result of the paucity of immune cells in the brain microenvironment, the healthy brain is “immune privileged”, and introduced antigens do not induce adaptive immune responses (Chow and Gu 2015). In addition, BBB EC are highly polarized with distinct luminal (blood side, apical) and abluminal (brain side, basolateral) compartments as a result of the presence of tight junctions. The luminal and abluminal plasma membranes are functionally distinct. As an example, the Na^+-dependent neutral amino acid transport is only present on the brain side of the EC, and not on the blood side, allowing for the transfer of selected amino acids against a concentration gradient to actively regulate the internal microenvironment of the brain (Betz and Goldstein 1978).

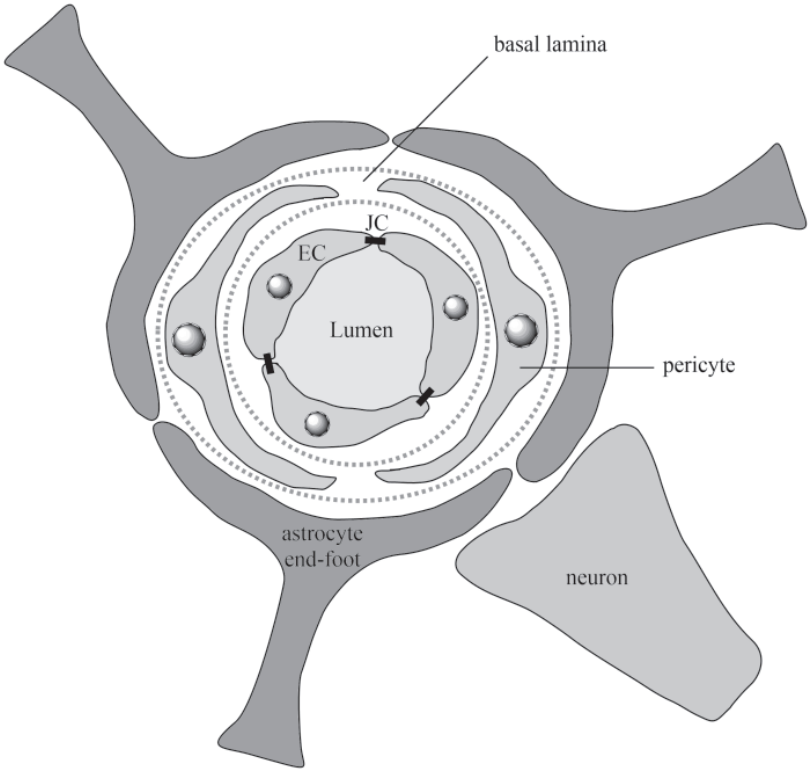

Figure 3-1: Schematic representation of the neurovascular unit (NVU). Endothelial cells (EC) surrounding the lumen are connected by junctional complexes (JC). EC are surrounded by pericytes embedded in the basal lamina, and the whole structure is further insulated by astrocytic end-feet processes. All these structures, together with the surrounding neurons, constitute a functional NVU.

Within the NVU, the EC lining the blood vessel lumen are covered by an extracellular matrix (ECM) known as basal lamina, in close contact with pericytes, a type of contractile cells able to control the capillary diameter and modulate the blood flow (Peppiatt et al. 2006). Pericytes also contribute to the development, maturation and support of EC, and play a role in angiogenesis and phagocytosis, providing an immunological defence by performing macrophage-like activities (Bergers and Song 2005, Sá-Pereira, Brites, and Brito 2012). EC and pericytes are interdependent, so a failure of proper communication between the two cell types can lead to pathological situations such as pericyte loss and

microaneurysms. It has been shown that the platelet-derived growth factor (PDGF) signaling pathway from EC recruits pericytes so that pericytes can migrate to growing vessels (Hammes et al. 2002). The basal lamina where the pericytes are embedded is composed of different extracellular matrix proteins such as laminin, collagen, fibronectin, agrin and perlecan (Wolburg and Lippoldt 2002, Pócsai, Bagyura, and Kálmán 2010, Yepes 2013). Approximately 95% of the basal lamina is covered by the end-feet processes of the astrocytes, constituting a second barrier known as the glia limitans. These astrocytic end-feet contribute to the induction and maintenance of the BBB's properties through paracrine interactions with EC and pericytes, essential for the regulation of metabolism and neuronal transmission, and CNS development and repair. One of the major roles of astrocytes within the NVU occurs during inflammation, when they tightly regulate BBB disruption to allow the penetration of immune cells into the brain, through activation of matrix metalloproteinases (MMP) that partially degrade the basal lamina (Harder, Zhang, and Gebremedhin 2002, Zozulya, Weidenfeller, and Galla 2008, Machida et al. 2015, Herndon, Tome, and Davis 2017, Quintana 2017). Also, in cerebral ischemia, both the MMP and their inhibitors, the tissue inhibitors of metalloproteinases (TIMP), are considered to be key factors in regulating BBB integrity (Cunningham, Wetzel, and Rosenberg 2005, Candelario-Jalil, Yang, and Rosenberg 2009).

Neuronal terminations also participate in the NVU, enabling neurovascular communication. These neurons detect minimal variations in the supply of nutrients and oxygen and communicate with the cells forming blood vessels through the astrocytes, either directly or via an interneuron that links a sensory neuron to a motor neuron. In this way, neurons can influence the vascular tone and blood supply. Although multiple pathways involved in this communication continue to be researched, it is known that astrocytes within the NVU detect variations in the levels of glutamate and the major inhibitory neurotransmitter γ-aminobutyric acid (GABA) released from neurons, and convert those signals into vasomotor commands (Zonta et al. 2003, Duchemin et al. 2012, Muoio, Persson, and Sendeski 2014).

Every constituent of the NVU makes an indispensable contribution to the integrity of the BBB, so if one fails, the barrier breaks down, potentially causing serious effects such as neuroinflammation or neurodegeneration (Obermeier, Daneman, and Ransohoff 2013).

Subcellular structures of the BBB

The principal structures responsible for the structural integrity and high resistance of the BBB are junctional complexes (JC) between adjacent EC, consisting of 1) tight junctions (TJ), and 2) adherens junctions (AJ) (Figure 3-2). Both types of junctional complexes are involved in the reorganization of the actin cytoskeleton.

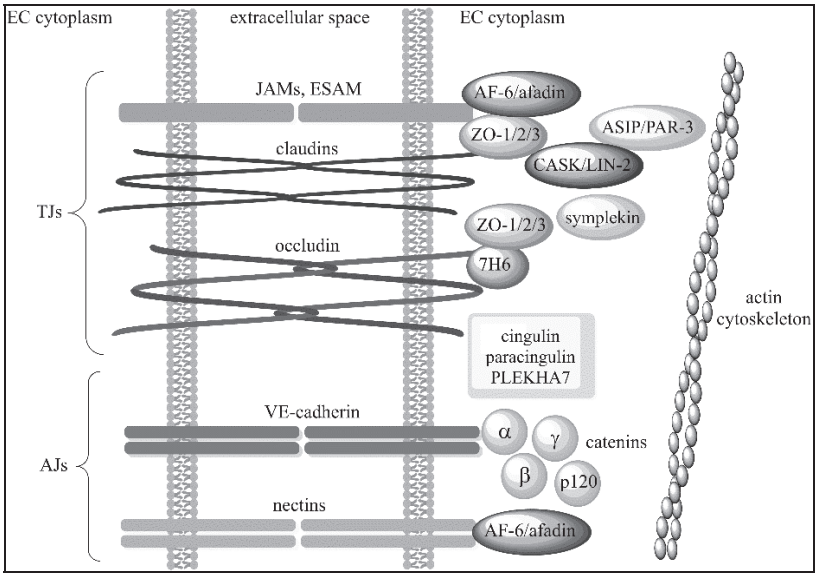

Figure 3-2: Schematic structure of the main proteins of the tight junctions (TJ) and adherens junctions (AJ) that constitute the junctional complexes between adjacent endothelial cells.

Tight junctions (TJ)

TJ are connected areas in the upper part of the apical section of the plasma membrane that stitch EC together. TJ constitute the first and main seal that regulates the paracellular transport between cells. Endothelial BBB TJ differ from the epithelial TJ in their morphological and molecular properties, and endothelial TJ are more sensitive to the microenvironment (Wolburg and Lippoldt 2002). TJ include claudins, TJ-associated MARVEL domain-containing proteins (TAM), junctional adhesion molecules (JAM) and cytoplasmic scaffolding proteins (Chiba et al. 2008,

Hartsock and Nelson 2008, Wolburg et al. 2009). The BBB TJ are very sensitive to CNS and circulating factors, which can modulate the properties and function of intercellular transport in a matter of minutes (Wolburg et al. 2003).

Claudins

Claudins are integral membrane proteins consisting of four transmembrane domains with the N- and C-terminals located in the cytoplasm, and two extracellular loops which show the highest degree of conservation between species (Tajes et al. 2014). The first detected claudins isolated from chicken liver junctional fractions were called claudin-1 and claudin-2. The number of these membrane proteins has increased to over 27 isoforms of claudins, among which claudin-3, -5 and -12 seem to have a more relevant presence and role in the BBB (Schrade et al. 2012, Jia et al. 2014). Functional studies have proven that the different combination and stoichiometry of the claudin isoforms directly determines the type and function of the barrier and varies between species and in pathological situations (Tsukita and Furuse 1999, Furuse et al. 2001). As an example, while rarely expressed in the BBB, claudin-1 is present in pathological conditions such as stroke, where the upregulation of claudin-1 mRNA corresponds to a downregulation in claudin-5, and the incorporation of claudin-1 in BBB TJ impedes BBB recovery and causes leakiness during post-stroke recovery (Sladojevic et al. 2018).

TJ-associated MARVEL domain-containing proteins (TAM)

TAM proteins are characterized by four transmembrane domains that share some homology with myelin and lymphocyte-associated protein (MAL) (Raleigh et al. 2010). The three members of this family that are involved in TJ formation are occludin, tricellulin and MarvelD3 (Van Itallie and Anderson 2014); they have both independent and overlapping functions (Raleigh et al. 2010).

Occludin was the first TJ transmembrane molecule discovered (Furuse et al. 1993). Like claudins, occludin also has four transmembrane domains with two extracellular loops and three intracytoplasmic domains, but it shares no sequence homology with claudins. Although occludin is mostly concentrated at TJ, a smaller fraction can be detected on the lateral membrane, depending on the degree of phosphorylation of the occludin (Wong 1997). It seems that, unlike claudins, occludin is not required for the formation of TJ strands, but it is needed for mature EC to regulate their

barrier permeability and obtain a proper sealing of the TJ (Lacaz-Vieira et al. 1999, Balda et al. 2000, Wolburg and Lippoldt 2002). This was proven by studying occludin-deficient mice and observing that the transepithelial resistance in intestinal epithelial cells and TJ morphology remained unaffected (Saitou et al. 2000). However, the occludin-deficient mice developed several abnormalities such as chronic inflammation and hyperplasia of the gastric epithelium (Saitou et al. 2000).

The occludin-related protein tricellulin was the first TJ protein found at tricellular TJ, the specialized structures that are formed where three cells meet together (Mariano et al. 2011), although it has been shown to also participate in the epithelial barrier and bicellular TJ. It consists of a four-pass transmembrane protein with a C-terminal sequence that shares 32% of sequence identity with that of occludin. However, the N-terminal cytoplasmic domain of tricellulin is longer than that of occludin (Chiba et al. 2008). The expression of the transcriptional repressor Snai1 represses the expression of tricellulin, as well as of claudins and occludin, contributing to the epithelial-to-mesenchymal transition (Ikenouchi et al. 2003, Ikenouchi et al. 2005). Tricellulin has been demonstrated to have an essential role in the epithelial barriers involved in normal hearing, and mutations in this gene lead to deafness (Riazuddin et al. 2006).

MarvelD3 is widely expressed in epithelial barriers and has a longer N-terminal and shorter C-terminal domain than occludin. MarvelD3 partially co-localizes with occludin and can be co-immunoprecipitated with both occludin and tricellulin, while the latter two do not co-immunoprecipitate with each other. As happens with occludin, MarvelD3 is not essential for the formation of functional TJ, although its depletion results in monolayers with decreased TEER (Steed et al. 2009, Raleigh et al. 2010, Van Itallie and Anderson 2014).

Junctional adhesion molecules (JAM)

JAM belong to the immunoglobulin superfamily, and consist of a single transmembrane domain, two large immunoglobulin-like loops, and one cytoplasmic tail of variable length containing a type II PDZ domain. They are localized at the TJ of polarized cells and on the cell surface of leukocytes (Garrido-Urbani, Bradfield, and Imhof 2014, Tajes et al. 2014). The JAM family is composed of seven members: three classical (JAM-A/JAM-1, JAM-B/JAM-2, JAM-C/JAM-3) and four non-classical JAM-related proteins (JAM-D/JAM-4, JAM-L, coxsackie adenovirus receptor (CAR) and endothelial selective adhesion molecule (ESAM). The three classical JAM share 32-38% of sequence identity among them (Aurrand-

Lions et al. 2000) while they share only 14-18 % with the non-classical JAM (Aurrand-Lions et al. 2000, Garrido-Urbani, Bradfield, and Imhof 2014). The main difference between the two groups resides in their cytoplasmic tail, which in non-classical JAM is longer and contains a type I PDZ binding motif (Garrido-Urbani, Bradfield, and Imhof 2014). They also differ in their location. JAM-1 is expressed in endothelial and epithelial cells, whereas JAM-2 and JAM-3 are expressed in most vascular EC (Wolburg and Lippoldt 2002). JAM-4, originally identified as a ligand of the membrane-associated guanylate kinase inverted 1 (MAGI-1) (Hirabayashi et al. 2003), is normally found at TJ in intestinal and mammary epithelial cells (Kansaku et al. 2006). JAM-L and CAR are predominantly expressed in epithelial cells lining the body cavities (Moog-Lutz et al. 2003, Raschperger et al. 2006, Chiba et al. 2008), where they interact with each other and mediate the activation of the epithelial γδ T cells during tissue repair (Witherden et al. 2010). Finally, ESAM is expressed in EC, megakaryocytes and platelets (Wolburg and Lippoldt 2002).

JAM play an important role in the regulation of cell polarity, the organization of the TJ structure and leukocyte extravasation and migration (Wolburg and Lippoldt 2002, Garrido-Urbani, Bradfield, and Imhof 2014). JAM can act as monomers, forming homophilic interactions via their intracellular domains, and can also form dimers with other JAM members or with integrins (Kummer and Ebnet 2018).

Submembrane TJ-associated proteins

The previously mentioned TJ proteins are further anchored and stabilized to the actin cytoskeleton through a number of cytoplasmic scaffolding and adaptor proteins, such as zonula occludens (ZO-1, ZO-2, ZO-3), cingulin, paracingulin, PLEKHA7, symplekin, 7H6, AF-6/afadin, ASIP and CASK/LIN-2 (Bazzoni et al. 2000, Schmidt et al. 2004, Abbott et al. 2010).

ZO proteins are members of the membrane-associated guanylate kinase (MAGuK) family, which contains one or more PDZ domains, an src-homology 3 (SH3) domain, and a guanylate kinase-like (GuK) domain (Wolburg and Lippoldt 2002, Schmidt et al. 2004). These domains are essential for signal transduction and to anchor the transmembrane TJ proteins to the cytoskeleton. Thus, while PDZ domains bind to the C-terminal cytoplasmic ends of transmembrane proteins, SH3-domains bind to signaling proteins and cytoskeletal elements, and GuK domains catalyze the ATP-dependent phosphorylation of GMP into GDP (Schmidt et al.

2004). ZO (especially ZO-1 and ZO-2) are the primary cytoplasmic actin and myosin binding proteins. In their absence, the formation of TJ is completely disrupted and claudins fail to polymerize in epithelial cells. The introduction of ZO-2 into cells lacking both ZO-1 and ZO-2 rescues TJ formation, whereas ZO-3 failed to be recruited to the JC in the same cells, suggesting that ZO-3 is not as indispensable for the formation of TJ (Shin and Margolis 2006, Umeda et al. 2006).

Cingulin, paracingulin, and PLEKHA7 are localized in the cytoplasmic region of the apical JC. While cingulin has been found at the TJ, PLEKHA7 has been detected at AJ, and paracingulin has been found at both TJ and AJ (Shah et al. 2016). Cingulin and paracingulin regulate the activity of the Rho and Rac1 GTPases, essential for the establishment and maintenance of JC, by interacting with their guanidine exchange factors (Wolburg and Lippoldt 2002, Guillemot, Paschoud, Jond, et al. 2008, Guillemot et al. 2014). PLEKHA7 and paracingulin are also part of a protein complex that links E-cadherin to the microtubule cytoskeleton at AJ (Citi, Pulimeno, and Paschoud 2012). Knock-out studies demonstrated that cingulin is dispensable for TJ structure and barrier function, although it plays a role in the control of claudin-2 expression. This could be due to the presence of paracingulin, which is structurally related to cingulin and has partially overlapping functions (Guillemot et al. 2012).

Another protein involved in the formation of JC is symplekin, a ubiquitously expressed protein with roles in mRNA polyadenylation, cell proliferation and differentiation, and tumorigenesis. In polarized epithelial cells, symplekin forms complexes with ZO-1, regulating the assembly of TJ and contributing to the maintenance of barrier integrity and cellular polarity (Keon et al. 1996, Guillemot, Paschoud, Pulimeno, et al. 2008, Chang, Zhang, and Cao 2012).

The Ras target ALL-1 fusion partner from chromosome 6 (AF-6/afadin) plays an essential role in the early polarization of the apical JC. AF-6 interacts with ZO-1 and JAM via its PDZ domain, acting as a peripheral component of TJ in epithelial cells. It is also present in the AJ, being recruited to nectin-based cell-cell adhesion sites (Yamamoto et al. 1997, Ebnet et al. 2000, Huang, Guilford, and Thiery 2012). MAGI-1 and -3 are also PDZ domain-containing scaffolding proteins that are found at epithelial TJ. As mentioned above, MAGI-1 interacts with JAM-4 (Hirabayashi et al. 2003), as well as with the actin-binding proteins α-actinin-4 and synaptopodin (Patrie et al. 2002). Another PDZ domain-containing scaffolding protein localized at TJ is the so-called multiple PDZ domain protein (MPDZ/MUPP-1). MPDZ contains 13 PDZ domains through which it interacts with multiple TJ proteins, including claudins

and JAM (Hamazaki et al. 2002, Jeansonne et al. 2003, Van Itallie and Anderson 2014).

Additional proteins linked to the submembranous cytoskeletal region of the TJ include: 7H6, essential for the regulation of paracellular barrier function in epithelial and vascular EC (Zhong et al. 1993, Satoh et al. 1996); ASIP/PAR-3, found in the apical part of TJ and that promotes their formation through interaction with atypical protein kinase C (Hirose et al. 2002); and calcium/calmodulin-dependent serine protein kinase (CASK/LIN-2), a protein related to membrane-associated guanylate kinases that interacts with JAM via a PDZ domain (Martínez-Estrada et al. 2001).

Adherens junctions (AJ)

AJ are the second component of JC present between EC within the BBB, and they differ from TJ in several characteristics. While TJ are found in the most apical part of the upper lateral membrane, AJ are organized in a lateral distribution joining the actin filaments of neighboring cells together (Garrido-Urbani, Bradfield, and Imhof 2014). Furthermore, TJ form a continuous tubular structure that seals the EC barrier, whereas AJ initiate and mediate the maturation and maintenance of the cell-cell contacts (Hartsock and Nelson 2008, Tajes et al. 2014).

AJ, similar to TJ, are organized into transmembrane cadherin and nectin proteins, mostly responsible for adhesion between cells, and a series of cytoplasmic/scaffolding proteins (Stamatovic et al. 2016). Hence, they include proteins from several functional families. The first includes classical cadherins (such as E-, N-, P-, and VE-cadherin), which consist of transmembrane proteins that form homodimers with cadherins from adjacent EC (Ivanov, Philippova, and Tkachuk 2001, Wei and Huang 2013). The intracellular domains of the classical cadherins provide a scaffold for the so-called armadillo family members, such as β-catenin, plakoglobin/γ-catenin, and p120-catenin. This second functional family is anchored to cytoskeletal components, such as actin filaments and microtubules, through cytoskeletal adapter proteins like α-catenin (Meng and Takeichi 2009, Green et al. 2010). In addition, several non-classical cadherins and the nectin family of IgG superfamily proteins are also localized in AJ (Takeichi 2006, Hartsock and Nelson 2008, Wei and Huang 2013).

Cadherins

Cadherins are calcium-dependent membrane-associated glycoproteins essential for cell-cell contact. Their extracellular portion consists of a variable number of highly homologous extracellular cadherin domains (EC domains) of approximately 110 amino acid residues each. Classical cadherins differ from non-classical cadherins in that they have five extracellular cadherin domains commonly designated as EC1-EC5 (Ivanov, Philippova, and Tkachuk 2001). The first three cadherins discovered were named according to the tissues where they were found: E-cadherin is present on mature epithelial cells; N-cadherin on nerves and muscles; and P-cadherin on placental and epidermal cells (Bruce Alberts 2002). In brain EC, the main transmembrane AJ protein is the vascular endothelial cadherin (VE-cadherin/CDH5/CD144), although some levels of N- and E-cadherins can also be found (Stamatovic et al. 2016). VE-cadherin not only mediates cell adhesion but is also essential for EC survival, which depends on the binding of the vascular endothelial growth factor (VEGF) to its receptor, that uses VE-cadherin as a co-receptor (Bruce Alberts 2002).

Catenins

In the BBB, VE-, N- and E-cadherins use their C-terminal membrane-distal regions to recruit β-, γ- and p120-catenins through their armadillo domains (Miller et al. 2013). These three catenins, in turn, bind to actin-binding proteins like α-catenin, vinculin, and epithelial protein lost in neoplasm (EPLIN) (Carisey and Ballestrem 2011, Chervin-Petinot et al. 2012, Gavard 2014). Hence, catenins serve as bridges between the cadherin multimers and the cytoskeleton (Gavard 2014).

Catenins are, however, multifaceted proteins that not only have a structural function in maintaining the stability of the BBB. For example, β-catenin is also an essential transducer of the Wnt signaling cascade, functioning as a transcription factor in the nucleus (Gavard 2014). Loss of β-catenin has induced BBB breakdown and downregulation of claudin-1 and -3 in adult brain EC (Tran et al. 2016). γ-catenin has some overlapping functions with β-catenin, and it also regulates transcription within the nucleus. Although β- and γ-catenin modulate many of the same genes, the role of β-catenin in the canonical Wnt pathway appears more direct (Miller et al. 2013). Thus, γ-catenin can compensate for β-catenin loss at AJ in the BBB maintenance but is unable to fulfil its functions in the Wnt signaling (Wickline et al. 2013). p120-catenin also regulates gene expression, and it

is a key regulator of VE-cadherin expression, as well as the trafficking thereof, and stability at the plasma membrane (Xiao et al. 2005). However, VE-cadherin rescue is not enough to recover endothelial barrier integrity in p120-depleted cells (Herron et al. 2011).

Regarding the so-called actin-binding proteins, it was traditionally accepted that α-catenin mediated the cadherin-actin bridge by directly binding to the actin cytoskeleton. Yet in 2005, Yamada and colleagues demonstrated that α-catenin binding to β-catenin and actin filaments were mutually exclusive. Furthermore, neither the contribution of vinculin was sufficient to mediate actin binding to the cadherin-catenin complex (Gates and Peifer 2005, Yamada et al. 2005, Chervin-Petinot et al. 2012). Several years of studies have revealed that the interactions of catenins with the cadherins and the cytoskeleton are much more flexible than initially expected. This led to a shift from the classical quaternary complex cadherins/β-catenin/α-catenin/actin to a model where α-catenin, alone or by forming homo/heterodimers, interacts with a range of actin regulators such as α-actinin, vinculin, EPLIN, spectrin, ZO-1, afadin, etc. to modulate actin dynamics and organization (Scott and Yap 2006, Pokutta et al. 2008, Wickline et al. 2016).

Nectins

Nectins are calcium-independent immunoglobulin-like intercellular adhesion molecules that compose a family of four members, named 1-4. Furthermore, each member has two or three splicing variants, differentiated by a Greek letter (e.g. nectin-1α) (Takai and Nakanishi 2003, Takai et al. 2008, Rikitake and Takai 2011). Each member of the nectin family initially forms homodimers by cis-dimerization through their extracellular domains. Cis-clusters of nectins homophilically and heterophilically interact in trans with each other, and can also heterophilically interact in trans with other immunoglobulin-like molecules, like Necls, CD96, CD226, and TIGIT (Rikitake and Takai 2011). The same way cadherins are bound to catenins, the intracellular regions of the nectins interact with the actin filament (F-actin)-binding protein afadin (Takai et al. 2008, Indra et al. 2013). In fact, nectin-afadin complexes are the first molecular constituents to be recruited to the AJ to initiate cell-cell contacts before the arrival of cadherin-catenin complexes (Takai et al. 2008, Rikitake and Takai 2011).

Several molecular interactions have been described between nectin and cadherin adhesive systems (Takai et al. 2008). In addition to the previously mentioned interaction of afadin with α-catenin, afadin has been

shown to form a complex with p120-catenin (Birukova et al. 2012), and cadherin and nectin could also interact through their ectodomains (Morita et al. 2010, Indra et al. 2013). Nevertheless, the cadherin and nectin-enriched clusters that form the AJ seem to be independent of one another, as they can be modified in size and even be completely assembled or disassembled without affecting the adjacent clusters (Indra et al. 2013).

Additional molecules can be found in the BBB cell-cell contacts outside TJ and AJ, such as PECAM-1/CD31 and MIC2/CD99 (Privratsky and Newman 2014, Tietz and Engelhardt 2015, Lertkiatmongkol et al. 2016). At the endothelial JC, CD31 functions as an adhesive stress-response mechanosensor, as a regulator of leukocyte trafficking, and in the maintenance of EC junctional integrity, as well as restoration of the vascular permeability after inflammation (Privratsky and Newman 2014, Lertkiatmongkol et al. 2016). CD99 is a highly O-glycosylated protein also involved in the regulation of the leukocyte transendothelial migration without affecting adhesion in BBB (Winger et al. 2014, Winger et al. 2016).

Barriergenesis of the BBB

The emergence of BBB characteristics during neurovascular development and maturation occurs gradually, due to a progressive tightening caused by increased expression of JC proteins and decreased transcellular transport (Butt, Jones, and Abbott 1990, Siegenthaler, Sohet, and Daneman 2013). In this progressive transformation, the presence of cells such as pericytes, neurons and microglia in the microenvironment plays an essential role (Abbott, Ronnback, and Hansson 2006, Nakagawa et al. 2009). Some of the earliest events observed during BBB development are the formation of TJ and the expression of glucose transporter Glut-1 (Siegenthaler, Sohet, and Daneman 2013).

It is widely accepted that BBB properties are not inherent to CNS EC, but that the neural microenvironment provides key inductive signals that confer the CNS EC with such BBB properties (Chow and Gu 2015). Hence, brain EC cells lose their barrier characteristics in cell culture and resemble peripheral EC (Wolburg and Lippoldt 2002). On the other hand, BBB properties such as high TEER and development of TJ can be induced by modifications in the microenvironment (Lippmann et al. 2012, Ribecco-Lutkiewicz et al. 2018). Such inducers from the microenvironment can be diverse in nature, as recently demonstrated using a BBB model of brain endothelial cells generated from human amniotic fluid-derived induced pluripotent stem cells (AF-iPSC), where the TEER

increased up to 1500 Ω cm^2 when induced by astrocyte-derived molecular cues and RA treatment, polarized expression of functional efflux transporters and receptor-mediated transcytosis triggered by antibodies against specific receptors (Ribecco-Lutkiewicz et al. 2018). In the 80s-90s, several studies suggested that humoral factors released by astrocytes contributed to TJ formation (Arthur, Shivers, and Bowman 1987, Dehouck et al. 1994, Wolburg et al. 1994), while others defended that direct contact between astrocytes and EC was required (Tao-Cheng, Nagy, and Brightman 1987). Finally, a series of transplantation experiments led to the conclusion that, although factors released from astrocytes were necessary, they were not sufficient to maintain BBB characteristics (Wolburg and Lippoldt 2002). Some authors also claim that pericytes can also induce BBB phenotype in EC via humoral factors (Cecchelli et al, 2014, Mossu et al, 2018).

There are multiple factors involved in the plasticity and genetic programming that leads to the development of the BBB phenotype, many of which are still under study. One of the classically accepted secreted factors is the glial cell line-derived neurotrophic factor (GDNF) (Igarashi et al. 1999, Utsumi et al. 2000). Astrocyte-derived RA has also been demonstrated to induce barrier properties in cultured human brain EC (Mizee et al. 2013). Another astrocyte-derived factor involved is the sonic hedgehog (Shh), essential for BBB integrity, as its disruption in EC causes BBB dysfunction through the suppressed expression of TJ proteins and extravasation of plasma proteins (Alvarez et al. 2011, Chow and Gu 2015). In addition, astrocytes produce angiotensinogen and cleave it into angiotensin. The latter binds and activates angiotensin receptors in brain EC, modifying proteins such as occludin to promote the efficient organization of TJ (Wosik et al. 2007).

Furthermore, crosstalk between components of TJ and AJ seems to play a role in JC regulation, including the expression of several components. As an example, extracellular calcium, which is essential for cadherins within AJ, was also shown to regulate TJ integrity (Brown and Davis 2002). VE-cadherin within AJ upregulates the expression of claudin-5 in the TJ, via activation of the transcription factor Foxo1 (Taddei et al. 2008, Weiss et al. 2009). Moreover, phosphorylation of proteins of JC, such as occludin and ZO-1, regulate TJ integrity and BBB permeability (Sakakibara et al. 1997, Yamamoto et al. 2008). In this regard, VEGF seems to alter TJ assembly and increase BBB permeability through occludin phosphorylation and/or degradation (Antonetti et al. 1999, Hirase et al. 2001, Wang, Dentler, and Borchardt 2001).

Many genetic programs and molecular pathways are also involved in BBB barriergenesis. The most well-characterized genetic program inducing BBB characteristics in CNS EC is the Wnt/β-catenin signaling, which in brain EC is involved in the early expression of the glucose transporter Glut-1 and CNS-specific angiogenesis (Liebner et al. 2008, Stenman et al. 2008, Daneman et al. 2009). TJ structure and function are also regulated by the Rho and Rac GTPases, whose activation leads to actin cytoskeleton rearrangements and increased paracellular permeability, as well as trans-endothelial migration of leukocytes (Jou, Schneeberger, and James Nelson 1998, Adamson et al. 1999, Weiss et al. 2009). Another gene that regulates BBB barriergenesis is the lipolysis-stimulated lipoprotein receptor (LSR). Although LSR knockout mice did not display vascular malformations or hemorrhage, they did show a marked BBB dysfunction (Chow and Gu 2015, Sohet et al. 2015). Finally, recent studies have focused on environmental factors extrinsic to the CNS that can impact BBB development and integrity as well. One surprising example is the gut microbiota, which has been shown to regulate the BBB through epigenetic control of TJ factors expression in CNS EC. Metabolic products from the microbiota, such as short-chain fatty acids, are released into the blood and can alter BBB integrity and modify BBB transport rates (Braniste et al. 2014, Logsdon et al. 2017, Wang et al. 2018).

Our understanding of BBB structure and function has greatly improved since the first experiments with aniline dyes were performed about a century ago. However, we have just started to understand many of the mechanisms involved in its regulation and how its disruption is involved in multiple diseases, such as cancer, Alzheimer's disease, and neurodegeneration in the ageing process.

References

Abbott, N. J., L. Ronnback, and E. Hansson. 2006. "Astrocyte-endothelial interactions at the blood-brain barrier." *Nat. Rev. Neurosci.* 7 (1):41-53. doi: 10.1038/nrn1824.

Abbott, N. Joan. 2005. "Dynamics of CNS Barriers: Evolution, Differentiation, and Modulation." *Cell Mol. Neurobiol.* 25 (1):5-23. doi: 10.1007/s10571-004-1374-y.

Abbott, N. Joan, Adjanie A. K. Patabendige, Diana E. M. Dolman, Siti R. Yusof, and David J. Begley. 2010. "Structure and function of the blood–brain barrier." *Neurobiol. Dis.* 37 (1):13-25. doi: https://doi.org/10.1016/j.nbd.2009.07.030.

Adamson, Peter, Sandrine Etienne, Pierre-Olivier Couraud, Virginia Calder, and John Greenwood. 1999. "Lymphocyte Migration Through Brain Endothelial Cell Monolayers Involves Signaling Through Endothelial ICAM-1 Via a Rho-Dependent Pathway." *J. Immunol.* 162 (5):2964-2973.

Alvarez, J. I., A. Dodelet-Devillers, H. Kebir, I. Ifergan, P. J. Fabre, S. Terouz, M. Sabbagh, K. Wosik, L. Bourbonniere, M. Bernard, J. van Horssen, H. E. de Vries, F. Charron, and A. Prat. 2011. "The Hedgehog pathway promotes blood-brain barrier integrity and CNS immune quiescence." *Science* 334 (6063):1727-1731. doi: 10.1126/science.1206936.

Antonetti, David A., Alistair J. Barber, Leigh Ann Hollinger, Ellen B. Wolpert, and Thomas W. Gardner. 1999. "Vascular Endothelial Growth Factor Induces Rapid Phosphorylation of Tight Junction Proteins Occludin and Zonula Occluden 1: A potential mechanism for vascular permeability in diabetic retinopathy and tumors." *J. Biol. Chem.* 274 (33):23463-23467. doi: 10.1074/jbc.274.33.23463.

Arthur, F. E., R. R. Shivers, and P. D. Bowman. 1987. "Astrocyte-mediated induction of tight junctions in brain capillary endothelium: an efficient in vitro model." *Brain Res.* 433 (1):155-159.

Aurrand-Lions, M. A., L. Duncan, L. Du Pasquier, and B. A. Imhof. 2000. "Cloning of JAM-2 and JAM-3: an emerging junctional adhesion molecular family?" *Curr. Top. Microbiol. Immunol.* 251:91-98.

Balda, Maria S., Catalina Flores-Maldonado, Marcelino Cereijido, and Karl Matter. 2000. "Multiple domains of occludin are involved in the regulation of paracellular permeability." *J. Cell. Biochem.* 78 (1):85-96. doi: doi:10.1002/(SICI)1097-4644(20000701)78:1<85::AID-JCB8>3.0.CO;2-F.

Bazzoni, Gianfranco, Ofelia Maria Martínez-Estrada, Fabrizio Orsenigo, Michelangelo Cordenonsi, Sandra Citi, and Elisabetta Dejana. 2000. "Interaction of Junctional Adhesion Molecule with the Tight Junction Components ZO-1, Cingulin, and Occludin." *J. Biol. Chem.* 275 (27):20520-20526. doi: 10.1074/jbc.M905251199.

Bergers, Gabriele, and Steven Song. 2005. "The role of pericytes in blood-vessel formation and maintenance." *Neuro-oncology* 7 (4):452-464. doi: 10.1215/S1152851705000232.

Betz, A. Lorris, and Gary W. Goldstein. 1978. "Polarity of the Blood-Brain Barrier: Neutral Amino Acid Transport into Isolated Brain Capillaries." *Science* 202 (4364):225-227.

Birukova, A. A., P. Fu, T. Wu, O. Dubrovskyi, N. Sarich, V. Poroyko, and K. G. Birukov. 2012. "Afadin controls p120-catenin-ZO-1 interactions

leading to endothelial barrier enhancement by oxidized phospholipids." *J. Cell. Physiol.* 227 (5):1883-1890. doi: 10.1002/jcp.22916.

Braniste, V., M. Al-Asmakh, C. Kowal, F. Anuar, A. Abbaspour, M. Toth, A. Korecka, N. Bakocevic, L. G. Ng, P. Kundu, B. Gulyas, C. Halldin, K. Hultenby, H. Nilsson, H. Hebert, B. T. Volpe, B. Diamond, and S. Pettersson. 2014. "The gut microbiota influences blood-brain barrier permeability in mice." *Sci. Transl. Med.* 6 (263):263ra158. doi: 10.1126/scitranslmed.3009759.

Brown, R. C., and T. P. Davis. 2002. "Calcium modulation of adherens and tight junction function: a potential mechanism for blood-brain barrier disruption after stroke." *Stroke* 33 (6):1706-1711.

Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and Peter Walter. 2002. *Molecular Biology of the Cell. 4th edition*. New York: Garland Science; 2002. Available from: https://www.ncbi.nlm.nih.gov/books/NBK21054/.

Butt, A. M., H. C. Jones, and N. J. Abbott. 1990. "Electrical resistance across the blood-brain barrier in anaesthetized rats: a developmental study." *J. Physiol.* 429:47-62.

Candelario-Jalil, E., Y. Yang, and G. A. Rosenberg. 2009. "Diverse roles of matrix metalloproteinases and tissue inhibitors of metalloproteinases in neuroinflammation and cerebral ischemia." *Neurosci.* 158 (3):983-994. doi: 10.1016/j.neuroscience.2008.06.025.

Carisey, Alex, and Christoph Ballestrem. 2011. "Vinculin, an adapter protein in control of cell adhesion signalling." *Eur. J. Cell Biol.* 90 (2-3):157-163. doi: 10.1016/j.ejcb.2010.06.007.

Chang, Hong, Chen Zhang, and Yi Cao. 2012. "Expression and distribution of symplekin regulates the assembly and function of the epithelial tight junction." *Histochem. Cell Biol.* 137 (3):319-327. doi: 10.1007/s00418-011-0906-z.

Chervin-Petinot, A., M. Courcon, S. Almagro, A. Nicolas, A. Grichine, D. Grunwald, M. H. Prandini, P. Huber, and D. Gulino-Debrac. 2012. "Epithelial protein lost in neoplasm (EPLIN) interacts with alpha-catenin and actin filaments in endothelial cells and stabilizes vascular capillary network in vitro." *J. Biol. Chem.* 287 (10):7556-7572. doi: 10.1074/jbc.M111.328682.

Chiba, Hideki, Makoto Osanai, Masaki Murata, Takashi Kojima, and Norimasa Sawada. 2008. "Transmembrane proteins of tight junctions." *Biochim. Biophys. Acta, Biomembranes* 1778 (3):588-600. doi: https://doi.org/10.1016/j.bbamem.2007.08.017.

Chow, Brian Wai, and Chenghua Gu. 2015. "The molecular constituents of the blood-brain barrier." *Trends Neurosci.* 38 (10):598-608. doi: 10.1016/j.tins.2015.08.003.

Citi, S., P. Pulimeno, and S. Paschoud. 2012. "Cingulin, paracingulin, and PLEKHA7: signaling and cytoskeletal adaptors at the apical junctional complex." *Ann. N. Y. Acad. Sci.* 1257:125-132. doi: 10.1111/j.1749-6632.2012.06506.x.

Cunningham, L. A., M. Wetzel, and G. A. Rosenberg. 2005. "Multiple roles for MMPs and TIMPs in cerebral ischemia." *Glia* 50 (4):329-339. doi: 10.1002/glia.20169.

Daneman, R., D. Agalliu, L. Zhou, F. Kuhnert, C. J. Kuo, and B. A. Barres. 2009. "Wnt/beta-catenin signaling is required for CNS, but not non-CNS, angiogenesis." *Proc. Natl. Acad. Sci. U.S.A.* 106 (2):641-646. doi: 10.1073/pnas.0805165106.

Dehouck, B., M. P. Dehouck, J. C. Fruchart, and R. Cecchelli. 1994. "Upregulation of the low density lipoprotein receptor at the blood-brain barrier: intercommunications between brain capillary endothelial cells and astrocytes." *J. Cell Biol.* 126 (2):465-473.

Duchemin, Sonia, Michael Boily, Nataliya Sadekova, and Helene Girouard. 2012. "The complex contribution of NOS interneurons in the physiology of cerebrovascular regulation." *Front. Neural Circuits* 6 (51). doi: 10.3389/fncir.2012.00051.

Ebnet, Klaus, C. U. Schulz, M. K. Meyer Zu Brickwedde, G. G. Pendl, and Dietmar Vestweber. 2000. "Junctional Adhesion Molecule Interacts with the PDZ Domain-containing Proteins AF-6 and ZO-1." *J. Biol. Chem.* 275. doi: 10.1074/jbc.M002363200.

Furuse, M, T Hirase, M Itoh, A Nagafuchi, S Yonemura, S Tsukita, and S Tsukita. 1993. "Occludin: a novel integral membrane protein localizing at tight junctions." *J. Cell Biol.* 123 (6):1777-1788. doi: 10.1083/jcb.123.6.1777.

Furuse, Mikio, Kyoko Furuse, Hiroyuki Sasaki, and Shoichiro Tsukita. 2001. "Conversion of Zonulae Occludentes from Tight to Leaky Strand Type by Introducing Claudin-2 into Madin-Darby Canine Kidney I Cells." *J. Cell Biol.* 153 (2):263-272. doi: 10.1083/jcb.153.2.263.

Garrido-Urbani, S., P. F. Bradfield, and B. A. Imhof. 2014. "Tight junction dynamics: the role of junctional adhesion molecules (JAM)." *Cell Tissue Res.* 355 (3):701-715. doi: 10.1007/s00441-014-1820-1.

Gates, Julie, and Mark Peifer. 2005. "Can 1000 Reviews Be Wrong? Actin, alpha-Catenin, and Adherens Junctions." *Cell* 123 (5):769-772. doi: 10.1016/j.cell.2005.11.009.

Gavard, Julie. 2014. "Endothelial permeability and VE-cadherin: a wacky comradeship." *Cell Adh. Migr.* 8 (2):158-164. doi: 10.4161/cam.29026.

Goldmann, E. E. 1909. "Die äussere und innere Sekretion des gesunden und kranken Organismus im Lichte der 'vitalen Färbung'." *Beiträg Klinische Chirurgie* 64:192–265.

Goldmann, E. E. 1913. "Vitalfärbung am Zentralnervensyatem. Beitrag zur Physio-Pathologie des plexus chorioideus und der Hirnhaute." *Abh Preuss, Akad Wiss Phys-Math Kl* 1:1–60.

Green, K. J., S. Getsios, S. Troyanovsky, and L. M. Godsel. 2010. "Intercellular junction assembly, dynamics, and homeostasis." *Cold Spring Harb. Perspect. Biol.* 2 (2):a000125. doi: 10.1101/cshperspect.a000125.

Guillemot, Laurent, Diego Guerrera, Domenica Spadaro, Rocio Tapia, Lionel Jond, Sandra Citi, and Benjamin Margolis. 2014. "MgcRacGAP interacts with cingulin and paracingulin to regulate Rac1 activation and development of the tight junction barrier during epithelial junction assembly." *Mol. Biol. Cell* 25 (13):1995-2005. doi: 10.1091/mbc.e13-11-0680.

Guillemot, Laurent, Serge Paschoud, Lionel Jond, Andrea Foglia, and Sandra Citi. 2008. "Paracingulin regulates the activity of Rac1 and RhoA GTPases by recruiting Tiam1 and GEF-H1 to epithelial junctions." *Mol. Biol. Cell* 19 (10):4442-4453. doi: 10.1091/mbc.E08-06-0558.

Guillemot, Laurent, Serge Paschoud, Pamela Pulimeno, Andrea Foglia, and Sandra Citi. 2008. "The cytoplasmic plaque of tight junctions: A scaffolding and signalling center." *Biochim. Biophys. Acta, Biomembranes* 1778 (3):601-613. doi: https://doi.org/10.1016/j.bbamem.2007.09.032.

Guillemot, Laurent, Yann Schneider, Paola Brun, Ignazio Castagliuolo, Daniela Pizzuti, Diego Martines, Lionel Jond, Massimo Bongiovanni, and Sandra Citi. 2012. "Cingulin is dispensable for epithelial barrier function and tight junction structure, and plays a role in the control of claudin-2 expression and response to duodenal mucosa injury." *J. Cell Sci.* 125 (21):5005-5014. doi: 10.1242/JC.101261.

Hamazaki, Y., M. Itoh, H. Sasaki, M. Furuse, and S. Tsukita. 2002. "Multi-PDZ domain protein 1 (MUPP1) is concentrated at tight junctions through its possible interaction with claudin-1 and junctional adhesion molecule." *J. Biol. Chem.* 277 (1):455-461. doi: 10.1074/jbc.M109005200.

Hammes, Hans-Peter, Jihong Lin, Oliver Renner, Moshe Shani, Andrea Lundqvist, Christer Betsholtz, Michael Brownlee, and Urban Deutsch. 2002. "Pericytes and the Pathogenesis of Diabetic Retinopathy." *Diabetes* 51 (10):3107-3112. doi: 10.2337/diabetes.51.10.3107.

Harder, D. R., C. Zhang, and D. Gebremedhin. 2002. "Astrocytes function in matching blood flow to metabolic activity." *News Physiol. Sci.* 17:27-31.

Hartsock, Andrea, and W. James Nelson. 2008. "Adherens and tight junctions: structure, function and connections to the actin cytoskeleton." *Biochim. Biophys. Acta* 1778 (3):660-669. doi: 10.1016/j.bbamem.2007.07.012.

Herndon, J. M., M. E. Tome, and T. P. Davis. 2017. "Chapter 9 - Development and Maintenance of the Blood–Brain Barrier." In *Primer on Cerebrovascular Diseases (Second Edition)*, edited by Louis R. Caplan, José Biller, Megan C. Leary, Eng H. Lo, Ajith J. Thomas, Midori Yenari and John H. Zhang, 51-56. San Diego: Academic Press.

Herron, Crystal R., Anthony M. Lowery, Patricia R. Hollister, Albert B. Reynolds, and Peter A. Vincent. 2011. "p120 regulates endothelial permeability independently of its NH2 terminus and Rho binding." *Am. J. Physiol. Heart Circ. Physiol.* 300 (1):H36-H48. doi: 10.1152/ajpheart.00812.2010.

Hirabayashi, S., M. Tajima, I. Yao, W. Nishimura, H. Mori, and Y. Hata. 2003. "JAM4, a junctional cell adhesion molecule interacting with a tight junction protein, MAGI-1." *Mol. Cell Biol.* 23 (12):4267-4282.

Hirase, Tetsuaki, Seinosuke Kawashima, Elaine Y. M. Wong, Tomomi Ueyama, Yoshiyuki Rikitake, Shoichiro Tsukita, Mitsuhiro Yokoyama, and James M. Staddon. 2001. "Regulation of Tight Junction Permeability and Occludin Phosphorylation by RhoA-p160ROCK-dependent and -independent Mechanisms." *J. Biol. Chem.* 276 (13):10423-10431. doi: 10.1074/jbc.M007136200.

Hirose, Tomonori, Yasushi Izumi, Yoji Nagashima, Yoko Tamai-Nagai, Hidetake Kurihara, Tatsuo Sakai, Yukari Suzuki, Tomoyuki Yamanaka, Atsushi Suzuki, Keiko Mizuno, and Shigeo Ohno. 2002. "Involvement of ASIP/PAR-3 in the promotion of epithelial tight junction formation." *J. Cell Sci.* 115 (12):2485-2495.

Huang, R. Y., P. Guilford, and J. P. Thiery. 2012. "Early events in cell adhesion and polarity during epithelial-mesenchymal transition." *J. Cell Sci.* 125 (Pt 19):4417-4422. doi: 10.1242/JC.099697.

Igarashi, Y., H. Utsumi, H. Chiba, Y. Yamada-Sasamori, H. Tobioka, Y. Kamimura, K. Furuuchi, Y. Kokai, T. Nakagawa, M. Mori, and N. Sawada. 1999. "Glial cell line-derived neurotrophic factor induces

barrier function of endothelial cells forming the blood-brain barrier." *Biochem. Biophys. Res. Commun.* 261 (1):108-112. doi: 10.1006/bbrc.1999.0992.

Ikenouchi, J., M. Matsuda, M. Furuse, and S. Tsukita. 2003. "Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail." *J. Cell Sci.* 116 (10):1959-1967. doi: 10.1242/JC.00389.

Ikenouchi, Junichi, Mikio Furuse, Kyoko Furuse, Hiroyuki Sasaki, Sachiko Tsukita, and Shoichiro Tsukita. 2005. "Tricellulin constitutes a novel barrier at tricellular contacts of epithelial cells." *J. Cell Biol.* 171 (6):939-945. doi: 10.1083/jcb.200510043.

Indra, Indrajyoti, Soonjin Hong, Regina Troyanovsky, Bernadett Kormos, and Sergey Troyanovsky. 2013. "The adherens junction: a mosaic of cadherin and nectin clusters bundled by actin filaments." *J. Invest. Dermatol.* 133 (11):2546-2554. doi: 10.1038/jid.2013.200.

Ivanov, D. B., M. P. Philippova, and V. A. Tkachuk. 2001. "Structure and functions of classical cadherins." *Biochemistry (Mosc)* 66 (10):1174-1186.

Jeansonne, B., Q. Lu, D. A. Goodenough, and Y. H. Chen. 2003. "Claudin-8 interacts with multi-PDZ domain protein 1 (MUPP1) and reduces paracellular conductance in epithelial cells." *Cell Mol Biol (Noisy-le-grand)* 49 (1):13-21.

Jia, W., R. Lu, T. A. Martin, and W. G. Jiang. 2014. "The role of claudin-5 in blood-brain barrier (BBB) and brain metastases (review)." *Mol. Med. Rep.* 9 (3):779-785. doi: 10.3892/mmr.2013.1875.

Jou, Tzuu-Shuh, Eveline E. Schneeberger, and W. James Nelson. 1998. "Structural and Functional Regulation of Tight Junctions by RhoA and Rac1 Small GTPases." *J. Cell Biol.* 142 (1):101-115. doi: 10.1083/jcb.142.1.101.

Kansaku, A., S. Hirabayashi, H. Mori, N. Fujiwara, A. Kawata, M. Ikeda, C. Rokukawa, H. Kurihara, and Y. Hata. 2006. "Ligand-of-Numb protein X is an endocytic scaffold for junctional adhesion molecule 4." *Oncogene* 25 (37):5071-5084. doi: 10.1038/sj.onc.1209468.

Keon, B. H., S. Schafer, C. Kuhn, C. Grund, and W. W. Franke. 1996. "Symplekin, a novel type of tight junction plaque protein." *J. Cell Biol.* 134 (4):1003-1018.

Kummer, Daniel, and Klaus Ebnet. 2018. "Junctional Adhesion Molecules (JAM): The JAM-Integrin Connection." *Cells* 7 (4):25. doi: 10.3390/cells7040025.

Lacaz-Vieira, F., M.M.M. Jaeger, P. Farshori, and B. Kachar. 1999. "Small Synthetic Peptides Homologous to Segments of the First

External Loop of Occludin Impair Tight Junction Resealing." *J. Membr. Biol.* 168 (3):289-297. doi: 10.1007/s002329900518.

Lertkiatmongkol, Panida, Danying Liao, Heng Mei, Yu Hu, and Peter J. Newman. 2016. "Endothelial functions of platelet/endothelial cell adhesion molecule-1 (CD31)." *Curr. Opin. Hematol.* 23 (3):253-259. doi: 10.1097/MOH.0000000000000239.

Liebner, S., M. Corada, T. Bangsow, J. Babbage, A. Taddei, C. J. Czupalla, M. Reis, A. Felici, H. Wolburg, M. Fruttiger, M. M. Taketo, H. von Melchner, K. H. Plate, H. Gerhardt, and E. Dejana. 2008. "Wnt/beta-catenin signaling controls development of the blood-brain barrier." *J. Cell Biol.* 183 (3):409-417. doi: 10.1083/jcb.200806024.

Lippmann, E. S., S. M. Azarin, J. E. Kay, R. A. Nessler, H. K. Wilson, A. Al-Ahmad, S. P. Palecek, and E. V. Shusta. 2012. "Derivation of blood-brain barrier endothelial cells from human pluripotent stem cells." *Nat. Biotechnol.* 30 (8):783-791. doi: 10.1038/nbt.2247.

Logsdon, Aric F., Michelle A. Erickson, Elizabeth M. Rhea, Therese S. Salameh, and William A. Banks. 2017. "Gut reactions: How the blood–brain barrier connects the microbiome and the brain." *Exp. Biol. Med.* 243 (2):159-165. doi: 10.1177/1535370217743766.

Machida, T., F. Takata, J. Matsumoto, H. Takenoshita, I. Kimura, A. Yamauchi, S. Dohgu, and Y. Kataoka. 2015. "Brain pericytes are the most thrombin-sensitive matrix metalloproteinase-9-releasing cell type constituting the blood-brain barrier in vitro." *Neurosci Lett* 599:109-114. doi: 10.1016/j.neulet.2015.05.028.

Mariano, Cibelle, Hiroyuki Sasaki, Dora Brites, and Maria Alexandra Brito. 2011. "A look at tricellulin and its role in tight junction formation and maintenance." *Eur. J. Cell Biol.* 90 (10):787-796. doi: https://doi.org/10.1016/j.ejcb.2011.06.005.

Martínez-Estrada, Ofelia Maria, Antonello Villa, Ferruccio Breviario, Fabrizio Orsenigo, Elisabetta Dejana, and Gianfranco Bazzoni. 2001. "Association of Junctional Adhesion Molecule with Calcium/calmodulin-dependent Serine Protein Kinase (CASK/LIN-2) in Human Epithelial Caco-2 Cells." *J. Biol. Chem.* 276 (12):9291-9296. doi: 10.1074/jbc.M006991200.

Meng, W., and M. Takeichi. 2009. "Adherens junction: molecular architecture and regulation." *Cold Spring Harb. Perspect. Biol.* 1 (6):a002899. doi: 10.1101/cshperspect.a002899.

Miller, Rachel K., Ji Yeon Hong, William A. Muñoz, and Pierre D. McCrea. 2013. "Beta-catenin versus the other armadillo catenins: assessing our current view of canonical Wnt signaling." *Prog. Mol.*

Biol. Transl. Sci. 116:387-407. doi: 10.1016/B978-0-12-394311-8.00017-0.

Mizee, M. R., D. Wooldrik, K. A. Lakeman, B. van het Hof, J. A. Drexhage, D. Geerts, M. Bugiani, E. Aronica, R. E. Mebius, A. Prat, H. E. de Vries, and A. Reijerkerk. 2013. "Retinoic acid induces blood-brain barrier development." *J. Neurosci.* 33 (4):1660-1671. doi: 10.1523/jneurosci.1338-12.2013.

Moog-Lutz, C., F. Cave-Riant, F. C. Guibal, M. A. Breau, Y. Di Gioia, P. O. Couraud, Y. E. Cayre, S. Bourdoulous, and P. G. Lutz. 2003. "JAML, a novel protein with characteristics of a junctional adhesion molecule, is induced during differentiation of myeloid leukemia cells." *Blood* 102 (9):3371-3378. doi: 10.1182/blood-2002-11-3462.

Morita, H., S. Nandadasa, T. S. Yamamoto, C. Terasaka-Iioka, C. Wylie, and N. Ueno. 2010. "Nectin-2 and N-cadherin interact through extracellular domains and induce apical accumulation of F-actin in apical constriction of Xenopus neural tube morphogenesis." *Development* 137 (8):1315-1325. doi: 10.1242/dev.043190.

Muoio, V., P. B. Persson, and M. M. Sendeski. 2014. "The neurovascular unit – concept review." *Acta Physiol.* 210 (4):790-798. doi: doi:10.1111/apha.12250.

Nakagawa, S., M. A. Deli, H. Kawaguchi, T. Shimizudani, T. Shimono, A. Kittel, K. Tanaka, and M. Niwa. 2009. "A new blood-brain barrier model using primary rat brain endothelial cells, pericytes and astrocytes." *Neurochem. Int.* 54 (3-4):253-263. doi: 10.1016/j.neuint.2008.12.002.

Obermeier, Birgit, Richard Daneman, and Richard M. Ransohoff. 2013. "Development, maintenance and disruption of the blood-brain barrier." *Nat. Med.* 19 (12):1584-1596. doi: 10.1038/nm.3407.

Patrie, K. M., A. J. Drescher, A. Welihinda, P. Mundel, and B. Margolis. 2002. "Interaction of two actin-binding proteins, synaptopodin and alpha-actinin-4, with the tight junction protein MAGI-1." *J. Biol. Chem.* 277 (33):30183-30190. doi: 10.1074/jbc.M203072200.

Peppiatt, Claire M., Clare Howarth, Peter Mobbs, and David Attwell. 2006. "Bidirectional control of CNS capillary diameter by pericytes." *Nature* 443:700. doi: 10.1038/nature05193.

Pócsai, Károly, Zsolt Bagyura, and Mihály Kálmán. 2010. "Components of the basal lamina and dystrophin-dystroglycan complex in the neurointermediate lobe of rat pituitary gland: different localizations of beta-dystroglycan, dystrobrevins, alpha1-syntrophin, and aquaporin-4." *J. Histochem. Cytochem.* 58 (5):463-479. doi: 10.1369/jhc.2010.954768.

Pokutta, Sabine, Frauke Drees, Soichiro Yamada, W. James Nelson, and William I. Weis. 2008. "Biochemical and structural analysis of α-catenin in cell–cell contacts." *Biochem. Soc. Trans.* 36 (2):141-147. doi: 10.1042/bst0360141.

Privratsky, Jamie R., and Peter J. Newman. 2014. "PECAM-1: regulator of endothelial junctional integrity." *Cell Tissue Res.* 355 (3):607-619. doi: 10.1007/s00441-013-1779-3.

Quintana, Francisco J. 2017. "Astrocytes to the rescue! Glia limitans astrocytic endfeet control CNS inflammation." *J. Clin. Investig.* 127 (8):2897-2899. doi: 10.1172/JCI95769.

Raleigh, D. R., A. M. Marchiando, Y. Zhang, L. Shen, H. Sasaki, Y. Wang, M. Long, and J. R. Turner. 2010. "Tight junction-associated MARVEL proteins marveld3, tricellulin, and occludin have distinct but overlapping functions." *Mol. Biol. Cell* 21 (7):1200-1213. doi: 10.1091/mbc.E09-08-0734.

Raschperger, Elisabeth, Johan Thyberg, Sven Pettersson, Lennart Philipson, Jonas Fuxe, and Ralf F. Pettersson. 2006. "The coxsackie- and adenovirus receptor (CAR) is an in vivo marker for epithelial tight junctions, with a potential role in regulating permeability and tissue homeostasis." *Exp. Cell Res.* 312 (9):1566-1580. doi: https://doi.org/10.1016/j.yexcr.2006.01.025.

Riazuddin, Saima, Zubair M. Ahmed, Alan S. Fanning, Ayala Lagziel, Shin-ichiro Kitajiri, Khushnooda Ramzan, Shaheen N. Khan, Parna Chattaraj, Penelope L. Friedman, James M. Anderson, Inna A. Belyantseva, Andrew Forge, Sheikh Riazuddin, and Thomas B. Friedman. 2006. "Tricellulin is a tight-junction protein necessary for hearing." *Am. J. Hum. Genet.* 79 (6):1040-1051. doi: 10.1086/510022.

Ribecco-Lutkiewicz, Maria, Caroline Sodja, Julie Haukenfrers, Arsalan S. Haqqani, Dao Ly, Peter Zachar, Ewa Baumann, Marguerite Ball, Jez Huang, Marina Rukhlova, Marzia Martina, Qing Liu, Danica Stanimirovic, Anna Jezierski, and Mahmud Bani-Yaghoub. 2018. "A novel human induced pluripotent stem cell blood-brain barrier model: Applicability to study antibody-triggered receptor-mediated transcytosis." *Sci. Rep.* 8 (1):1873. doi: 10.1038/s41598-018-19522-8.

Rikitake, Yoshiyuki, and Yoshimi Takai. 2011. "Chapter three - Directional Cell Migration: Regulation by Small G Proteins, Nectin-like Molecule-5, and Afadin." In *Int. Rev. Cell Mol. Biol.*, edited by Kwang W. Jeon, 97-143. Academic Press.

Sá-Pereira, Inês, Dora Brites, and Maria Alexandra Brito. 2012. "Neurovascular Unit: a Focus on Pericytes." *Mol. Neurobiol.* 45 (2):327-347. doi: 10.1007/s12035-012-8244-2.

Saitou, Mitinori, Mikio Furuse, Hiroyuki Sasaki, Jörg-Dieter Schulzke, Michael Fromm, Hiroshi Takano, Tetsuo Noda, Shoichiro Tsukita, and W. James Nelson. 2000. "Complex Phenotype of Mice Lacking Occludin, a Component of Tight Junction Strands." *Mol. Biol. Cell* 11 (12):4131-4142. doi: 10.1091/mbc.11.12.4131.

Sakakibara, Akira, Mikio Furuse, Mitinori Saitou, Yuhko Ando-Akatsuka, and Shoichiro Tsukita. 1997. "Possible Involvement of Phosphorylation of Occludin in Tight Junction Formation." *J. Cell Biol.* 137 (6):1393-1401. doi: 10.1083/jcb.137.6.1393.

Satoh, H., Y. Zhong, H. Isomura, M. Saitoh, K. Enomoto, N. Sawada, and M. Mori. 1996. "Localization of 7H6 tight junction-associated antigen along the cell border of vascular endothelial cells correlates with paracellular barrier function against ions, large molecules, and cancer cells." *Exp. Cell Res.* 222 (2):269-274. doi: 10.1006/excr.1996.0034.

Schmidt, Anke, Darkhan I. Utepbergenov, Gerd Krause, and Ingolf E. Blasig. 2004. "Capter 2 - Direct Demonstration of Association of the Blood-Brain Barrier Proteins ZO-1 and Occludin Using Surface Plasmon Resonance Spectroscopy; Effect of SIN-1." In *Blood-Spinal Cord and Brain Barriers in Health and Disease*, edited by Hari Shanker Sharma and Jan Westman, 11-17. San Diego: Academic Press.

Schrade, Anja, Hadassah Sade, Pierre-Olivier Couraud, Ignacio A. Romero, Babette B. Weksler, and Jens Niewoehner. 2012. "Expression and localization of claudins-3 and -12 in transformed human brain endothelium." *Fluids Barriers CNS* 9:6-6. doi: 10.1186/2045-8118-9-6.

Scott, Jeanie A., and Alpha S. Yap. 2006. "Cinderella no longer: α-catenin steps out of cadherin's shadow." *J. Cell Sci.* 119 (22):4599-4605. doi: 10.1242/JC.03267.

Shah, J., D. Guerrera, E. Vasileva, S. Sluysmans, E. Bertels, and S. Citi. 2016. "PLEKHA7: Cytoskeletal adaptor protein at center stage in junctional organization and signaling." *Int. J. Biochem. Cell. Biol.* 75:112-116. doi: 10.1016/j.biocel.2016.04.001.

Shin, Kunyoo, and Ben Margolis. 2006. "ZOning out Tight Junctions." *Cell* 126 (4):647-649. doi: https://doi.org/10.1016/j.cell.2006.08.005.

Siegenthaler, Julie A., Fabien Sohet, and Richard Daneman. 2013. "'Sealing off the CNS': cellular and molecular regulation of blood-brain barriergenesis." *Curr. Opin. Neurobiol.* 23 (6):1057-1064. doi: 10.1016/j.conb.2013.06.006.

Sladojevic, Nikola, Svetlana M Stamatovic, Allison M Johnson, Jennifer Choi, Anna Hu, Sophie Dithmer, Ingolf E Blasig, Richard F Keep, and Anuska V Andjelkovic. 2018. "Claudin-1 dependent destabilization of

the blood-brain barrier in chronic stroke." *J. Neurosci.*:1432-1418. doi: 10.1523/jneurosci.1432-18.2018.

Sohet, F., C. Lin, R. N. Munji, S. Y. Lee, N. Ruderisch, A. Soung, T. D. Arnold, N. Derugin, Z. S. Vexler, F. T. Yen, and R. Daneman. 2015. "LSR/angulin-1 is a tricellular tight junction protein involved in blood-brain barrier formation." *J. Cell Biol.* 208 (6):703-711. doi: 10.1083/jcb.201410131.

Stamatovic, Svetlana M., Allison M. Johnson, Richard F. Keep, and Anuska V. Andjelkovic. 2016. "Junctional proteins of the blood-brain barrier: New insights into function and dysfunction." *Tissue barriers* 4 (1):e1154641-e1154641. doi: 10.1080/21688370.2016.1154641.

Steed, E., N. T. Rodrigues, M. S. Balda, and K. Matter. 2009. "Identification of MarvelD3 as a tight junction-associated transmembrane protein of the occludin family." *BMC Cell Biol.* 10:95. doi: 10.1186/1471-2121-10-95.

Stenman, J. M., J. Rajagopal, T. J. Carroll, M. Ishibashi, J. McMahon, and A. P. McMahon. 2008. "Canonical Wnt signaling regulates organ-specific assembly and differentiation of CNS vasculature." *Science* 322 (5905):1247-1250. doi: 10.1126/science.1164594.

Taddei, Andrea, Costanza Giampietro, Annarita Conti, Fabrizio Orsenigo, Ferruccio Breviario, Valentina Pirazzoli, Michael Potente, Christopher Daly, Stefanie Dimmeler, and Elisabetta Dejana. 2008. "Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5." *Nat. Cell Biol.* 10:923. doi: 10.1038/ncb1752.

Tajes, Marta, Eva Ramos-Fernández, Xian Weng-Jiang, Mònica Bosch-Morató, Biuse Guivernau, Abel Eraso-Pichot, Bertrán Salvador, Xavier Fernàndez-Busquets, Jaume Roquer, and Francisco J. Muñoz. 2014. "The blood-brain barrier: Structure, function and therapeutic approaches to cross it." *Mol. Membr. Biol.* 31 (5):152-167. doi: 10.3109/09687688.2014.937468.

Takai, Yoshimi, Wataru Ikeda, Hisakazu Ogita, and Yoshiyuki Rikitake. 2008. "The Immunoglobulin-Like Cell Adhesion Molecule Nectin and Its Associated Protein Afadin." *Annu. Rev. Cell Dev. Biol.* 24 (1):309-342. doi: 10.1146/annurev.cellbio.24.110707.175339.

Takai, Yoshimi, and Hiroyuki Nakanishi. 2003. "Nectin and afadin: novel organizers of intercellular junctions." *J. Cell Sci.* 116 (1):17-27. doi: 10.1242/JC.00167.

Takeichi, Masatoshi. 2006. "The cadherin superfamily in neuronal connections and interactions." *Nat. Rev. Neurosci.* 8:11. doi: 10.1038/nrn2043.

Tao-Cheng, J. H., Z. Nagy, and M. W. Brightman. 1987. "Tight junctions of brain endothelium in vitro are enhanced by astroglia." *J. Neurosci.* 7 (10):3293-3299.

Tietz, Silvia, and Britta Engelhardt. 2015. "Brain barriers: Crosstalk between complex tight junctions and adherens junctions." *J. Cell Biol.* 209 (4):493-506. doi: 10.1083/jcb.201412147.

Tran, K. A., X. Zhang, D. Predescu, X. Huang, R. F. Machado, J. R. Gothert, A. B. Malik, T. Valyi-Nagy, and Y. Y. Zhao. 2016. "Endothelial beta-Catenin Signaling Is Required for Maintaining Adult Blood-Brain Barrier Integrity and Central Nervous System Homeostasis." *Circulation* 133 (2):177-186. doi: 10.1161/circulationaha.115.015982.

Tsukita, Shoichiro, and Mikio Furuse. 1999. "Occludin and claudins in tight-junction strands: leading or supporting players?" *Trends Cell Biol.* 9 (7):268-273. doi: https://doi.org/10.1016/S0962-8924(99)01578-0.

Umeda, K., J. Ikenouchi, S. Katahira-Tayama, K. Furuse, H. Sasaki, M. Nakayama, T. Matsui, S. Tsukita, M. Furuse, and S. Tsukita. 2006. "ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation." *Cell* 126 (4):741-754. doi: 10.1016/j.cell.2006.06.043.

Utsumi, H., H. Chiba, Y. Kamimura, M. Osanai, Y. Igarashi, H. Tobioka, M. Mori, and N. Sawada. 2000. "Expression of GFRalpha-1, receptor for GDNF, in rat brain capillary during postnatal development of the BBB." *Am. J. Physiol. Cell Physiol.* 279 (2):C361-368. doi: 10.1152/ajpcell.2000.279.2.C361.

Van Itallie, Christina M., and James M. Anderson. 2014. "Architecture of tight junctions and principles of molecular composition." *Semin. Cell Dev. Biol.* 36:157-165. doi: 10.1016/j.semcdb.2014.08.011.

Wang, Shugui, Louise Harvey, Rocio Martin, Eline M. van der Beek, Jan Knol, John F. Cryan, and Ingrid B. Renes. 2018. "Targeting the gut microbiota to influence brain development and function in early life." *Neurosci. Biobehav. Rev.* 95:191-201. doi: https://doi.org/10.1016/j.neubiorev.2018.09.002.

Wang, Wen, William L. Dentler, and Ronald T. Borchardt. 2001. "VEGF increases BMEC monolayer permeability by affecting occludin expression and tight junction assembly." *Am. J. Physiol. Heart Circ. Physiol.* 280 (1):H434-H440. doi: 10.1152/ajpheart.2001.280.1.H434.

Wei, Qi, and Hayden Huang. 2013. "Chapter Five - Insights into the Role of Cell–Cell Junctions in Physiology and Disease." In *Int. Rev. Cell Mol. Biol.*, edited by Kwang W. Jeon, 187-221. Academic Press.

Weiss, Nicolas, Florence Miller, Sylvie Cazaubon, and Pierre-Olivier Couraud. 2009. "The blood-brain barrier in brain homeostasis and neurological diseases." *Biochim. Biophys. Acta, Biomembranes* 1788 (4):842-857. doi: https://doi.org/10.1016/j.bbamem.2008.10.022.

Wickline, Emily D., Ian W. Dale, Chelsea D. Merkel, Jonathon A. Heier, Donna B. Stolz, and Adam V. Kwiatkowski. 2016. "Alpha-T-Catenin Is a Constitutive Actin-Binding α-Catenin That Directly Couples the Cadherin-Catenin Complex to Actin Filaments." *J. Biol. Chem.* 291(30):15687-15699. doi: 10.1074/jbc.M116.735423.

Wickline, Emily Diane, Yu Du, Donna B. Stolz, Michael Kahn, and Satdarshan P. S. Monga. 2013. "γ-Catenin at adherens junctions: mechanism and biologic implications in hepatocellular cancer after β-catenin knockdown." *Neoplasia* 15 (4):421-434.

Wilhelm, Imola, Ádám Nyúl-Tóth, Maria Suciu, Anca Hermenean, and István A. Krizbai. 2016. "Heterogeneity of the blood-brain barrier." *Tissue barriers* 4 (1):e1143544-e1143544. doi: 10.1080/21688370.2016.1143544.

Winger, Ryan C., Christopher T. Harp, Ming-Yi Chiang, David P. Sullivan, Richard L. Watson, Evan W. Weber, Joseph R. Podojil, Stephen D. Miller, and William A. Muller. 2016. "Cutting Edge: CD99 Is a Novel Therapeutic Target for Control of T Cell-Mediated Central Nervous System Autoimmune Disease." *J. Immunol.* 196 (4):1443-1448. doi: 10.4049/jimmunol.1501634.

Winger, Ryan C., Jennifer E. Koblinski, Takashi Kanda, Richard M. Ransohoff, and William A. Muller. 2014. "Rapid remodeling of tight junctions during paracellular diapedesis in a human model of the blood-brain barrier." *J. Immunol.* 193 (5):2427-2437. doi: 10.4049/jimmunol.1400700.

Witherden, Deborah A., Petra Verdino, Stephanie E. Rieder, Olivia Garijo, Robyn E. Mills, Luc Teyton, Wolfgang H. Fischer, Ian A. Wilson, and Wendy L. Havran. 2010. "The junctional adhesion molecule JAML is a costimulatory receptor for epithelial gammadelta T cell activation." *Science* 329 (5996):1205-1210. doi: 10.1126/science.1192698.

Wolburg, H., J. Neuhaus, U. Kniesel, B. Krauss, E. M. Schmid, M. Ocalan, C. Farrell, and W. Risau. 1994. "Modulation of tight junction structure in blood-brain barrier endothelial cells. Effects of tissue culture, second messengers and cocultured astrocytes." *J. Cell Sci.* 107 (5):1347-1357.

Wolburg, H., K. Wolburg-Buchholz, J. Kraus, G. Rascher-Eggstein, S. Liebner, S. Hamm, F. Duffner, E. H. Grote, W. Risau, and B. Engelhardt. 2003. "Localization of claudin-3 in tight junctions of the

blood-brain barrier is selectively lost during experimental autoimmune encephalomyelitis and human glioblastoma multiforme." *Acta Neuropathol.* 105 (6):586-592. doi: 10.1007/s00401-003-0688-z.

Wolburg, Hartwig, and Andrea Lippoldt. 2002. "Tight junctions of the blood–brain barrier: development, composition and regulation." *Vasc. Pharmacol.* 38 (6):323-337. doi: https://doi.org/10.1016/S1537-1891(02)00200-8.

Wolburg, Hartwig, Susan Noell, Andreas Mack, Karen Wolburg-Buchholz, and Petra Fallier-Becker. 2009. "Brain endothelial cells and the glio-vascular complex." *Cell Tissue Res.* 335 (1):75-96. doi: 10.1007/s00441-008-0658-9.

Wong, V. 1997. "Phosphorylation of occludin correlates with occludin localization and function at the tight junction." *Am. J. Physiol.* 273 (6(1)):C1859-1867.

Wosik, K., R. Cayrol, A. Dodelet-Devillers, F. Berthelet, M. Bernard, R. Moumdjian, A. Bouthillier, T. L. Reudelhuber, and A. Prat. 2007. "Angiotensin II controls occludin function and is required for blood brain barrier maintenance: relevance to multiple sclerosis." *J. Neurosci.* 27 (34):9032-9042. doi: 10.1523/jneurosci.2088-07.2007.

Xiao, Kanyan, Jennifer Garner, Kathleen M. Buckley, Peter A. Vincent, Christine M. Chiasson, Elisabetta Dejana, Victor Faundez, and Andrew P. Kowalczyk. 2005. "p120-Catenin Regulates Clathrin-dependent Endocytosis of VE-Cadherin." *Mol. Biol. Cell* 16 (11):5141-5151. doi: 10.1091/mbc.e05-05-0440.

Yamada, Soichiro, Sabine Pokutta, Frauke Drees, William I. Weis, and W. James Nelson. 2005. "Deconstructing the cadherin-catenin-actin complex." *Cell* 123 (5):889-901. doi: 10.1016/j.cell.2005.09.020.

Yamamoto, Masaru, Servio H. Ramirez, Shinji Sato, Tomomi Kiyota, Ronald L. Cerny, Kozo Kaibuchi, Yuri Persidsky, and Tsuneya Ikezu. 2008. "Phosphorylation of Claudin-5 and Occludin by Rho Kinase in Brain Endothelial Cells." *Am. J. Pathol.* 172 (2):521-533. doi: 10.2353/ajpath.2008.070076.

Yamamoto, T., N. Harada, K. Kano, S. Taya, E. Canaani, Y. Matsuura, A. Mizoguchi, C. Ide, and K. Kaibuchi. 1997. "The Ras target AF-6 interacts with ZO-1 and serves as a peripheral component of tight junctions in epithelial cells." *J. Cell Biol.* 139 (3):785-795.

Yepes, Manuel. 2013. "TWEAK and Fn14 in the Neurovascular Unit." *Front. Immunol.* 4:367. doi: 10.3389/fimmu.2013.00367.

Zhong, Y., T. Saitoh, T. Minase, N. Sawada, K. Enomoto, and M. Mori. 1993. "Monoclonal antibody 7H6 reacts with a novel tight junction-

associated protein distinct from ZO-1, cingulin and ZO-2." *J. Cell Biol.* 120 (2):477-483.

Zonta, Micaela, María Cecilia Angulo, Sara Gobbo, Bernhard Rosengarten, Konstantin- A. Hossmann, Tullio Pozzan, and Giorgio Carmignoto. 2003. "Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation." *Nat. Neurosci.* 6:43. doi: 10.1038/nn980.

Zozulya, Alla, Christian Weidenfeller, and Hans-Joachim Galla. 2008. "Pericyte–endothelial cell interaction increases MMP-9 secretion at the blood–brain barrier in vitro." *Brain Research* 1189:1-11. doi: https://doi.org/10.1016/j.brainres.2007.10.099.

CHAPTER FOUR

CELLULAR INTERACTIONS BETWEEN THE IMMUNE SYSTEM AND THE BLOOD-BRAIN BARRIER

CAROLINE COISNE

Introduction

The central nervous system (CNS) has traditionally been considered an absolutely "immunologically-privileged site" (Billingham and Boswell 1953) in which any blood-borne component from the periphery is not allowed to enter the CNS compartment to avoid any immune surveillance of the brain and spinal cord tissue. This concept appeared to be commonly accepted in regard to the homeostasis of the CNS to ensure proper neuron activity. Circulating immune cells frequently entering the CNS in search of any suitable antigens would have been very damaging. Besides, several experimental outcomes supported the immune privilege concept based on the fact that the CNS was shown to lack lymphatic vessels and MHC class I and class II expressing cells, preventing any proper access of circulating antigens to the CNS and their subsequent presentation to T cells via professional antigen-presenting cells (APC) respectively. Early heterotopic tissue transplantation experiments to mammalian brain strengthened this idea, as grafting skin to brain tissue did not produce an immune reaction (reviewed by Carson et al. 2006). Moreover, the presence of brain barriers, i.e. the endothelial blood-brain barrier (BBB) and to a lesser extent the epithelial blood-cerebrospinal fluid barrier (BCSFB), whose function is to limit the blood-borne molecules that enter, has also been extended to prevent immune cell migration from circulating into the CNS. This has been supported by the presence of a complex network of tight junctions (TJ) and TJ-associated proteins between adjacent cells that tightly close any paracellular route of access for immune cells across the BBB, as well as reduced pinocytic activity within BBB endothelial cells, also limiting

any transcellular route for leukocyte migration, in contrast to other endothelial cells from the periphery (Reese and Karnovsky 1967).

Nonetheless, absolute immune privilege has now become rather relative, due to the fact that lymphocytes, activated in the periphery, could readily cross the BBB and mount an immune response inside the CNS (Medawar 1948). Microglial cells display APC functions and efferent lymphatic drainage from the CNS towards deep cervical lymph nodes has been reported, indicating that immune surveillance takes place in the CNS compartment (reviewed by Hickey 2001, Engelhardt and Coisne 2011). Yet the role of the BBB remains the same, as this barrier acts in protecting the CNS from harmful peripheral immune events, modulating physical and chemical aspects of the interaction between the effector cells of the immune response and the BBB-forming endothelial cells. Upon inflammation, the BBB becomes impaired and this is referred to as "BBB breakdown". High numbers of circulating immune cells can readily cross the inflamed BBB endothelium and infiltrate the CNS parenchyma, as has been observed in neuroinflammatory and/or neurodegenerative disorders such as multiple sclerosis (MS), and its animal model experimental autoimmune encephalomyelitis (EAE), but also in Alzheimer's disease and during viral or bacterial infections or stroke (Lopes-Pinheiro et al. 2016).

Immune cell recruitment across any vascular bed requires the sequential interaction of several adhesion and signaling molecules localized on the surface of the circulating immune cell and of the respective endothelial cell. This recruitment represents a multi-step cascade in which each step involves diverse and numerous receptor-ligand pairs, giving rise to a high number of molecular combinations that allow a variety of leukocyte subtypes from the bloodstream to infiltrate any tissue (Springer 1995, Ley et al. 2007). Based on the properties of the CNS microvessels, one can easily emphasize the specificity of such molecular interplays between adhesion and signaling mechanisms occurring between leukocytes and the specialized blood-brain barrier endothelium that may differ from those observed in the periphery.

Our current knowledge on the molecular mechanisms involved in immune cell traffic into the CNS was mostly documented from in vivo studies in animal models, mainly from MS, but also from simple to more complex in vitro migration set-ups, either in static conditions or in the presence of flow mimicking shear forces applied in blood circulation. From in vivo and in vitro experimental findings, aiming at discriminating each molecular partner involved in the multi-step cascade paradigm, targeting the recruitment of leukocytes across the BBB to treat human

diseases gave birth to a new therapeutic area. This constantly needs to adjust the balance between real benefits and potential risks when interfering with the immune system.

The multi-step adhesion cascade of immune cell recruitment

The recruitment of immune cells into any tissue requires circulating cells to transmigrate across the endothelium lining the vessel wall. With this aim, closely regulated sequential interactions involving diverse molecular partners (cell surface adhesion molecules, signaling molecules, carbohydrate ligands, chemoattractant mechanisms) take place on the cell surface of both the leukocyte and endothelial cell of the blood vessel wall. Each step was specifically characterized thanks to blocking antibodies, selective inhibitors, or targeted-gene manipulations that made it possible to inhibit each sequence of the multistep cascade (Butcher 1991, Springer 1995, Ley et al. 2007). Moreover, development of in vitro devices adding the effect of the blood flow, as well as development of intravital microscopy making it possible to directly view the interaction of circulating immune cells with the vessel wall under physiological blood flow, allowed a better characterization of the dynamics of immune cell recruitment into any tissue of the body (Von Andrian and N'Rini 1998, Coisne and Engelhardt 2009, Coisne, Lyck, and Engelhardt 2013, Haghayegh Jahromi et al. 2017).

First in the migration cascade, an immune cell circulating with a high speed slows down within the bloodstream by initiating transient and weak contacts between carbohydrate ligands such as P-selectin glycoprotein ligand (PSGL)-1 on the leukocyte surface and their counter receptors of the selectin family (E- or P-selectin) expressed on the endothelium. This initial step, called "tethering", characterized by low-affinity cellular interactions, aims at reducing leukocyte velocity in circulation to facilitate the subsequent "rolling" of the immune cell on the vessel wall towards the direction of the blood flow. This later step is mediated by leukocyte α4-integrins, α4β1- (Very Late Antigen-4; VLA-4) and α4β7-integrins, binding to their endothelial ligands of cell adhesion molecules (CAM) of the immunoglobulin superfamily, i.e. vascular cell adhesion molecule-1 (VCAM-1/CD106) and mucosal vascular addressin cell adhesion molecule (MAdCAM)-1, respectively. When rolling along the luminal surface of the blood vessel at a lower speed, the leukocyte is exposed to chemotactic factors of the chemokine family. These chemokines of endothelial origin bind to G-protein coupled receptors (GPCR) expressed on the leukocyte

surface. Upon activation of the GPCR, a pertussis toxin-sensitive "inside-out-signal" is delivered into the leukocyte, resulting in the subsequent activation of cell surface adhesion molecules of the integrin family. Integrins are heterodimers composed of an α- and a β-chain that are constitutively expressed on the leukocyte surface in an inactive conformation. Upon activation, they modify their conformation and cluster on the leukocyte surface. In their activated form, integrins mediate "the firm arrest" and "adhesion strengthening" of the leukocyte to the vascular wall by binding to their endothelial CAM ligands. After polarizing and spreading its cell body over the endothelial surface, the immune cell starts to "crawl" on the endothelium through adhesive integrins/CAM interactions in search of an appropriate site for subsequent "diapedesis". This represents the last stage of the recruitment cascade when the immune cell crosses the vascular wall and is likely to be mediated by integrins/CAM binding and signaling events through chemokines. The leukocyte cell body squeezes across the endothelium either following a paracellular route (i.e. between adjacent endothelial cells through cell-cell contacts) or a transcellular route (i.e. through the entire endothelial cell body, as defined as emperipolesis) via pore formation (Carman and Springer 2004, Carman 2009, Barzilai et al. 2017). The molecular events that regulate leukocyte diapedesis across the vessel wall, and the route of the trans-endothelial migration (TEM) being preferred over the other one, remain partially understood. After their endothelial transmigration, leukocytes face additional mechanical barriers to cross the sub-endothelial basement membrane, the pericyte layer and then the interstitial space between stromal cells, which represents the *glia limitans perivascularis* in the BBB context.

Immune cell recruitment across the blood-brain barrier

The BBB generally refers to capillaries draining the brain parenchyma. Some BBB features such as the presence of efficient TJ in capillary endothelial cells do, however, extend to the endothelium of pre-capillary arterioles and post-capillary venules, not limited to the brain but also to the spinal cord (Bechmann, Galea, and Perry 2007). In capillaries, the perivascular space delineated between the endothelial basement membrane embedding high numbers of pericytes and the *glia limitans perivascularis*, formed by a layer of astrocytic end-feet and their parenchymal basement membrane, is narrow (Thal 2009). Conversely, in pre-capillary arterioles and post-capillary venules, the perivascular space becomes enlarged and filled with perivascular CSF where host APC are found (Bechmann,

Galea, and Perry 2007). Leukocyte transmigration into the CNS does not take place at capillary-level but rather at the level of CNS post-capillary venules (Owens, Bechmann, and Engelhardt 2008). Based on the specificity of the BBB architecture and function, one can easily speculate on the specificity of immune cell traffic into the CNS across the BBB.

Initial contact: capture/tether and rolling

In EAE, self-reactive CD4+, and alternatively CD8+ T cells, recognizing myelin-derived antigens, are activated outside the CNS and have to cross brain barriers to gain access to the CNS, where they start the molecular events leading to clinical manifestation of the disease (Huseby et al. 2001, Cabarrocas et al. 2003). Live-cell imaging techniques have provided direct evidence of the migration of encephalitogenic CD4+ T cells through the BBB of the spinal cord white matter, which represents a unique phenomenon in healthy mice. To initiate interaction with the healthy BBB, encephalitogenic CD4+ T cells do not roll on the endothelium but are instead abruptly captured from circulation by their high-affinity α4-integrins binding to the endothelial VCAM-1. Pretreatment with antibodies against either α4-integrins or VCAM-1, but not with pertussis toxin, have almost abrogated the capture of CD4+ T cells, indicating a G-protein coupled chemokine receptor-independent event (Vajkoczy, Laschinger, and Engelhardt 2001), i.e. independent of the activation of cell surface integrins.

In contrast, upon neuroinflammation, tethering and rolling have been observed first in superficial brain and meningeal microvessels by performing intravital videomicroscopy via a cranial window in mice afflicted with EAE (Kerfoot and Kubes 2002, Kerfoot et al. 2006) or through the intact skull of TNF-α challenged mice (Battistini et al. 2003), as well as in spinal cord white matter post-capillary venules (Coisne and Engelhardt, 2009). Encephalitogenic CD4+ T cells prefer to roll on the inflamed BBB endothelium with few alternative capture events. Blocking either α4-integrins or β7- or β1-integrins with specific blocking antibodies or depleting β1-integrin on the T cell surface did not interfere with initial contact of encephalitogenic CD4+ T cells and activated CD8+ T cells with the inflamed BBB (Bauer et al. 2009, Coisne, Mao, and Engelhardt 2009, Coisne, Lyck, and Engelhardt 2013). Another surface molecule, P-selectin, upregulated in CNS microvessel endothelial cells during EAE, has been reported to mediate PSGL-1 dependent CD8+ T cell rolling in inflamed leptomeningeal brain vessels (Battistini et al. 2003). Additionally, E-selectin has been implicated in leukocyte rolling in inflamed superficial

brain vessels (Piccio et al. 2002, 2005). However, activated T cells did not express L-selectin on their surface, and selective blockade of L-selectin with antibodies did not influence lymphocyte rolling on CNS vessels (Ley and Kansas 2004, Piccio et al. 2005). In contrast, at the inflamed BBB, Sathiyanadan et al. (2014) used intravital videomicroscopy to observe that leukocyte PSGL-1 mediating both endothelial E- and P-selectin interaction is essential for T cell rolling on inflamed spinal cord microvascular endothelium in EAE. Abolishing T cell rolling resulted in a reduction of T cells firmly adhering to the spinal cord microvasculature. In contrast, the functional absence of either PSGL-1 or E- and P selectin did not ameliorate the course of EAE in both C57BL/6 and SJL/ J mouse strains (Engelhardt et al. 2005, Osmers, Bullard, and Barnum 2005, Bill et al. 2011). Although it is essential for T cell rolling on the inflamed BBB, the interaction of PSGL-1 with E/P-selectins may not be essential for T cells entering the CNS.

Firm adhesion: arrest and adhesion strengthening

After initial contact, the subsequent arrest and adhesion strengthening of encephalitogenic T cells on the BBB endothelium are mediated by integrins binding to low amounts of constitutively expressed endothelial VCAM-1 and ICAM-1 on the healthy BBB (Lee and Benveniste 1999, Laschinger, Vajkoczy, and Engelhardt 2002). The expression of both is up-regulated in EAE and MS pathologies (Bö et al. 1996, Peterson et al. 2002). ICAM-2, expressed on intact BBB, is not upregulated upon inflammation. In contrast, MAdCAM-1 expression has not been observed in impaired BBB in EAE, except in the Biozzi EAE model (O'Neill et al., 1991, Baron et al. 1993, Steffen, Butcher, and Engelhardt 1994). α4-integrins LFA-1/αLβ2 have been expressed on the surface of the majority of infiltrating lymphocytes and monocytes within or close to MS lesions (Cannella and Raine 1995).

The reduced velocity of a lymphocyte that rolls on the BBB endothelium facilitates interaction of G-protein coupled chemokine receptors on the leukocyte with chemokines expressed at the surface of the vascular wall. This leads to the activation of the integrins α4β1 and αLβ2 on T cell surface through an inside-out signaling. Activation changes the conformation of integrins into an upright position in which endothelial ligand binding is possible, and induces a clustering of integrins, increasing both their affinity and avidity for endothelial CAM ligands (Luo and Springer 2006).

The involvement of α4-integrins in mediating leukocyte arrest and

adhesion strengthening to inflamed vessels was first reported by Yednock and colleagues (1992) in frozen brain sections of mice afflicted with EAE ex vivo. The authors demonstrated that blocking antibodies against the α4-integrin subunit was able to inhibit the development of EAE and the accumulation of inflammatory cells inside the CNS. Since then, numerous in vitro and in vivo studies have accumulated evidence confirming the role of α4-integrins in T cell adhesion on the BBB (Steffen, Butcher, and Engelhardt 1994, Bauer et al. 2009, Coisne, Lyck, and Engelhardt 2013). α4β1- and α4β7-integrins expressed on lymphocyte surface preferentially bind to VCAM-1 and MAdCAM-1 respectively. MAdCAM-1 expression was not found in CNS microvessels, in contrast to VCAM-1. The binding partner of VCAM-1, α4β1-integrin, was considered the main integrin involved in leukocyte migration into the CNS, although in vitro both α4β1- and α4β7-integrins could bind to VCAM-1 (Engelhardt et al. 1998). In vivo discrepant experimental read-outs have been reported. Blocking α4β7-integrin function with antibodies did not affect EAE development in SJL mice (Engelhardt et al. 1998), whereas β7-integrin deficient mice developed a less severe EAE (Kanwar et al. 2000). Combining intravital videomicroscopy technology in EAE mice with the use of blocking antibodies against α4β1-, β7- or α4-integrins or β1-integrin deficient CD4+ T cells, we and others have observed that encephalitogenic CD4+ T cells use α4β1- but not α4β7-integrins to adhere to the inflamed BBB in EAE mice (Vajkoczy, Laschinger, and Engelhardt 2001, Coisne et al., 2009; Bauer et al. 2009, Coisne, Mao, and Engelhardt 2009).

Using the same experimental settings, immature dendritic cells (DC) but not LPS-matured DC have been shown to efficiently roll, capture and then adhere to the inflamed BBB endothelium of SJL mice with EAE in an α4-integrin-dependent manner (Jain et al. 2010). The functional inhibition of β1-integrins but not of β7- nor α4β7-integrins decreased DC firm adhesion to the inflamed BBB, involving α4β1- but not α4β7-integrins in this process.

Similarly, CD8+ T cells relied on α4β1-integrins to cross the inflamed BBB in EAE mice (Coisne, Lyck, and Engelhardt 2013, Martin-Blondel et al. 2015). α4β1-integrins on CD8+ T cell surface could alternatively bind to junctional adhesion molecule (JAM)-B, in addition to VCAM-1, and promote CD8+ T cell migration across the inflamed BBB (Martin-Blondel et al. 2015). However, α4β1-integrins/JAM-B interaction did not play any role in mediating encephalitogenic CD4+ T cell firm adhesion and subsequent migration across the inflamed BBB in vitro, as well as in vivo in EAE animals (Tietz et al. 2018).

Crawling

Development of live cell imaging (e.g. in vitro time-lapse imaging, intravital videomicroscopy in rodents) has made it possible to investigate the post-arrest dynamic behavior of immune cells under physiological flow (Coisne, Lyck, and Engelhardt 2013). Steiner et al. (2010) have observed that, upon arrest, the adherent immune cell spreads its cell body and modifies its shape towards a polarized morphology (i.e. lamellipodium formation at the cell front and a rear uropod) within 1-3 min in the case of encephalitogenic CD4+ T lymphocytes on BBB endothelium in vitro. Immune cell polarization facilitates the palpation of the endothelium surface by invasive podosome-like protrusions of the leukocyte in search of a permissive site for its subsequent extravasation (Steiner et al. 2010). Directional crawling against the direction of the blood flow has been described for activated CD4+ T lymphocytes on inflamed mouse BBB, as well as in meningeal spinal cord vessels of EAE rats, with a reported speed of 3 to 12 μm/min and a covered distance of 25 to 188 μm before diapedesis (Bartholomaus et al. 2009, Steiner et al. 2010 and 2011, Lyck and Engelhardt 2012). Endothelial ICAM-1 and ICAM-2 are the exclusive integrin ligands binding to LFA-1 involved in CD4+ T cell crawling (Steiner et al. 2010, Valignat et al. 2013). T cell crawling distances on top of primary BBB endothelium in vitro, displaying high paracellular tightness, get shorter compared with those reported on immortalized brain endothelium exhibiting leakier barrier function (Steiner et al. 2011). This has been linked to the amount of endothelial ICAM-1 on the cell surface. The higher the amount of ICAM-1 is, the shorter the crawling distance gets (Abadier et al. 2015). In contrast to CD4+ T cells, activated CD8+ T lymphocytes, once arrested on primary mouse BBB cells under flow conditions, did not crawl on the endothelium but rather remain stalled, meaning that these cells stand on their diameter while actively probing endothelium in search of the ideal spot for their rapid diapedesis (Rudolph et al. 2016). Elegant gene-depletion studies, abrogating either neutrophil integrins or endothelial CAM or both, demonstrated that neutrophils under physiological flow crawl via the interaction of both β2 integrins, LFA-1 and CD11b/CD18, with endothelial ICAM-1 and ICAM-2 on the inflamed BBB in vitro (Gorina et al. 2013, Li et al. 2018). In contrast to the unique crawling behavior of encephalitogenic CD4+ T cells against the direction of the blood flow, neutrophils exclusively crawl towards the bloodstream.

Diapedesis

Referring to the complex and unique architecture of the BBB, mostly based on the presence of TJ between adjacent endothelial cells, diapedesis may differ from that observed in other vascular beds. TJ sealing the paracellular space was supposed to stop any transmigration of immune cells via the paracellular route (Engelhardt and Wolburg, 2004). Nevertheless, during immune surveillance, CNS T cells preferentially take the paracellular pathway. Under inflammatory conditions, the transcellular route is also chosen to cross the BBB (Winger et al. 2014; Abadier et al. 2015, Lutz et al. 2017). In this respect, cell adhesion molecules of the immunoglobulin superfamily represent essential key molecular partners responsible for efficient diapedesis across the BBB.

ICAM-1 was the first identified in the extravasation process of lymphocytes into the CNS (Greenwood, Wang, and Calder 1995, Reiss et al. 1998). Abadier et al. (2015) investigated whether the expression levels of endothelial ICAM-1 would influence the cellular route chosen by CD4+ T cells in their diapedesis across inflamed BBB under physiological flow in vitro. Depending on the inflammatory stimulus, ICAM-1 levels expressed by BBB cells differ. IL1-β stimulation, inducing a high amount of endothelial ICAM-1, favored transcellular diapedesis of encephalitogenic CD4+ T cells across the BBB. In contrast, intermediate levels of ICAM-1, upon TNFα stimulation, induced the paracellular route. Regardless of the route chosen by T cells to cross the endothelium, this was not accompanied by a loss of BBB integrity. Unexpectedly, few T cells were still able to diapedese across ICAM-1- and ICAM-2-depleted endothelial cells via an alternative transcellular pathway, independent of VCAM-1. Therefore, this study indicates that the amount of ICAM-1 expressed on the BBB endothelial cell surface directly depends on the nature of the inflammatory stimulus. This is of prime importance to discriminate between paracellular and transcellular routes in encephalitogenic CD4+ T cell transmigration into the CNS. Diapedesis represents a dynamic process that requires the formation of endothelial docking structures in which endothelial cell membrane protrusions, enriched in F- actin, engulf adherent leukocyte pseudopodia (Carman and Springer, 2004). In addition, reorganization of the cytoskeleton, disassembly of TJ architecture and pore formation take place within the endothelial cell (Barreiro et al. 2002, Barzilai et al. 2017). These depend on the activation of several intracellular signaling pathways of great complexity. Intracellular downstream signaling induced through LFA-1 binding to ICAM-1 was investigated by Durieu-Trautmann et al. (1994) in cerebral endothelial cells. The authors performed cross-linking

experiments with an antibody that mimics LFA-1/ICAM-1 adhesion on RBE4 cells, a rat brain endothelial cell line. This resulted in outside-in signaling via the cytoplasmic tail of ICAM-1 that activated the small GTPase Rho, a key player in actin cytoskeleton remodeling through the tyrosine kinase p60src in addition to intracellular calcium mobilization. The cortactin got further phosphorylated and formed cortical actin filaments (Etienne et al. 1998, Adamson et al. 1999, Etienne-Manneville et al. 2000). ICAM-1 engagement was involved in tyrosine phosphorylation in the cytoplasmic domain of VE-cadherin, localized at adherens junctions. This resulted in the paracellular transmigration of CD4+ T cells across the endothelial cells of cerebral origin in vitro (Turowski et al. 2008). Other binding partners have been suggested, such as caveolin-1, filamin B and α-actinin 4 enabled lymphocyte transcellular transmigration through non-CNS endothelium (Kanters et al. 2008, Millán et al. 2008, Sverdlov et al. 2009).

Other CAM were involved in the signaling process of extravasation of different leukocyte subpopulations across the BBB, such as VCAM-1, platelet endothelial CAM (PECAM-1/CD31) and activated leukocyte CAM (ALCAM/CD166). VCAM-1 is involved in regulating monocyte diapedesis (Floris et al. 2002). PECAM-1 is expressed on neutrophils, monocytes, platelets and lymphocyte subpopulations as well as on endothelial cells (Muller et al. 1989, Stockinger et al. 1990, Bird et al. 1993). Until recently, the role of PECAM-1 in leukocyte transmigration was barely understood and only described in the extravasation of monocytes and neutrophils through homophilic interactions between endothelial cells and migrating cells at the junctions (Muller et al. 1993, Mamdouh et al. 2003, Schenkel, Chew, and Muller 2004). PECAM-1 involvement in encephalitogenic CD4+T cell diapedesis was recently addressed by Wimmer et al. (2019) across primary mouse brain microvascular endothelial cells with live-cell imaging. The authors observed that PECAM-1-depleted brain endothelial cells exhibited impaired BBB properties (higher permeability to integrity markers and lower transendothelial electrical resistance). Lack of PECAM-1 did not affect arrest or polarization, or crawling and/or diapedesis of encephalitogenic CD4+ T cells. However, the route of migration across the endothelium was modified, shifting from the favored paracellular route to the transcellular one, without having any effect on the amount of T cells crossing the BBB. Therefore, PECAM-1 may affect one transmigration route versus the other depending on its availability on the BBB endothelium.

ALCAM, enriched in transmigratory cups in BBB endothelial cells,

has also been involved in TJ assembly (Lécuyer et al. 2017). ALCAM is constitutively expressed at the BBB and is upregulated upon inflammation in vitro and in vivo in EAE and MS disorders (Cayrol et al. 2008, Lyck et al. 2017). ALCAM was localized in lipid rafts where it binds to the leukocyte CD6 through heterophilic interactions, but also to the leukocyte ALCAM through weaker homophilic interactions (Skonier et al. 1996, van Kempen et al. 2001). Upon binding, endothelial ALCAM induced transmigration of T cells, CD19+ B cells and monocytes, but had no effect on CD8+ T cell extravasation across the BBB (Cayrol et al. 2008). Inhibiting ALCAM downstream signaling with blocking antibodies, decreased human CD4+ Th1 cell extravasation but not of CD4+Th17 cells across a static human in vitro BBB model. Nonetheless, in the same study with the use of a mouse in vitro BBB setting, ALCAM did mediate the diapedesis of T cells under static conditions, but no longer under physiological shear (Lyck et al. 2017), indicating a limited involvement of ALCAM in T cell diapedesis across the BBB. In contrast, ALCAM was instead described as supporting rolling, firm adhesion, and transmigration of human CD14+ monocytes across human in vitro BBB under both static and flow conditions. Similarly, a role for ALCAM in monocyte interaction and diapedesis across the BBB has been described during human immunodeficiency virus type 1 (HIV-1) infection (Yao et al. 2011, Williams et al. 2013 and 2015). The human T-lymphotropic virus type 1 (HTLV-1) is the infectious agent accountable for the development of HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), a progressive neurodegenerative disorder. In vitro, human HTLV-1-infected lymphocytes overexpressed ALCAM, but not CD6, on their cell surface. In addition, ALCAM levels were found unchanged on microvascular endothelia in thoracic spinal cord sections from HTLV-1 infected patients compared to healthy subjects. Upon downregulation of their ALCAM levels, the migration of CD4+ T cells from HAM/TSP patients was dampened across HCMEC/D3 cells compared to those from healthy donors (Curis et al. 2016).

Therapeutic approaches modulating immune cell interactions at the blood-brain barrier

Therapies aiming at blocking the access of immune cells to the CNS have been developed based on the pre-clinical findings previously described in this chapter, i.e. the role of α4-integrins in pathogenic T lymphocyte extravasation across the BBB in EAE animal models. Therefore, targeting α4-integrins with the humanized monoclonal antibody

natalizumab (Tysabri®), developed by Biogen Idec/Elan, has demonstrated effective benefits in treating MS patients in reducing the risk of sustained disability progression by 42% and the annualized relapse rate by 68% over a two-year clinical study as a monotherapy (Polman et al. 2006, Kappor et al. 2018). By means of intravital microscopy, we have demonstrated how natalizumab modulates human T cell migration into the inflamed CNS. Natalizumab specifically inhibited firm adhesion but not rolling or capture of human T cells on inflamed BBB in EAE mice (Coisne, Mao, and Engelhardt 2009). However, when combined with IFN-β, the first line of treatment for relapsing-remitting MS (Rudick et al. 2006), natalizumab was associated with the unexpected and rare occurrence of progressive multifocal leukoencephalopathy (PML) in 1/1000 treated-patients (Kleinschmidt-Demasters and Tyler 2005, Mills and Mao-Draayer, 2018). PML is caused by polyomavirus (previously known as John Cunningham or JC virus) infection of oligodendrocytes and is associated with fatal outcomes. This raised concerns about the safety of modulating immune cell functions and migration to treat diseases as α4-integrins control multiple immunological processes elsewhere in the body, such as hematopoietic stem cell maturation (Bungartz et al. 2006), T lymphocyte activation and polarization (Mittelbrunn et al. 2004) or retention of memory T cells in their niches (Sixt et al. 2006). Therefore, blocking antibody therapy against α4-integrins targets essential parts of the immune system that result in broader immunosuppressive side effects, showing the limitations of such therapy. Natalizumab is currently being used as a second line of treatment when standard treatments fail or in case of poor disease prognosis. Observation of natalizumab-treated MS patients is controlled to prevent or at least monitor for the occurrence of PML.

Small molecules antagonizing α4-integrin interacting with VCAM-1 at the BBB represent an appealing substitute for chronic diseases, as compared to infused medication like monoclonal antibodies, as they can be orally delivered and have a relatively lower cost. CDP-323, co-developed by UCB and Biogen Idec, is an anti-α4-integrin antagonist displaying efficient inhibition of leukocyte binding to VCAM-1 and no toxicity in preclinical trials (Simmons 2005). However, a phase II clinical trial in relapsing-remitting MS patients was discontinued at mid-stage due to the lack of clinically relevant benefit (http://www.clinicaltrials.gov). The orally bioavailable small molecule antagonist of α4β-integrins developed by GlaxoSmithKline and Tanabe, SB-683699 (Firategrast), was evaluated in a phase II clinical trial including a small number of participants (343). This study demonstrated proven reduction in new MS lesion formation, without any occurrence of PML or JC virus reactivation

recorded during the duration of the treatment (Miller et al. 2012). Neumann and colleagues (2015) implicated α4-integrins in the migration process of neutrophils into the CNS during ischemic stroke, in which these cells have been involved in disease progression and neuronal damage (Emerich et al. 2002, Neumann et al. 2015). However, blocking α4β1-integrin (VLA4)/VCAM-1 interaction at the BBB during acute ischemia led to discrepant results in animal models (Becker et al. 2001, Liesz et al. 2011, Langhauser et al. 2014). A preclinical randomized controlled trial gathering data from several research centers was launched to evaluate an anti-VLA4 treatment in acute brain ischemia. Combined experimental findings in different animal models revealed that VLA-4 blockade at the BBB reduced leukocyte infiltration into the brain parenchyma, although its effect in reducing the infarct volume merely depended on the severity and localization of the infarct (Llovera et al. 2015). Besides VCAM-1, ICAM-1 represents another major target in therapeutic strategies. Only a few clinical trials for MS or stroke of either ICAM-1 or αLβ2 blocking antibodies have been evaluated so far. A phase II clinical trial investigating the humanized anti-αLβ2 antibody Hu23F2G (LeukoArrest or Rovelizumab by ICOS Corporation), at 1 or 2 mg/kg as compared to placebo, in MS patients with acute exacerbations of the disease, was interrupted due to treatment inefficiency in the 2000 recipients (Lublin et al. 1999, Jones et al. 2000). When ischemic stroke was applied to mice, ICAM-1 KO animals displayed reduced size of ischemic lesions and a better functional outcome as compared to their wild-type littermates (Connolly Jr. et al. 1996). Blockade of CD11b/ICAM-1 interaction with antibodies (Chopp et al. 1994, Chen et al. 1994, Bednar et al. 1996) or antisense oligonucleotides (Vemuganti, Dempsey, and Bowen 2004) has been effective in pre-clinical studies in reducing infarct volume and improving neurological functions in several animal models of stroke. These promising outcomes raise hope for translation into the clinic. The monoclonal anti-human ICAM-1 antibody, Enlimomab (Boehringer Ingelheim), has been investigated in phase III clinical trial in patients with acute ischemic stroke. Unfortunately, this antibody developed in mice induced a severe immune response in humans, as patients were diagnosed with severe side effects, such as infections, neurological deficits and higher mortality rate as compared to the placebo group (Furuya et al. 2001). Other clinical trial attempts have also failed, including administration of recombinant neutrophil inhibiting factor (rNIF) in acute stroke patients (Krams et al. 2003), as well as the infusion of anti-CD11 antibody in patients 12 hours after onset of ischemic stroke (Becker 2002).

Conclusion

Considerable efforts have been applied to understanding the mechanisms involved in immune cell trafficking into the CNS, across the BBB, to inhibit, or at least modulate, such processes responsible for the development or exacerbation of many neuropathological disorders. Technological advances using live-cell imaging have greatly enhanced our understanding by directly viewing the behavior of a moving cell on top of the BBB and how this cell transmigrates across the endothelium. In this context, the active role of the BBB has been highlighted in initiating crosstalk with immune cells to ensure transmigration success. As a highly specialized vascular bed, the unicity of the BBB has been connected, to a certain extent, with its mode of interaction with the immune system. As compared to other vascular beds, immune cell recruitment takes longer across the BBB, the para- versus transcellular route can be preferred based on the immune cell sub-type or the inflammatory context.

Attempts to inhibit immune cell migration across the BBB have demonstrated proven benefits in treating CNS disorders such as MS but have also been associated with severe side effects leading to some deadly outcomes when translated into the clinic. Modulating immune cell functions and migration to treat CNS diseases can be risky as it directly affects other components and processes of the immune response. Therefore, further understanding the interplays and dynamics involved in immune cell migration into the CNS would greatly favor the discovery of more specific targets to minimize side effects and provide safer therapies.

References

Abadier, M., N. Haghayegh Jahromi, L. Cardoso Alves, R. Boscacci, D. Vestweber, S. Barnum, U. Deutsch, B. Engelhardt and R. Lyck. 2015. "Cell surface levels of endothelial ICAM-1 influence the transcellular or paracellular T-cell diapedesis across the blood-brain barrier." *Eur J Immunol* no. 45:1043–58. doi: 10.1002/eji.201445125.

Adamson, P., S. Etienne, P.O. Couraud, V. Calder and J. Greenwood. 1999. "Lymphocyte migration through brain endothelial cell monolayers involves signaling through endothelial ICAM-1 via a Rho-dependent pathway." *J Immunol* no. 162(5):2964-73.

Barreiro, O., M. Yanez-Mo, J.M. Serrador, M.C. Montoya, M. Vicente-Manzanares, R. Tejedor, H. Furthmayr and F. Sanchez-Madrid. 2002. "Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes." *J*

Cell Biol no. 157(7):1233-45.

Barzilai, S., S.K. Yadav, S. Morrell, F. Roncato, E. Klein, L. Stoler-Barak, O. Golani, S.W. Feigelson, A. Zemel, S. Nourshargh and R. Alon. 2017. "Leukocytes breach endothelial barriers by insertion of nuclear lobes and disassembly of endothelial actin filaments." *Cell Rep* no. 18(3):685-699. doi: 10.1016/j.celrep.2016.12.076.

Battistini, L., L. Piccio, B. Rossi, S. Bach, S. Galgani, C. Gasperini, L. Ottoboni, D. Ciabini, M.D. Caramia, G. Bernardi, C. Laudanna, E. Scarpini, R.P. McEver, E.C. Butcher, G. Borsellino and G. Constantin. 2003. "CD8+ T cells from patients with acute multiple sclerosis display selective increase of adhesiveness in brain venules: a critical role for P-selectin glycoprotein ligand-1." *Blood* no. 101(12):4775-82.

Baron, J.L., J.A. Madri, N.H. Ruddle, G. Hashim, C.A. Jr. Janeway. 1993. "Surface expression of alpha 4 integrin by CD4 T cells is required for their entry into brain parenchyma." *J Exp Med* no. 177(1):57-68.

Bartholomaus, I., N. Kawakami, F. Odoardi, C. Schlager, D. Miljkovic, J.W. Ellwart, W.E. Klinkert, C. Flugel-Koch, T.B. Issekutz, H. Wekerle and A. Flugel. 2009. "Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions." *Nature* no. 462:94–98.

Bauer, M., C. Brakebusch, C. Coisne, M. Sixt, H. Wekerle, B. Engelhardt and R. Fassler. 2009. "{beta}1 integrins differentially control extravasation of inflammatory cell subsets into the CNS during autoimmunity." *Proc Natl Acad Sci U S A* no. 106:1920–1925.

Bechmann, I., I. Galea and V.H. Perry. 2007. "What is the blood-brain barrier (not)? " *Trends Immunol* no. 28(1):5-11.

Bednar, M.M., S.D. Wright, S.J. Raymond-Russell, J.J. Kohut and C.E. Gross. 1996. "IB4, a monoclonal antibody against the CD18 leukocyte adhesion protein, reduces intracranial pressure following thromboembolic stroke in the rabbit." *Neurol Res* no. 18:171-5.

Becker, K.J. 2001. "Targeting the central nervous system inflammatory response in ischemic stroke." *Curr Opin Neurol* no. 14(3):349-53.

Becker, K.J. 2002. "Anti-leukocyte antibodies: LeukArrest (Hu23F2G) and Enlimomab (R6.5) in acute stroke." *Curr Med Res Opin* no. 18 Suppl 2:s18-22.

Billingham, R.E. and T. Boswell. 1953. "Studies on the problem of corneal homografts." *Proc R Soc Lond B Biol Sci* no.141(904):392-406.

Bill, R., A. Döring, U. Deutsch and B. Engelhardt. 2011. "PSGL-1 is dispensible for the development of active experimental autoimmune encephalomyelitis in SJL/J mice." *J. Neuroimmunol* no. 232: 207 208.

Bird, I.N., J.H. Spragg, A. Ager and N. Matthews. 1993. "Studies of

lymphocyte transendothelial migration: analysis of migrated cell phenotypes with regard to CD31 (PECAM-1), CD45RA and CD45RO." *Immunology* no. 80:553–60.

Bö, L., J.W. Peterson, S. Mørk, P.A. Hoffman, W.M. Gallatin, R.M. Ransohoff and B.D. Trapp. 1996. "Distribution of immunoglobulin superfamily members ICAM-1, -2, -3, and the beta 2 integrin LFA-1 in multiple sclerosis lesions." *J. Neuropathol. Exp. Neurol* no. 55(10):1060-72.1060–1072.

Butcher, E.C. 1991. "Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity." *Cell* no. 67(6):1033-6.

Bungartz, G., S. Stiller, M. Bauer, W. Muller, A. Schippers, N. Wagner, R. Fassler, and C. Brakebusch. 2006. "Adult murine hematopoiesis can proceed without β1 and β7 integrins." *Blood* no. 108: 1857–1864.

Cabarrocas, J., J. Bauer, E. Piaggio, R. Liblau and H. Lassmann. 2003."Effective and selective immune surveillance of the brain by MHC class I-restricted cytotoxic T lymphocytes." *Eur J Immunol* no. 33(5):1174-82.

Cannella, B. and C.S. Raine. 1995. "The adhesion molecule and cytokine profile of multiple sclerosis lesions." *Ann. Neurol* no. 37(4):424-35.

Carman, C.V. and T.A. Springer. 2004. "A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them." *J Cell Biol* no. 167:377–88.

Carman, C.V. 2009. "Mechanisms for transcellular diapedesis: probing and pathfinding by 'invadosome-like protrusions'." *J Cell Sci* no. 122(Pt 17):3025-35. doi: 10.1242/jcs.047522.

Carson, M.J., J.M. Doose, B. Melchior, C.D. Schmid and C.C. Ploix. 2006. "CNS immune privilege: hiding in plain sight." *Immunol Rev* no. 213:48–65. doi:10.1111/j.1600-065X.2006.00441.x.

Cayrol, R., K. Wosik, J.L. Berard, A. Dodelet-Devillers, I. Ifergan, H. Kebir, A.S. Haqqani, K. Kreymborg, S. Krug, R. Moumdjian, A. Bouthillier, B. Becher, N. Arbour, S. David, D. Stanimirovic and A. Prat. 2008. "Activated leukocyte cell adhesion molecule promotes leukocyte trafficking into the central nervous system." *Nat Immunol* no. 9:137-45.

Chen, H., M. Chopp, R.L. Zhang, G. Bodzin, Q. Chen, J.R. Rusche and R.F.3rd. Todd. 1994. "Anti-CD11b monoclonal antibody reduces ischemic cell damage after transient focal cerebral ischemia in rat." *Ann Neurol* no. 35(4):458-63.

Chopp, M., R.L. Zhang, H. Chen, Y. Li, N. Jiang and J.R. Rusche. 1994."Postischemic administration of an anti-Mac-1 antibody reduces

ischemic cell damage after transient middle cerebral artery occlusion in rats." *Stroke* no. 25(4):869-75.

Coisne, C., W. Mao and B. Engelhardt. 2009. "Cutting edge: Natalizumab blocks adhesion but not initial contact of human T cells to the blood-brain barrier in vivo in an animal model of multiple sclerosis." *J Immunol* no. 182(10):5909-13. doi: 10.4049/jimmunol.0803418.

Coisne, C., and B. Engelhardt. 2009. "Preclinical testing of strategies for therapeutic targeting of human T-cell trafficking in vivo." *Methods Mol Biol* no. 616:268-81. doi: 10.1007/978-1-60761-461-6_17.

Coisne, C., R. Lyck and B. Engelhardt. 2013. "Live cell imaging techniques to study T cell trafficking across the blood-brain barrier in vitro and in vivo." *Fluids Barriers CNS* no. 10(1):7. doi: 10.1186/2045-8118-10-7.

Connolly, E.S. Jr., C.J. Winfree, T.A. Springer, Y. Naka, H, Liao, S.D. Yan, D.M. Stern, R.A. Solomon, J.C. Gutierrez-Ramos and D.J. Pinsky. 1996. "Cerebral protection in homozygous null ICAM-1 mice after middle cerebral artery occlusion. Role of neutrophil adhesion in the pathogenesis of stroke." *J Clin Invest* no. 97(1):209-16.

Curis, C., F. Percher, P. Jeannin, T. Montange, S.A. Chevalier, D. Seilhean, L. Cartier, P.O. Couraud, O. Gout, A. Gessain, P.E. Ceccaldi and P.V. Afonso. 2016. "Human T-Lymphotropic Virus Type 1-Induced Overexpression of Activated Leukocyte Cell Adhesion Molecule (ALCAM) Facilitates Trafficking of Infected Lymphocytes through the Blood-Brain Barrier." *J Virol* no. 90(16):7303-7312. doi: 10.1128/JVI.00539-16.

Durieu-Trautmann, O., N. Chaverot, S. Cazaubon, A.D. Strosberg and P.O. Couraud. 1994. " Intercellular adhesion molecule-1 activation induces tyrosine phosphorylation of the cytoskeleton associated protein cortactin in brain microvessel endothelial cells." *J Biol Chem* no. 269:12536–40.

Engelhardt, B., M. Laschinger, M. Schulz, U. Samulowitz, D. Vestweber and G. Hoch.1998. "The development of experimental autoimmune encephalomyelitis in the mouse requires alpha4-integrin but not alpha4beta7-integrin." *J Clin Invest* no. 102(12):2096-105.

Engelhardt, B. and Wolburg, H. 2004. "Mini-review: transendothelial migration of leukocytes: through the front door or around the side of the house?" *Eur J Immunol* no. 34:2955–63. doi: 10.1002/eji.200425327.

Engelhardt, B., B. Kempe, S. Merfeld-Clauss, M. Laschinger, B. Furie, M. K. Wild and D. Vestweber. 2005. "P-selectin glycoprotein ligand 1 is not required for the development of experimental autoimmune

encephalomyelitis in SJL and C57BL/6 mice." *J. Immunol* no. 175: 1267–1275.

Engelhardt, B. and C. Coisne. 2011. "Fluids and barriers of the CNS establish immune privilege by confining immune surveillance to a two-walled castle moat surrounding the CNS castle." *Fluids Barriers CNS* no. 8(1):4. doi: 10.1186/2045-8118-8-4.

Engelhardt, B. and R.M. Ransohoff. 2012. "Capture, crawl, cross: the T cell code to breach the blood–brain barriers. " *Trends Immunol* no. 33:579–589.

Emerich, D.F., R.L.3rd. Dean and R.T. Bartus. 2002. "The role of leukocytes following cerebral ischemia: pathogenic variable or bystander reaction to emerging infarct? " *Exp Neurol* no. 173(1):168-81.

Etienne, S., P. Adamson, J. Greenwood, A.D. Strosberg, S. Cazaubon and P.O. Couraud. 1998. "ICAM-1 signaling pathways associated with Rho activation in microvascular brain endothelial cells." *J Immunol* no.161: 5755–61.

Etienne-Manneville, S., J.B. Manneville, P. Adamson, B. Wilbourn, J. Greenwood and P.O. Couraud. 2000. "ICAM-1-coupled cytoskeletal rearrangements and transendothelial lymphocyte migration involve intracellular calcium signaling in brain endothelial cell lines." *J Immunol* no. 165:3375–83.

Floris, S., S.R. Ruuls, A. Wierinckx, S.M. van der Pol, E. Döpp, P.H. van der Meide, C.D. Dijkstra and H.E. De Vries. 2002. "Interferon-β directly influences monocyte infiltration into the central nervous system." *J Neuroimmunol* no. 127: 69–79.

Furuya, K., H. Takeda, S. Azhar, R.M. McCarron, Y. Chen, C.A. Ruetzler, K.M. Wolcott, T.J. DeGraba, R. Rothlein, T.E. Hugli, G.J. del Zoppo and J.M. Hallenbeck. 2001. "Examination of several potential mechanisms for the negative outcome in a clinical stroke trial of enlimomab, a murine anti-human intercellular adhesion molecule-1 antibody: a bedside-to- bench study." *Stroke* no. 32(11):2665-74.

Greenwood, J., Y. Wang and V. Calder. 1995. "Lymphocyte adhesion and transendothelial migration in the CNS: the role of LFA-1, ICAM-1, VLA-4 and VCAM-1." *Immunology* no. 86: 408–15.

Gorina, R., R. Lyck, D. Vestweber and B. Engelhardt. 2014."β2 integrin-mediated crawling on endothelial ICAM-1 and ICAM-2 is a prerequisite for transcellular neutrophil diapedesis across the inflamed blood-brain barrier."*J Immunol* no.192(1):324-37. doi: 10.4049/jimmunol.1300858.

Haghayegh Jahromi, N., H. Tardent, G. Enzmann, U. Deutsch, N. Kawakami, S. Bittner, D. Vestweber, F. Zipp, J.V. Stein and B. Engelhardt. 2017."A Novel Cervical Spinal Cord Window Preparation Allows for Two-Photon Imaging of T-Cell Interactions with the Cervical Spinal Cord Microvasculature during Experimental Autoimmune Encephalomyelitis." *Front Immunol* no. 8:406. doi: 10.3389/fimmu.2017.00406.

Hickey, W.F., 2001. "Basic principles of immunological surveillance of the normal central nervous system." *Glia* no. 36:118-24.

Huseby, E.S., D. Liggitt, T. Brabb, B. Schnabel, C. Ohlén and J. Goverman. 2001. "A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis." *J Exp Med* no. 194(5): p. 669-76.

Jain, P., C. Coisne, G. Enzmann, R. Rottapel and B. Engelhardt. 2010. "Alpha4beta1 integrin mediates the recruitment of immature dendritic cells across the blood-brain barrier during experimental autoimmune encephalomyelitis." *J Immunol* no. 184(12):7196-206. doi: 10.4049/jimmunol.0901404.

Jones, R. 2000. "*Rovelizumab (ICOS Corp).*" *IDrugs* no. **3**(4):442-6.

Kanters, E., J. van Rijssel, P.J. Hensbergen, D. Hondius, F.P. Mul, A.M. Deelder, A. Sonnenberg, J.D. van Buul and P.L. Hordijk. 2008. "Filamin B. Mediates ICAM-1-driven leukocyte transendothelial migration." *J Biol Chem* no. 283: 31830–9.

Kanwar, J.R., J.E. Harrison, D. Wang, E. Leung, W. Mueller, N. Wagner and G.W. Krissansen. 2000. "Beta7 integrins contribute to demyelinating disease of the central nervous system." *J Neuroimmunol* no. 103:146-52.

Kapoor, R., P.R. Ho, N. Campbell, I. Chang, A. Deykin, F. Forrestal, N. Lucas, B. Yu, D.L. Arnold, M.S. Freedman, M.D. Goldman, H.P. Hartung, E.K. Havrdová, D. Jeffery, A. Miller, F. Sellebjerg, D. Cadavid, D. Mikol and D. Steiner; ASCEND investigators. 2018. "Effect of natalizumab on disease progression in secondary progressive multiple sclerosis (ASCEND): a phase 3, randomised, double-blind, placebo-controlled trial with an open-label extension." *Lancet Neurol* no.17(5):405-415. doi: 10.1016/S1474-4422(18)30069-3.

Kerfoot, S. and P. Kubes. 2002. "Overlapping roles of P-selectin and alpha 4 integrin to recruit leukocytes to the central nervous system in experimental autoimmune encephalomyelitis." *J. Immunol* no. 169:1000-6.

Kerfoot, S., M.M.U. Norman, B.M. Lapointe, C.S. Bonder, L. Zbytnuik and P. Kubes. 2006. "Reevaluation of P-selectin and alpha 4 integrin as

targets for the treatment of experimental autoimmune encephalomyelitis." *J Immunol* no. **176**:6225–34

Kleinschmidt-Demasters, B.K. and K.L. Tyler. 2005. "Progressive Multifocal Leukoencephalopathy Complicating Treatment with Natalizumab and Interferon Beta-1a for Multiple Sclerosis." *N Engl J Med* no. 353(4):369-74.

Krams, M., K.R. Lees, W. Hacke, A.P. Grieve, J.M. Orgogozo and G.A. Ford; ASTIN Study Investigators. 2003. "Acute Stroke Therapy by Inhibition of Neutrophils (ASTIN): an adaptive dose-response study of UK-279,276 in acute ischemic stroke." *Stroke* no. 34(11):2543-8.

Langhauser, F., P. Kraft, E. Göb, J. Leinweber, M.K. Schuhmann, K. Lorenz, M. Gelderblom, S. Bittner, S.G. Meuth, H. Wiendl, T. Magnus and C. Kleinschnitz. 2014. "Blocking of α4 integrin does not protect from acute ischemic stroke in mice." *Stroke* no. 45(6):1799-806. doi: 10.1161/STROKEAHA.114.005000.

Laschinger, M., P. Vajkoczy, and B. Engelhardt. 2002. "Encephalitogenic T cells use LFA-1 for transendothelial migration but not during capture and initial adhesion strengthening in healthy spinal cord microvessels in vivo." *Eur J Immunol* no. 32(12):3598-606.

Lécuyer, M.A., O. Saint-Laurent, L. Bourbonnière, S. Larouche, C. Larochelle, L. Michel, M. Charabati, M. Abadier, S. Zandee, N. Haghayegh Jahromi, E. Gowing, C. Pittet, R. Lyck, B. Engelhardt and A. Prat. 2017. "Dual role of ALCAM in neuroinflammation and blood-brain barrier homeostasis." *Proc Natl Acad Sci U S A* no. 114(4):E524-E533.

Lee, S.J., and E.N. Benveniste. 1999. "Adhesion molecule expression and regulation on cells of the central nervous system." *J Neuroimmunol* no. 98:77-88.

Ley, K., and G. Kansas. 2004. "Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation." *Nat. Rev. Immunol* no. 4:325.

Ley, K., C. Laudanna, M.I. Cybulsky and S. Nourshargh. 2007. "Getting to the site of inflammation: the leukocyte adhesion cascade updated". *Nat Rev Immunol* no. 7:678–89.

Li, N., H. Yang, M. Wang, S. Lü, Y. Zhang and M. Long. 2018. "Ligand-specific binding forces of LFA-1 and Mac-1 in neutrophil adhesion and crawling." *Mol Biol Cell* no. 29(4):408-418. doi: 10.1091/mbc.E16-12-0827.

Liesz A, Sun L, Zhou W, Schwarting S, Mracsko E, Zorn M, Bauer H, Sommer C, Veltkamp R. 2011."FTY720 reduces post-ischemic brain

lymphocyte influx but does not improve outcome in permanent murine cerebral ischemia." *PLoS One* no. 6(6):e21312. doi: 10.1371/journal.pone.0021312.

Llovera, G., K. Hofmann, S. Roth, A. Salas-Pérdomo, M. Ferrer-Ferrer, C. Perego, E.R. Zanier, U. Mamrak, A. Rex, H. Party, V. Agin, C. Fauchon, C. Orset, B. Haelewyn, M.G. De Simoni, U. Dirnagl, U. Grittner, A.M. Planas, N. Plesnila, D. Vivien and A. Liesz. 2015. "Results of a preclinical randomized controlled multicenter trial (pRCT): Anti-CD49d treatment for acute brain ischemia." *Sci Transl Med* no. 7(299):299ra121. doi: 10.1126/scitranslmed.aaa9853.

Lopes-Pinheiro, M.A., G. Kooij, M.R. Mizee, A. Kamermans, G. Enzmann, R. Lyck, M. Schwaninger, B. Engelhardt and H.E. de Vries. 2016. "Immune cell trafficking across the barriers of the central nervous system in multiple sclerosis and stroke." *Biochim Biophys Acta* no. 1862(3):461-71. doi: 10.1016/j.bbadis.2015.10.018.

Lublin, F.t.H.F.G.M.S.G. 1999. "A Phase II Trial of Anti-CD11/CD18 Monoclonal Antibody in Acute Exacerbations of Multiple Sclerosis." *Neurology* no. **52**((6) (Supplement 2)): p. A290-A291.

Luo, B.H., and T.A. Springer. 2006. "Integrin structures and conformational signaling." *Curr Opin Cell Biol* no. 18(5):579-86.

Lutz, S.E., J.R. Smith, D.H. Kim, C.V.L. Olson, K. Ellefsen, J.M. Bates, S.P. Gandhi and D. Agalliu. 2017. "Caveolin1 is required for Th1 cell infiltration, but not tight junction remodeling, at the blood-brain barrier in autoimmune neuroinflammation." *Cell Rep* no. 21(8):2104-2117. doi: 10.1016/j.celrep.2017.10.094.

Lyck, R., and B. Engelhardt. 2012. "Going against the tide--how encephalitogenic T cells breach the blood-brain barrier." *J Vasc Res* no. 49(6):497-509. doi: 10.1159/000341232.

Lyck, R., M.A. Lécuyer, M. Abadier, C.B. Wyss, C. Matti, M. Rosito, G. Enzmann, T. Zeis, L. Michel, A.B. García Martín, F. Sallusto, F. Gosselet, U. Deutsch, J.A. Weiner, N. Schaeren-Wiemers, A. Prat and B. Engelhardt. 2017. "ALCAM (CD166) is involved in extravasation of monocytes rather than T cells across the blood-brain barrier." *J Cereb Blood Flow Metab* no. 37(8):2894-2909. doi: 10.1177/0271678X16678639.

Mamdouh, Z., X. Chen, L.M. Pierini, F.R. Maxfield and W.A. Muller. 2003. "Targeted recycling of PECAM from endothelial surface-connected compartments during diapedesis." *Nature* no. 421:748–53. doi: 10.1038/nature01300.

Martin-Blondel, G., B. Pignolet, S. Tietz, L. Yshii, C. Gebauer, T. Perinat, I. Van Weddingen, C. Blatti, B. Engelhardt and R. Liblau. 2015.

"Migration of encephalitogenic CD8 T cells into the central nervous system is dependent on the α4β1-integrin." *Eur J Immunol* no. 45(12):3302- 12. doi: 10.1002/eji.201545632.

Medawar, P.B. 1948. "Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye." *Br J Exp Pathol* no. 29(1):58-69.

Millán, J., L. Hewlett, M. Glyn, D. Toomre, P. Clark and A.J. Ridley. 2006. "Lymphocyte transcellular migration occurs through recruitment of endothelial ICAM-1 to caveola- and F-actin-rich domains." *Nat Cell Biol* no. 8: 113–23.

Miller, D.H., T. Weber, R. Grove, C. Wardell, J. Horrigan, 0. Graff, G. Atkinson, P. Dua, T. Yousry, D. Macmanus and X. Montalban. 2012. "Firategrast for relapsing remitting multiple sclerosis: a phase 2, randomised, double-blind, placebo-controlled trial." *Lancet Neurol* no. 11:131-9. doi: 10.1016/S1474-4422(11)70299-X.

Mills, E.A., and Y. Mao-Draayer. 2018. "Understanding Progressive Multifocal Leukoencephalopathy risk in multiple sclerosis patients treated with immunomodulatory therapies: a bird's eye view." *Front Immunol* no. 9:138. doi: 10.3389/fimmu.2018.00138.

Mittelbrunn, M., A. Molina, M. M. Escribese, M. Yanez-Mo, E. Escudero, A. Ursa, R. Tejedor, F. Mampaso, and F. Sanchez-Madrid. 2004. "VLA-4 integrin concentrates at the peripheral supramolecular activation complex of the immune synapse and drives T helper 1 responses." *Proc. Natl. Acad. Sci. USA* no. 101: 11058–11063.

Muller, W.A., C.M. Ratti, S.L. McDonnell and Z.A. Cohn. 1989. "A human endothelial cell-restricted, externally disposed plasmalemmal protein enriched in intercellular junctions." *J Exp Med* no. 170:399–414. doi: 10.1084/jem.170.2.399.

Muller, W.A., S.A. Weigl, X. Deng and D.M. Phillips. 1993. "PECAM-1 is required for transendothelial migration of leukocytes." *J Exp Med* no. 178:449–60. doi: 10.1084/jem.178.2.449.

Neumann ,J., M. Riek-Burchardt, J. Herz, T.R. Doeppner, R. König, H. Hütten, E. Etemire, L. Männ, A. Klingberg, T. Fischer, M.W. Görtler, H.J. Heinze, P. Reichardt, B. Schraven, D.M. Hermann, K.G.

Reymann and M. Gunzer. 2015. "Very-late-antigen-4 (VLA-4)-mediated brain invasion by neutrophils leads to interactions with microglia, increased ischemic injury and impaired behavior in experimental stroke." *Acta Neuropathol* no. 129:259-77. doi: 10.1007/s00401-014-1355-2.

O'Neill, J.K., C. Butter, D. Baker, S.E. Gschmeissner, G. Kraal, E.C. Butcher and J.L. Turk. 1991. "Expression of vascular addressins and

ICAM-1 by endothelial cells in the spinal cord during chronic relapsing experimental allergic encephalomyelitis in the Biozzi AB/H mouse." *Immunology* no. 72(4):520-5.

Osmers, I., D. C. Bullard and S. R. Barnum. 2005. "PSGL-1 is not required for development of experimental autoimmune encephalomyelitis." *J. Neuroimmunol* no. 166: 193–196.

Owens, T., I. Bechmann and B. Engelhardt. 2008. "Perivascular spaces and the two steps to neuroinflammation." *J Neuropathol Exp Neurol* no. 67(12):1113-21. doi: 10.1097/NEN.0b013e31818f9ca8.

Peterson, J.W., L. Bö, S. Mörk, A. Chang, R.M. Ransohoff, B.D. Trapp. 2002. "VCAM-1-positive microglia target oligodendrocytes at the border of multiple sclerosis lesions." *J. Neuropathol. Exp. Neurol* no. 61(6):539-46.

Piccio, L., B. Rossi, E. Scarpini, C. Laudanna, C. Giagulli, A.C. Issekutz, D. Vestweber, E.C. Butcher, and G. Constantin. 2002. "Molecular mechanisms involved in lymphocyte recruitment in inflamed brain microvessels: critical roles for P-selectin glycoprotein ligand-1 and heterotrimeric G(i)-linked receptors." *J Immunol* no. 168(4):1940-9.

Piccio, L., B. Rossi, L. Colantonio, R. Grenningloh, A. Gho, L. Ottoboni, J.W. Homeister, E. Scarpini, M. Martinello, C. Laudanna, D. D'Ambrosio, J.B. Lowe and G. Constantin. 2005. "Efficient recruitment of lymphocytes in inflamed brain venules requires expression of cutaneous lymphocyte antigen and fucosyltransferase-VII." *J Immunol* no. 174(9):5805-13.

Polman, C.H., P.W. O'Connor, E. Havrdova, M. Hutchinson, L. Kappos, D.H. Miller, J.T. Phillips, F.D. Lublin, G. Giovannoni, A. Wajgt, M. Toal, F. Lynn, M.A. Panzara, A.W. Sandrock; AFFIRM Investigators. 2006. "A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis." *N Engl J Med* no. 354(9):899-910.

Reese, T.S. and M.J. Karnovsky. 1967. "Fine structural localization of a blood-brain barrier to exogenous peroxidase." *J Cell Biol* no. 34(1):207-17.

Reiss, Y., G. Hoch, U. Deutsch and B. Engelhardt. 1998. "T cell interaction with ICAM-1-deficient endothelium in vitro: essential role for ICAM-1 and ICAM-2 in transendothelial migration of T cells." *Eur J Immunol* no. **28**:3086–99.

Rudick, R.A., W.H. Stuart, P.A. Calabresi, C. Confavreux, S.L. Galetta, E.W. Radue, F.D. Lublin, B. Weinstock-Guttman, D.R. Wynn, F. Lynn, M.A. Panzara and A.W. Sandrock; SENTINEL Investigators. 2006. "Natalizumab plus interferon beta-1a for relapsing multiple sclerosis." *N Engl J Med* no. 354(9):911-23.

Rudolph, H., A. Klopstein, I. Gruber, C. Blatti, R. Lyck, B. Engelhardt. 2016. "Postarrest stalling rather than crawling favors CD8(+) over CD4(+) T-cell migration across the blood-brain barrier under flow in vitro." *Eur J Immunol* no. 46(9):2187-203. doi: 10.1002/eji.201546251

Sathiyanadan, K., C. Coisne, G. Enzmann, U. Deutsch and B. Engelhardt. 2014. "PSGL-1 and E/P-selectins are essential for T-cell rolling in inflamed CNS microvessels but dispensable for initiation of EAE." *Eur J Immunol* no. 44(8):2287-94. doi: 10.1002/eji.201344214.

Schenkel, A.R., T.W. Chew and W.A. Muller. 2004. "Platelet endothelial cell adhesion molecule deficiency or blockade significantly reduces leukocyte emigration in a majority of mouse strains." *J Immunol* no. 173:6403–8. doi: 10.4049/jimmunol.173.10.6403.

Simmons, D.L. 2005. "Anti-adhesion therapies." *Curr Opin Pharmacol* no. 5(4):398-404.

Sixt, M., M. Bauer, T. Lammermann, and R. Fassler. 2006. "β1 integrins: zip codes and signaling relay for blood cells." *Curr. Opin. Cell Biol* no. 18: 482–490.

Skonier, J.E., M.A. Bowen, J. Emswiler, A. Aruffo and J. Bajorath. 1996. "Recognition of diverse proteins by members of the immunoglobulin superfamily: delineation of the receptor binding site in the human CD6 ligand ALCAM." *Biochemistry* no. **35**:12287–91.

Springer, T. A. 1995. "Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration." *Annu. Rev. Physiol* no. 57, 827–872.

Steffen, B.J., E.C. Butcher and B. Engelhardt. 1994. "Evidence for involvement of ICAM-1 and VCAM-1 in lymphocyte interaction with endothelium in experimental autoimmune encephalomyelitis in the central nervous system in the SJL/J mouse." *Am J Pathol* no.145,189–201.

Steiner, O., C. Coisne, R. Cecchelli, R. Boscacci, U. Deutsch, B. Engelhardt and R. Lyck. 2010. "Differential roles for endothelial ICAM-1, ICAM-2, and VCAM-1 in shear-resistant T cell arrest, polarization, and directed crawling on blood-brain barrier endothelium." *J Immunol* no. 185(8):4846-55. doi: 10.4049/jimmunol.0903732.

Steiner, O., C. Coisne, B. Engelhardt and R. Lyck. 2011. "Comparison of immortalized bEnd5 and primary mouse brain microvascular endothelial cells as in vitro blood-brain barrier models for the study of T cell extravasation." *J Cereb Blood Flow Metab* no. 31(1):315-27. doi: 10.1038/jcbfm.2010.96.

Stockinger, H., S.J. Gadd, R. Eher, O. Majdic, W. Schreiber, W.

Kasinrerk, B. Strass, E. Schnabl, W. Knapp. 1990. "Molecular characterization and functional analysis of the leukocyte surface protein CD31." *J Immunol* no.45:3889–97.

Sverdlov, M., V. Shinin, A.T. Place, M. Castellon and R.D. Minshall. 2009. "Filamin A regulates caveolae internalization and trafficking in endothelial cells." *Mol Biol Cell* no. 20:4531–40.

Thal, D.R. 2009. "The pre-capillary segment of the blood-brain barrier and its relation to perivascular drainage in Alzheimer's disease and small vessel disease." *Scientific World Journal* no. 9:557-63. doi: 10.1100/tsw.2009.72.

Tietz, S., T. Périnat, G. Greene, G. Enzmann, U. Deutsch, R. Adams, B. Imhof, M. Aurrand-Lions and B. Engelhardt. 2018. "Lack of junctional adhesion molecule (JAM)-B ameliorates experimental autoimmune encephalomyelitis." *Brain Behav Immun* no. 73:3-20. doi: 10.1016/j.bbi.2018.06.014.

Turowski, P., R. Martinelli, R. Crawford, D. Wateridge, A-P. Papagiorgiou, M.G. Lampugnani, A. Gamp, D. Vestweber, P. Adamson, E. Dejana and J. Greenwood. 2008. "Phosphorylation of vascular endothelial cadherin controls lymphocyte emigration." *J Cell Sci* no. 121: 29–37.

Vajkoczy, P., M. Laschinger and B. Engelhardt. 2001. "Alpha4-integrin-VCAM-1 binding mediates G protein-independent capture of encephalitogenic T cell blasts to CNS white matter microvessels." *J Clin Invest* no. 108(4):557-65.

Valignat, M-P., O. Theodoly, A. Gucciardi, N. Hogg and A.C. Lellouch. 2013. "T lymphocytes orient against the direction of fluid flow during LFA-1-mediated migration." *Biophys J.* 104:322-31. doi: 10.1016/j.bpj.2012.12.007.

van Kempen, L.C., J.M. Nelissen, W.G. Degen, R. Torensma, U.H. Weidle, H.P. Bloemers, C.G. Figdor and G.W. Swart. 2001. "Molecular basis for the homophilic activated leukocyte cell adhesion molecule (ALCAM)-ALCAM interaction." *J Biol Chem* no. 276: 25783–90.

Vemuganti, R., R.J. Dempsey and K.K. Bowen. 2004. "Inhibition of intercellular adhesion molecule-1 protein expression by antisense oligonucleotides is neuroprotective after transient middle cerebral artery occlusion in rat." *Stroke* no. 35(1):179-84.

von Andrian, U.H. and C. M'Rini. 1998. "In situ analysis of lymphocyte migration to lymph nodes. " *Cell Adhes Commun* no. 6:85–96.

Williams, D.W., K. Anastos, S. Morgello and J.W. Berman. 2015. "JAM-A and ALCAM are therapeutic targets to inhibit diapedesis across the

BBB of CD14_CD16_ monocytes in HIV-infected individuals." *J Leukoc Biol* no.97:401–412.

Williams, D.W., T.M. Calderon, L. Lopez, L. Carvallo-Torres, P.J. Gaskill, E.A. Eugenin, S. Morgello and J.W. Berman. 2013. "Mechanisms of HIV entry into the CNS: increased sensitivity of HIV infected CD14_CD16_monocytes to CCL2 and key roles of CCR2, JAM-A, and ALCAM in diapedesis." *PLoS One* no. 8:e69270.

Wimmer, I., S. Tietz, H. Nishihara, U. Deutsch, F. Sallusto, F. Gosselet, R. Lyck, W.A. Muller, H. Lassmann and B. Engelhardt. 2019. "PECAM-1 stabilizes blood-brain barrier integrity and favors paracellular T-cell diapedesis across the blood-brain barrier during neuroinflammation." *front Immunol* no. 10:711. doi: 10.3389/fimmu.2019.00711.

Winger, R.C., J.E. Koblinski, T. Kanda, R.M. Ransohoff and W.A. Muller. 2014. "Rapid remodeling of tight junctions during paracellular diapedesis in a human model of the blood-brain barrier." *J Immunol* no. 193(5): 2427–2437. doi: 10.4049/jimmunol.1400700.

Yao, H., K. Kim, M. Duan, T. Hayashi, M. Guo, S. Morgello, A. Prat, J. Wang, T.P. Su and S. Buch. 2011. "Cocaine hijacks sigma1 receptor to initiate induction of activated leukocyte cell adhesion molecule: implication for increased monocyte adhesion and migration in the CNS." *J Neurosci* no. 31:5942–5955.

Yednock, T.A., C. Cannon, L.C. Fritz, F. Sanchez-Madrid, L. Steinman and N. Karin. 1992. "Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha4beta 1 integrin." *Nature* no. 356(6364):63-6.

CHAPTER FIVE

ACUTE NEUROVASCULAR PATHOLOGIES AND DYSFUNCTIONS

RAQUEL FERREIRA
AND MIGUEL CASTELO-BRANCO

Introduction

According to the World Health Organization (WHO), stroke or cerebrovascular accidents are the second main cause of mortality and the third cause of morbidity worldwide; in many countries, stroke is still the main cause of mortality (WHO 2012). Cerebrovascular accidents occur when the integrity of the brain vasculature and/or blood flow become compromised. In particular, the interruption of blood flow affects oxygen and nutrient supply to brain cells, impairing several neurological and motor functions. In this context, acute events either refer to the obstruction of a blood vessel (ischemic stroke), or a ruptured vessel (causing intracerebral hemorrhage or subarachnoid hemorrhage) (Grysiewicz, Thomas, and Pandey 2008); they are triggered by several different factors, which can be caused or further aggravated by detrimental lifestyle behaviors. These types of injury may unleash profound neurological deficits and physical disability that amount to a significant socioeconomic burden on family members, care providers, health systems and society. In that sense, low- and middle-income countries are particularly affected, but preventive measures targeting these factors have proved effective (Feigin, Roth, et al. 2016). However, prevention of stroke involves a call for action from governments, which have the power to tightly regulate environmental, social, medical and lifestyle factors through appropriate legislation and research funding (Feigin, Norrving, and Mensah 2017).

The introduction of thrombolysis and thrombectomy are intervention priorities in ischemic stroke (Hacke et al. 1998). However, full neurological recovery or rehabilitation can be challenging given the

narrow therapeutic window after stroke onset and the potentially serious side effects of most treatments if administered beyond that period. To counteract these issues, the main approach (apart from thrombolysis and thrombectomy) to treating acute neurovascular pathologies and dysfunctions has been primarily focused on symptom management, highlighting the need for new pharmaceuticals and/or medical devices. However, stroke research continues to be underfunded compared to other pathologies, delaying progress in this field (Rothwell 2007, Feigin, Norrving, and Mensah 2017). The present chapter will review current data on ischemic stroke, intracerebral and subarachnoid hemorrhage and discuss the therapeutic targets and experimental approaches used to design more efficient treatments for acute neurovascular pathologies and dysfunctions.

Ischemic Stroke

Ischemic stroke is the most common subtype, representing 80 to 85% of stroke cases, and can be triggered by an obstructing clot that causes an embolism, or by local plaque causing a thrombotic stroke (Dirnagl, Iadecola, and Moskowitz 1999). Commonly, a stroke will trigger lateralized symptoms such as hemiparesis or loss of sensation, aphasia, visual impairment, amongst other indicators. Symptoms that last less than 24 hours typically indicate a temporary ischemic event or transient ischemic attack (TIA). Approximately half of stroke survivors will still display hemiparesis and dysphagia six months after onset, while a quarter will remain institutionalized (Rego, Duarte, and Oliveira 2017). Lesions on strategic areas (e.g. hippocampus), white matter lesions, cerebral microbleeds and the occurrence of neurodegenerative conditions (e.g. Alzheimer's disease) may cause post-stroke cognitive impairment or dementia, affecting to various degrees language, memory, executive function, visuoconstruction, calculation and comprehension (Sun, Tan, and Yu 2014, Kalaria, Akinyemi, and Ihara 2016).

In overall terms, blood flow disruption lowers glucose and oxygen levels. Initially, blood vessels dilate to counteract this effect. However, as the supply of nutrient and oxygen continues to decrease, adenosine triphosphate (ATP) levels significantly drop, lactate accumulates, ionic homeostasis is disrupted, and metabolic pathways are inhibited, leading to unregulated inflammatory activity and cytotoxicity (Dirnagl, Iadecola, and Moskowitz 1999, Dirnagl 2012, Hu and Song 2017). The ischemic core designates the region of irreversible damage in which most neural cells have died by necrosis. The penumbra area or peri-infarct region presents

reduced collateral blood circulation, but ionic homeostasis is still maintained, and the majority of neuronal cells become sensitive to apoptosis (Hu and Song 2017).

In terms of incidence, there are significant differences between men and women, with 133 per 100,000 person-years for men, and 99 per 100,000 person-years for women (Feigin, Norrving, and Mensah 2017). These numbers likely reflect a greater life expectancy for women and the fact that female hormone estrogen seems to award protection in vitro (Gu et al. 2010, Nakamura et al. 2006). However, a clinical trial testing the effect of 1 mg of estradiol-17β daily in postmenopausal women who had an ischemic stroke or transient ischemic attack showed no reduction in mortality or recurrence (Viscoli et al. 2001).

There are several risk factors involved in ischemic stroke, namely hypertension, atrial fibrillation, smoking, abdominal obesity, poor diet and limited physical activity, diabetes mellitus, increased alcohol intake and psychosocial factors, and higher levels of apolipoproteins (O'Donnell et al. 2010). Non-modifiable factors that increase the risk of stroke include age (being over 80 years old), race (black) and sex (males) (Grysiewicz (Grysiewicz, Thomas, and Pandey 2008).

Thrombolytic therapy (intravenous administration of a clot-dissolving agent such as recombinant tissue plasminogen activator or rtPA) is a well-established method, depending on clot size. Larger clots require endovascular approaches such as retrievable stents and large bore aspiration catheters (Jacquin and van Adel 2015). A common complication of ischemic stroke (10 to 40% of patients) is hemorrhagic transformation, which may also arise from the administration of thrombolytic therapy to restore blood flow (Jickling et al. 2014, Terruso et al. 2009, Beslow et al. 2011). While the extravasation of protein-rich fluids into ischemic tissue may cause edema, delayed reperfusion of brain tissue may also enhance ionic and later vasogenic swelling. The detection of ionic and vasogenic edema is effectively achieved by computed tomography, which can quantify changes in water content. A complementary tool of diagnosis is diffusion-weighted magnetic resonance imaging (MRI) (von Kummer and Dzialowski 2017). A review of hemorrhagic transformation occurring in clinical trials of acute ischemic stroke revealed a trend towards higher hemorrhage rates after endovascular intervention or delayed recanalization (Sussman and Connolly 2013).

Guidelines for the early management of patients with acute ischemic stroke have been recently made available to improve to work of prehospital care providers, physicians, health professionals, and even hospital administrators (Powers et al. 2018). This document details

prehospital care, urgent and emergency evaluation as well as intravenous and intra-arterial treatment, and in-hospital management.

Intracerebral hemorrhage

Intracerebral hemorrhage (ICH) is defined by vessel spillage into the brain parenchyma (Caceres and Goldstein 2012). The absence of a pre-existing lesion defines primary ICH, while hemorrhaging caused by an underlying lesion is termed secondary ICH (Ikram, Wieberdink, and Koudstaal 2012). ICH usually manifests as a result of hypertensive vasculopathy that degenerates the vessel wall and later leads to smooth muscle cell loss, wall thickening and consequent luminal reduction, and small-scale bleeding (Caceres and Goldstein 2012). The initial hematoma produces injury to the brain parenchyma and unleashes an inflammatory and coagulation cascade.

ICH has a reported incidence of 24.6 per 100,000 person-years and is most common in older individuals (over 85 years old) and/or in those of Asian background (van Asch et al. 2010).

ICH is caused by the association of several risk factors, which include medical conditions such as hypertension, cerebral amyloid angiopathy and diabetes mellitus, use of oral anticoagulants and low levels of cholesterol, poor lifestyle habits (smoking, drug use and increased alcohol intake), and genetic factors such as the presence of the ε2 and ε4 alleles of the apolipoprotein E gene (Caceres and Goldstein 2012, Ikram, Wieberdink, and Koudstaal 2012).

A recent document discussing guidelines for the management of spontaneous ICH concluded that lowering blood pressure can be considered safe and possibly effective (Hemphill et al. 2015). Patients can also be subjected to surgical intervention such as external ventricular drain placement, intraventricular thrombolysis, and hematoma evacuation (Caceres and Goldstein 2012). Hematoma evacuation, a minimally invasive procedure was shown to reduce perihematomal edema volume; rtPA administration did not influence edema size (Mould et al. 2013). The application of neuroprotective agents is also considered but translating data from animal models to patients is a challenging and time-consuming process.

Subarachnoid hemorrhage

Subarachnoid hemorrhage (SAH) is defined by a ruptured vessel into the subarachnoid space, with or without intraparenchymal hemorrhage. It

can be caused by a traumatic event or, in nontraumatic cases, by a bursting aneurysm (which accounts for approximately 80% of spontaneous SAH) (Raya and Diringer 2014, Caceres and Goldstein 2012).

SAH incidence is 9 per 100,000 patients-years (de Rooij et al. 2007, Rinkel and Algra 2011) and it is most common in women over the age of 55, particularly with a history of hypertension, smoking and alcohol intake (de Rooij et al. 2007, Caceres and Goldstein 2012).

SAH results from wall shear stress and transmural pressures on the vessel wall, which lead to the formation and growth of aneurysms. Fatal consequences of SAH are vasospasm (arterial spasm that leads to vasoconstriction) and delayed cerebral ischemia.

Patients often present a severe headache, neck pain or stiffness, and high blood pressure. In this case, they are often subjected to a neurological exam and endotracheal intubation, to blood pressure management, seizure prophylaxis, and glycemic and temperature control. If applicable, surgical interventions may require endovascular coiling or surgical clipping (Caceres and Goldstein 2012).

Therapeutic targets and experimental approaches

An important tool to pinpoint the underlying mechanisms of disease and putative targets for the development of more effective therapies is the use of animal models. Simpler in vitro or ex vivo approaches resort to exposing cells to oxygen and glucose deprivation (OGD) to mimic the ischemic environment (Holloway and Gavins 2016). More complex ventures using the whole animal can more accurately reproduce some of the pathophysiological traits of the disease and provide a deeper insight on early events and the effects of reperfusion and intravascular administration of molecules of interest (Fluri, Schuhmann, and Kleinschnitz 2015). Briefly, models based on intraluminal occlusion of the middle cerebral artery are thought to mimic human ischemic stroke with higher reproducibility although it is not suitable for studying thrombolysis. Conversely, the embolic stroke model (microsphere-/macrosphere-induced or thromboembolic clot approaches) may be used to study the administration of thrombolytic agents. Nevertheless, infarcts are difficult to reproduce, and spontaneous recanalization may occur in this model. The craniotomy model, which is highly invasive, presents high long-term survival rates but requires a high degree of surgical skill. The photothrombosis model produces a well-defined ischemic lesion, however, it is not believed to be suitable for investigating neuroprotective agents. The endothelin-1 model induces ischemia in cortical or subcortical

regions, but the main disadvantages pertain to difficulty in controlling the duration of ischemia; data interpretation is compromised by the occurrence of astrocytosis and axonal sprouting.

These experimental approaches enable the study of basic mechanisms underlying stroke, namely excitotoxicity, oxidative stress and inflammation, and other processes that contemplate the diverse cell-cell interactions taking place within the neurovascular unit (Terasaki et al. 2014). Excessive exposure to glutamate is connected to several acute and neurodegenerative brain disorders by causing significant neuronal damage or death (excitotoxicity). Glutamate accumulation in the synaptic cleft keeps N-methyl-D-aspartate receptors active, causing an abnormal calcium influx that activates phospholipases and proteases that trigger membrane damage, endonucleases that unleash nuclear damage, ATPase activation and mitochondrial dysfunction that lower ATP levels, further potentiating cell death (Hu and Song 2017).

Neuronal cells are particularly sensitive to oxidative stress: neurons have high oxygen consumption, high content of polyunsaturated fatty acids, catecholamines, and transition metals (Fe^{2+}, Cu^{+}), and low levels of anti-oxidant enzymes (Rego, Duarte, and Oliveira 2017). In that sense, an efficient therapeutic approach may focus on inhibiting/lowering free radical production, scavenging free radicals (or enhancing their degradation), and upregulating endogenous antioxidants (Shirley, Ord, and Work 2014). Edaravone (free radical scavenger) is commonly used in Japan to treat brain infarct and although infarct size appears to be significantly smaller in stroke patients with small-vessel occlusion, the improvement of neurological outcomes is still unclear (Nakase, Yoshioka, and Suzuki 2011). ROS are crucial targets that significantly contribute to vascular damage and lesion progression. However, the lack of randomization and blinding leads to an overestimation of drug efficacy. Moreover, for better clinical trial design and to enhance the chances of success, it is advisable to test different animal models since no one model alone can accurately portray the heterogenic nature of stroke (Shirley, Ord, and Work 2014).

The cascade of mediators such as ions, neurotransmitters, free radicals and nitric oxide encourages production of the inflammatory molecules that signal neighboring cells like microglia and astrocytes and peripheral cells such as leukocytes (Dirnagl, Iadecola, and Moskowitz 1999). Doll and colleagues have reviewed the role and potential use of cytokines in stroke. Tumor necrosis factor-alpha (TNF-α) expression has been documented in post-mortem brain tissue: around the second to the third day, there is an increased detection of TNF-positive microglia/macrophages, neurons and

infiltrating immune cells. Stroke severity seems to influence the pattern of TNF-α expression. Similarly, interleukin (IL)-6 detection is dependent on stroke severity and type. Studies show IL-6 increases during the week after stroke onset. Anti-inflammatory cytokine IL-10 levels tend to be lower shortly after a stroke, although with high variability. Data on IL-1β are still unclear (Doll, Barr, and Simpkins 2014). Chemokines, or chemotactic cytokines, guide leucocyte infiltration and are actively involved in angiogenesis and neuronal survival, which are key processes to safeguard after a stroke. In post-mortem brains of patients with ischemic stroke secondary to middle cerebral artery occlusion, chemokine (C-C motif) ligand (CCL)1 and CCL2 expression was found to be higher in neuronal cells than in blood vessels, while CCL5 and CCL22 were found to be lower in infarct areas. However, only CCL22 levels were significantly decreased in the blood during the acute phase (García-Berrocoso et al. 2014). Some blocking molecules such as a CCL2 peptide antagonist are being evaluated for their anti-inflammatory and anti-angiogenic potential (Speyer and Ward 2011). The immunoneutralization of CCL2 would reduce the recruitment and infiltration of macrophages and T cells.

Inflammatory response can significantly impact vascular activity and function (Machado-Pereira et al. 2017). In that sense, there are several endothelial cell-derived mediators and vascular targets that are either produced, released or altered upon injury. Endothelial dysfunction can also further affect the inflammatory setting and hinder repair.

Matrix metalloproteinases (MMP) degrade extracellular matrix components in a process that restructures the surrounding tissue, and that modulates endothelial migration and leukocyte invasion (Wang et al. 2006, Goldman and Chen 2011). The MMP family includes members of a constitutive or inducible nature. In the brain, constitutive MMP-2 and MMP-14 act locally, maintaining basement membrane integrity, while inducible MMP-3 and MMP-9 are mainly expressed by immune cells and become active as part of the inflammatory cascade; other inducible forms include MMP-8 and MMP-13 (Yang and Rosenberg 2015). MMP actions are regulated by endogenous tissue inhibitors of metalloproteinases (TIMP 1-4) (Griffioen and Molema 2000). TIMP also exert other functions, namely on cell proliferation, apoptosis, tumor angiogenesis, metastasis or inflammation (Murphy 2011). MMP dysregulation has been associated with blood-brain barrier (BBB) disruption and leukocyte infiltration, edema and hemorrhage (Lee et al. 2004, Lenglet, Montecucco, and Mach 2015, Rosenberg and Yang 2007, Si-Tayeb et al. 2006, Wasserman and Schlichter 2007, Yang et al. 2010). Conversely, the use of MMP inhibitors, such as SB-3CT (selective MMP-9 inhibitor), has shown

protective effects (Yang and Rosenberg 2015). However, the timing of this approach is important to allow angiogenesis to occur in later stages of post-ischemic injury. For instance, MMP-9 was found to be upregulated one to two weeks after focal cerebral ischemia. However, treatment with MMP inhibitors suppressed neurovascular remodeling, increased injury and impaired functional recovery (Zhao et al. 2006). Hence, an approach targeting MMP activity should take into consideration early (BBB injury) and late (angiogenesis) events.

MMP-2 and MMP-9 levels were measured in the plasma of patients with cardioembolic stroke. MMP-2 levels were found normal and MMP-9 increased irrespective of hemorrhagic transformation. Nevertheless, the highest baseline MMP-9 levels were related to patients that presented late hemorrhagic transformation, suggesting that this parameter could be used to predict late hemorrhagic transformation and early parenchymal hematoma (Montaner et al. 2001). Clinically, MMP-2, MMP-3 and MMP-9 have been found overexpressed in human spontaneous ICH, although only MMP-9 was correlated with ICH and perihematomal edema growth. Both MMP-3 and MMP-9 were associated with neurological deterioration (Florczak-Rzepka et al. 2012). In an animal model of ICH, induced by the striatal injection of a saline solution containing 0.05U of collagenase, there were significant alterations in mRNA expression of several MMP and TIMP, particularly MMP-12. This marker was then specifically detected in nonphagocytic monocytes cells surrounding the parenchymal hematoma (Power et al. 2003). Infiltration of inflammatory cells is commonly associated with brain damage and activation of endothelial and glial cells. In this context, the endothelium enhances expression of adhesion molecules (e.g. vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecules (ICAM)-1, platelet/endothelial cell adhesion molecule (PECAM)-1, vascular endothelial (VE)-cadherin, P- and E-selectins) to facilitate cell migration (Shuvaev, Brenner, and Muzykantov 2015, Vestweber 2015). The downregulation or immunoneutralization of adhesion molecules reduces tissue damage and function. However, two clinical trials evaluating the effect of LeukArrest (Hu23F2G) and Enlimomab (R6.5) in acute stroke showed no benefit (Becker 2002). Neutrophils and/or lymphocytes tend to gather in the brain before or during injury; and accordingly, neutrophil deletion reduces infarct size and neurological deficits (Yilmaz and Granger 2008).

The recovery process occurring after brain injury commonly involves the release of growth factors that stimulate cell proliferation and migration towards vascular remodeling and tissue regeneration (Su 2015). The most well-characterized molecules include insulin-like growth factors (IGF),

basic and acidic fibroblast growth factor (FGF), transforming growth factor-beta (TGF)-β, platelet-derived growth factor (PDGF) and vascular endothelial growth factors (VEGF). SAH patients have augmented PDGF levels—these positively correlate with cerebral vasospasm and delayed cerebral ischemia (Gaetani et al. 1997, Lad et al. 2012, Yanamoto et al. 2012). In that sense, several compounds that interfere with PDGF-β-mediated signaling have been tested, such as statins, serine protease inhibitors (e.g. nafamostat mesylate, argatroban) and inhibitors of proliferation (e.g. imatinib, trapidil, fasudil, cilostazol). These molecules lower PDGF-β levels and consequently prevent or treat SAH-related vasospasm (Ghali et al. 2018). VEGF family members participate and regulate a series of processes, namely vasculogenesis, angiogenesis and vascular permeability, arteriogenesis and neuroprotection. Experimental studies have shown that the administration of VEGF-A alone, of VEGF-A in combination with stem cells, or the induction of VEGF-A release by transplanted cells enhances repair (Greenberg and Jin 2013). Serum VEGF-A levels were found to be significantly higher in patients with more severe ICH supporting its potential value as a marker for ICH severity (Zheng et al. 2017). Regarding ischemic stroke, plasma VEGF-A levels were elevated in patients with atherothrombotic infarction, lacunar infarction and cardioembolic infarction (Matsuo et al. 2013). After an injury, VEGF-A promotes the recruitment of bone marrow-derived endothelial progenitor cells to the injury site to initiate neovascularization (Zampetaki, Kirton, and Xu 2008). However, VEGF can also promote vascular permeability and associated brain edema. Additionally, VEGF-A enhances a peripheral immune cell response, which can aggravate an injury if leukocyte infiltration becomes excessive (Angelo and Kurzrock 2007).

Angiopoietins (ANGPT1-4) are other members of the growth factor family that regulate angiogenesis (Griffioen and Molema 2000, Fagiani and Christofori 2013). These proteins bind to specific tyrosine-protein kinase receptors expressed by endothelial cells and act in a complementary manner. Specifically, angiopoietin-1 ensures vessel integrity and stability while angiopoietin-2 acts as an angiopoietin-1 antagonist (Fiedler et al. 2004; Wakui et al. 2006). Rats that had their middle cerebral artery blocked by autologous thrombi were treated with cartilage oligomeric protein (COMP)-angiopoietin-1 protein through a catheter in the inguinal vein. These ischemic rats showed significant inhibition of hemorrhagic transformation and cerebral edema (Kawamura et al. 2014). A more recent study with more than three hundred stroke patients revealed that angiopoietin-1 levels were lower in the plasma of patients with a poorer

outcome (Golledge et al. 2014). A study with mice subjected to permanent middle cerebral artery occlusion after intracerebroventricular injection of angiopoietin-2 and/or VEGF concluded that while VEGF aggravated damage (increased permeability and infarct size), angiopoietin-2 diminished ischemic volume (even in the presence of VGEF) (Marteau et al. 2013). Additionally, brain endothelial cells from angiopoietin-2-gain-of-function mice showed decreased expression of tight/adherens junction molecules and upregulation of caveolin-1. These mice showed enlarged infarct size and higher vessel permeability after occlusion due to less pericyte coverage and compromised intra-endothelial junctions (Gurnik et al. 2016).

Hemostasis, or the maintenance of blood fluidity, is a complex process regulated by coagulating and thrombolytic mechanisms. If this tight balance is disrupted by surgery, trauma, infection or hypoxia, the patient may suffer hemorrhage or thrombosis (Palta, Saroa, and Palta 2014). This occurs whenever the procoagulant activity of factors like collagen, tissue factor, prothrombin, platelets, clotting factor, Von Willebrand factor, fibrinogen or exposed endothelium is increased, or when the activity of inhibitors such as heparin, thrombomodulin, tissue plasminogen activator, antithrombin, protein C and S or plasminogen is increased (Previtali et al. 2011). Prothrombin, or clotting factor II, generates thrombin, which is a protease that converts fibrinogen (soluble) into fibrin (insoluble). Thrombin is involved in immune responses by triggering oxidative stress in the brain endothelium and by potentiating the expression of key interleukins (IL-1β, IL-6 and TNF-α) by surrounding glia (Choi, Lee, Chung, et al. 2005, Choi, Lee, Kim, et al. 2005). Since the 1950s, thrombin generation tests have been used to assess the state of pro- and anticoagulant pathways and infer about plasma coagulability related to hemorrhagic and bleeding disorders (Castoldi and Rosing 2011). In a recent article, thrombolysis in acute ischemic stroke patients attenuated the thrombin generation profile showing that rtPA can change blood coagulation (Goldman et al. 2017). Conversely, thrombomodulin, a membrane glycoprotein, is an anti-thrombogenic molecule that acts as a cofactor for thrombin (Boehme et al. 1996). The actions of thrombomodulin and tissue plasminogen activator are counteracted by protease inhibitor plasminogen activator inhibitor-1 (Tran et al. 1999).

Prostaglandins H2 are lipid mediators derived from arachidonic acid by the initial action of three different phospholipases A2 and then by cyclooxygenases (COX)-1 and -2 (Kishimoto et al. 2010). Subsequently, prostaglandins PGE2, PGI2, PGD2, PGF2α and TxA2 are obtained via specific synthases. These mediators inhibit endothelial cell activation

(Birukova et al. 2007). Prostaglandin I2 inhibits platelet activation and causes vasodilation (Pober and Sessa 2007). Hypoxia stimulates both the expression of COX-2 and prostaglandin PGE2 by neuronal, glial and endothelial cells. Prostaglandins activate different receptors (DP1–2, EP1–4, FP, IP, and TP), which respond differently to cerebral ischemia by causing either neurotoxic or neuroprotective effects (Andreasson 2010). The intracerebroventricular injection of BW245C (a DP1-selective agonist) has markedly reduced middle cerebral artery occlusion-induced brain infarction size (Ahmad et al. 2010). Prostaglandin receptors have been found to trigger different responses in ICH and ischemic stroke (Mohan et al. 2012).

The correlation between neuroinflammation and vascular dysfunction has been evaluated in ongoing or recent clinical trials. In October 2010, a phase I clinical trial (NCT01066572) led by Dr Christopher Price (Northumbria Healthcare NHS Foundation Trust), Dr Anand Dixit (Newcastle-upon-Tyne Hospitals) and Dr Ann Fox (North East Ambulance Service NHS Trust) initiated a study on the use of lisinopril, an angiotensin-converting-enzyme inhibitor, to lower blood pressure in stroke patients by research-trained paramedics. Only four of the 14 participants completed the period of study medication, which led to inconclusive results (although a reduction in blood pressure was observed) (Shaw et al. 2014).

In November 2011, a phase II clinical trial (NCT01439555) led by Dr Elizabeth DeGrush (UMass MedicalSchool), and sponsored by University of Massachusetts, started the combined administration of simvastatin, L-arginine and tetrahydrobiopterin (nitric oxide synthase cofactor) over 4 months to increase blood flow to the brain of Alzheimer's disease patients. A total of 10 participants enrolled but no results have been made available, to the best of our knowledge.

In February 2013, phase I and II clinical trials (NCT01805895) led by Dr Jeffrey Switzer (Augusta University), and sponsored by Augusta University in collaboration with the American Heart Association, evaluated the role of minocycline in acute intracerebral hemorrhage patients. The first dose consisted of 400 mg intravenously within 12 hours of symptom onset—the next doses were 400 mg orally, given daily on days two to five (total of five doses). Control patients received standard of care treatment. A total of 16 participants enrolled, with 8 randomized to minocycline. Results were recently published stating that oral administration caused delayed absorption and this form of administration is not recommended for rapid effect. The group concluded that a 400 mg dose of minocycline was safe, achieved neuroprotective levels in the

serum of intracerebral hemorrhage patients and, therefore, its intravenous administration could be used for pre-hospital treatment trial (Fouda et al. 2017).

In May 2015, a phase III clinical trial (NCT02430350) led by Dr Yongjun Wang (Beijing Tiantan Hospital), sponsored by Jiangsu Simcere Pharmaceutical Co., evaluated the safety and efficacy of intravenous injection of the compound Edaravone, a free radical scavenger, on patients with acute ischemic stroke. Patients were administered the compound Edaravone at 37.5 mg/dose (Edaravone 30 mg, (+)-Borneol 7.5 mg), one dose every 12 hours for two weeks, or Edaravone 30 mg/dose, one dose every 12 hours for two weeks. A total of 1,200 participants enrolled and although the study completion date was December 2016, no results have been posted yet, to the best of our knowledge.

Overall, data from clinical trials are still insufficiently available or inconclusive to promote the implementation of new therapies.

Conclusion

Prognosis for most stroke patients is still quite poor: approximately 40% of those with ICH die after one month (van Asch et al. 2010), around 35% of SAH cases are fatal (Rinkel and Algra 2011) and, after a year, only about 65% of ischemic stroke patients survive (Zhang, He, and Chen 2014). Additionally, stroke survivors experience considerable cognitive and motor deficits, posing a considerable burden on healthcare systems and care providers, while still having an added risk for recurrent stroke. Prevention is therefore of utmost importance and a change in lifestyle factors was estimated to halve the number of all strokes (Tikk et al. 2014). In the meantime, obtaining relevant in vitro and in vivo data is dependent on funding, which is still insufficient. Additionally, clinical trials show difficulty in translating basic science data into efficient bench side solutions. Recently, within all noncommunicable diseases, stroke was announced as one of the main priorities by the United Nations and the WHO (Feigin, Roth, et al. 2016, Feigin, Norrving, and Mensah 2017, Feigin, Norrving, et al. 2016). Accordingly, there will be an important effort in improving guidelines and developing new strategies to identify new therapeutic targets/approaches and more effectively treat stroke.

References

Ahmad, A. S., M. Ahmad, T. Maruyama, S. Narumiya, and S. Dore. 2010. "Prostaglandin D2 DP1 receptor is beneficial in ischemic stroke and in acute exicitotoxicity in young and old mice." *Age (Dordr)* no. 32 (3):271-82. doi: 10.1007/s11357-010-9135-y.

Andreasson, K. 2010. "Prostaglandin signalling in cerebral ischaemia." *Br J Pharmacol* no. 160 (4):844-6. doi: 10.1111/j.1476-5381.2010.00715.x.

Angelo, L. S., and R. Kurzrock. 2007. "Vascular endothelial growth factor and its relationship to inflammatory mediators." *Clinical Cancer Research* no. 13 (10):2825-30. doi: 10.1158/1078-0432.CCR-06-2416.

Becker, K. J. 2002. "Anti-leukocyte antibodies: LeukArrest (Hu23F2G) and Enlimomab (R6.5) in acute stroke." *Curr Med Res Opin* no. 18 Suppl 2:s18-22.

Beslow, L. A., S. E. Smith, A. Vossough, D. J. Licht, S. E. Kasner, C. G. Favilla, A. R. Halperin, D. M. Gordon, C. I. Jones, A. J. Cucchiara, and R. N. Ichord. 2011. "Hemorrhagic transformation of childhood arterial ischemic stroke." *Stroke* no. 42 (4):941-6. doi: 10.1161/STROKEAHA.110.604199.

Birukova, A. A., T. Zagranichnaya, P. Fu, E. Alekseeva, W. Chen, J. R. Jacobson, and K. G. Birukov. 2007. "Prostaglandins PGE(2) and PGI(2) promote endothelial barrier enhancement via PKA- and Epac1/Rap1-dependent Rac activation." *Experimental Cell Research* no. 313 (11):2504-20. doi: 10.1016/j.yexcr.2007.03.036.

Boehme, M. W., Y. Deng, U. Raeth, A. Bierhaus, R. Ziegler, W. Stremmel, and P. P. Nawroth. 1996. "Release of thrombomodulin from endothelial cells by concerted action of TNF-alpha and neutrophils: in vivo and in vitro studies." *Immunology* no. 87 (1):134-40.

Caceres, J. A., and J. N. Goldstein. 2012. "Intracranial hemorrhage." *Emerg Med Clin North Am* no. 30 (3):771-94. doi: 10.1016/j.emc.2012.06.003.

Castoldi, E., and J. Rosing. 2011. "Thrombin generation tests." *Thromb Res* no. 127 Suppl 3:S21-5. doi: 10.1016/S0049-3848(11)70007-X.

Choi, S. H., D. Y. Lee, E. S. Chung, Y. B. Hong, S. U. Kim, and B. K. Jin. 2005. "Inhibition of thrombin-induced microglial activation and NADPH oxidase by minocycline protects dopaminergic neurons in the substantia nigra in vivo." *Journal of Neurochemistry* no. 95 (6):1755-65. doi: 10.1111/j.1471-4159.2005.03503.x.

Choi, S. H., D. Y. Lee, S. U. Kim, and B. K. Jin. 2005. "Thrombin-induced oxidative stress contributes to the death of hippocampal

neurons in vivo: role of microglial NADPH oxidase." *Journal of Neuroscience* no. 25 (16):4082-90. doi: 10.1523/JNEUROSCI.4306-04.2005.

de Rooij, N. K., F. H. Linn, J. A. van der Plas, A. Algra, and G. J. Rinkel. 2007. "Incidence of subarachnoid haemorrhage: a systematic review with emphasis on region, age, gender and time trends." *J Neurol Neurosurg Psychiatry* no. 78 (12):1365-72. doi: 10.1136/jnnp.2007.117655.

Dirnagl, U. 2012. "Pathobiology of injury after stroke: the neurovascular unit and beyond." *Annals of the New York Academy of Sciences* no. 1268:21-5. doi: 10.1111/j.1749-6632.2012.06691.x.

Dirnagl, U., C. Iadecola, and M. A. Moskowitz. 1999. "Pathobiology of ischaemic stroke: an integrated view." *Trends Neurosci* no. 22 (9):391-7.

Doll, D. N., T. L. Barr, and J. W. Simpkins. 2014. "Cytokines: their role in stroke and potential use as biomarkers and therapeutic targets." *Aging Dis* no. 5 (5):294-306. doi: 10.14336/AD.2014.0500294.

Fagiani, E., and G. Christofori. 2013. "Angiopoietins in angiogenesis." *Cancer Letters* no. 328 (1):18-26. doi: 10.1016/j.canlet.2012.08.018.

Feigin, V. L., B. Norrving, M. G. George, J. L. Foltz, G. A. Roth, and G. A. Mensah. 2016. "Prevention of stroke: a strategic global imperative." *Nat Rev Neurol* no. 12 (9):501-12. doi: 10.1038/nrneurol.2016.107.

Feigin, V. L., B. Norrving, and G. A. Mensah. 2017. "Global Burden of Stroke." *Circ Res* no. 120 (3):439-448. doi: 10.1161/CIRCRESAHA.116.308413.

Feigin, V. L., G. A. Roth, M. Naghavi, P. Parmar, R. Krishnamurthi, S. Chugh, G. A. Mensah, B. Norrving, I. Shiue, M. Ng, K. Estep, K. Cercy, C. J. L. Murray, M. H. Forouzanfar, Injuries Global Burden of Diseases, Study Risk Factors, and Group Stroke Experts Writing. 2016. "Global burden of stroke and risk factors in 188 countries, during 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013." *Lancet Neurol* no. 15 (9):913-924. doi: 10.1016/S1474-4422(16)30073-4.

Florczak-Rzepka, M., C. Grond-Ginsbach, J. Montaner, and T. Steiner. 2012. "Matrix metalloproteinases in human spontaneous intracerebral hemorrhage: an update." *Cerebrovasc Dis* no. 34 (4):249-62. doi: 10.1159/000341686.

Fluri, F., M. K. Schuhmann, and C. Kleinschnitz. 2015. "Animal models of ischemic stroke and their application in clinical research." *Drug Des Devel Ther* no. 9:3445-54. doi: 10.2147/DDDT.S56071.

Fouda, A. Y., A. S. Newsome, S. Spellicy, J. L. Waller, W. Zhi, D. C. Hess, A. Ergul, D. J. Edwards, S. C. Fagan, and J. A. Switzer. 2017. "Minocycline in Acute Cerebral Hemorrhage: An Early Phase Randomized Trial." *Stroke* no. 48 (10):2885-2887. doi: 10.1161/STROKEAHA.117.018658.

Gaetani, P., F. Tancioni, G. Grignani, F. Tartara, E. M. Merlo, A. Brocchieri, and R. Rodriguez y Baena. 1997. "Platelet derived growth factor and subarachnoid haemorrhage: a study on cisternal cerebrospinal fluid." *Acta Neurochir (Wien)* no. 139 (4):319-24.

García-Berrocoso, T, D Giralt, V Llombart, A Bustamante, A Penalba, A Flores, M Ribó, CA Molina, A Rosell, and A Montaner. 2014. "Chemokines after human ischemic stroke: From neurovascular unit to blood using protein arrays." *Translational Proteomics* no. 3:1-9.

Ghali, M. G. Z., V. M. Srinivasan, J. Johnson, P. Kan, and G. Britz. 2018. "Therapeutically Targeting Platelet-Derived Growth Factor-Mediated Signaling Underlying the Pathogenesis of Subarachnoid Hemorrhage-Related Vasospasm." *J Stroke Cerebrovasc Dis* no. 27 (9):2289-2295. doi: 10.1016/j.jstrokecerebrovasdis.2018.02.017.

Goldman, S. A., and Z. Chen. 2011. "Perivascular instruction of cell genesis and fate in the adult brain." *Nat Neurosci* no. 14 (11):1382-9. doi: 10.1038/nn.2963.

Goldman, S., S. M. Prior, J. P. Bembenek, M. Niewada, E. Broniatowska, A. Czlonkowska, S. Butenas, and A. Undas. 2017. "Activation of blood coagulation and thrombin generation in acute ischemic stroke treated with rtPA." *J Thromb Thrombolysis* no. 44 (3):362-370. doi: 10.1007/s11239-017-1544-7.

Golledge, J., P. Clancy, J. Maguire, L. Lincz, S. Koblar, M. McEvoy, J. Attia, C. Levi, J. Sturm, O. P. Almeida, B. B. Yeap, L. Flicker, P. E. Norman, and G. J. Hankey. 2014. "Plasma angiopoietin-1 is lower after ischemic stroke and associated with major disability but not stroke incidence." *Stroke* no. 45 (4):1064-8. doi: 10.1161/STROKEAHA.113.004339.

Greenberg, D. A., and K. Jin. 2013. "Vascular endothelial growth factors (VEGFs) and stroke." *Cell Mol Life Sci* no. 70 (10):1753-61. doi: 10.1007/s00018-013-1282-8.

Griffioen, A. W., and G. Molema. 2000. "Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation." *Pharmacological Reviews* no. 52 (2):237-68.

Grysiewicz, R. A., K. Thomas, and D. K. Pandey. 2008. "Epidemiology of ischemic and hemorrhagic stroke: incidence, prevalence, mortality, and risk factors." *Neurol Clin* no. 26 (4):871-95, vii. doi: 10.1016/j.ncl.2008.07.003.

Gu, Y., G. Xi, W. Liu, R. F. Keep, and Y. Hua. 2010. "Estrogen reduces iron-mediated brain edema and neuronal death." *Acta Neurochir Suppl* no. 106:159-62. doi: 10.1007/978-3-211-98811-4_29.

Gurnik, S., K. Devraj, J. Macas, M. Yamaji, J. Starke, A. Scholz, K. Sommer, M. Di Tacchio, R. Vutukuri, H. Beck, M. Mittelbronn, C. Foerch, W. Pfeilschifter, S. Liebner, K. G. Peters, K. H. Plate, and Y. Reiss. 2016. "Angiopoietin-2-induced blood-brain barrier compromise and increased stroke size are rescued by VE-PTP-dependent restoration of Tie2 signaling." *Acta Neuropathol* no. 131 (5):753-73. doi: 10.1007/s00401-016-1551-3.

Hacke, W., M. Kaste, C. Fieschi, R. von Kummer, A. Davalos, D. Meier, V. Larrue, E. Bluhmki, S. Davis, G. Donnan, D. Schneider, E. Diez-Tejedor, and P. Trouillas. 1998. "Randomised double-blind placebo-controlled trial of thrombolytic therapy with intravenous alteplase in acute ischaemic stroke (ECASS II). Second European-Australasian Acute Stroke Study Investigators." *Lancet* no. 352 (9136):1245-51.

Hemphill, J. C., 3rd, S. M. Greenberg, C. S. Anderson, K. Becker, B. R. Bendok, M. Cushman, G. L. Fung, J. N. Goldstein, R. L. Macdonald, P. H. Mitchell, P. A. Scott, M. H. Selim, D. Woo, Council American Heart Association Stroke, Cardiovascular Council on, Nursing Stroke, and Cardiology Council on Clinical. 2015. "Guidelines for the Management of Spontaneous Intracerebral Hemorrhage: A Guideline for Healthcare Professionals From the American Heart Association/ American Stroke Association." *Stroke* no. 46 (7):2032-60. doi: 10.1161/STR.0000000000000069.

Holloway, P. M., and F. N. Gavins. 2016. "Modeling Ischemic Stroke In Vitro: Status Quo and Future Perspectives." *Stroke* no. 47 (2):561-9. doi: 10.1161/STROKEAHA.115.011932.

Hu, H. J., and M. Song. 2017. "Disrupted Ionic Homeostasis in Ischemic Stroke and New Therapeutic Targets." *J Stroke Cerebrovasc Dis* no. 26 (12):2706-2719. doi: 10.1016/j.jstrokecerebrovasdis.2017.09.011.

Ikram, M. A., R. G. Wieberdink, and P. J. Koudstaal. 2012. "International epidemiology of intracerebral hemorrhage." *Curr Atheroscler Rep* no. 14 (4):300-6. doi: 10.1007/s11883-012-0252-1.

Jacquin, G. J., and B. A. van Adel. 2015. "Treatment of acute ischemic stroke: from fibrinolysis to neurointervention." *J Thromb Haemost* no. 13 Suppl 1:S290-6. doi: 10.1111/jth.12971.

Jickling, G. C., D. Liu, B. Stamova, B. P. Ander, X. Zhan, A. Lu, and F. R. Sharp. 2014. "Hemorrhagic transformation after ischemic stroke in animals and humans." *J Cereb Blood Flow Metab* no. 34 (2):185-99. doi: 10.1038/jcbfm.2013.203.

Kalaria, R. N., R. Akinyemi, and M. Ihara. 2016. "Stroke injury, cognitive impairment and vascular dementia." *Biochim Biophys Acta* no. 1862 (5):915-25. doi: 10.1016/j.bbadis.2016.01.015.

Kawamura, K., T. Takahashi, M. Kanazawa, H. Igarashi, T. Nakada, M. Nishizawa, and T. Shimohata. 2014. "Effects of angiopoietin-1 on hemorrhagic transformation and cerebral edema after tissue plasminogen activator treatment for ischemic stroke in rats." *PLoS One* no. 9 (6):e98639. doi: 10.1371/journal.pone.0098639.

Kishimoto, K., R. C. Li, J. Zhang, J. A. Klaus, K. K. Kibler, S. Dore, R. C. Koehler, and A. Sapirstein. 2010. "Cytosolic phospholipase A2 alpha amplifies early cyclooxygenase-2 expression, oxidative stress and MAP kinase phosphorylation after cerebral ischemia in mice." *J Neuroinflammation* no. 7:42. doi: 10.1186/1742-2094-7-42.

Lad, S. P., H. Hegen, G. Gupta, F. Deisenhammer, and G. K. Steinberg. 2012. "Proteomic biomarker discovery in cerebrospinal fluid for cerebral vasospasm following subarachnoid hemorrhage." *J Stroke Cerebrovasc Dis* no. 21 (1):30-41. doi: 10.1016/j.jstrokecerebrovasdis.2010.04.004.

Lee, H. S., K. Namkoong, D. H. Kim, K. J. Kim, Y. H. Cheong, S. S. Kim, W. B. Lee, and K. Y. Kim. 2004. "Hydrogen peroxide-induced alterations of tight junction proteins in bovine brain microvascular endothelial cells." *Microvasc Res* no. 68 (3):231-8. doi: 10.1016/j.mvr.2004.07.005.

Lenglet, S., F. Montecucco, and F. Mach. 2015. "Role of matrix metalloproteinases in animal models of ischemic stroke." *Curr Vasc Pharmacol* no. 13 (2):161-6.

Machado-Pereira, M., T. Santos, L. Bernardino, and R. Ferreira. 2017. "Vascular inter-regulation of inflammation: molecular and cellular targets for CNS therapy." *J Neurochem* no. 140 (5):692-702. doi: 10.1111/jnc.13914.

Marteau, L., S. Valable, D. Divoux, S. A. Roussel, O. Touzani, E. T. MacKenzie, M. Bernaudin, and E. Petit. 2013. "Angiopoietin-2 is vasoprotective in the acute phase of cerebral ischemia." *J Cereb Blood Flow Metab* no. 33 (3):389-95. doi: 10.1038/jcbfm.2012.178.

Matsuo, R., T. Ago, M. Kamouchi, J. Kuroda, T. Kuwashiro, J. Hata, H. Sugimori, K. Fukuda, S. Gotoh, N. Makihara, M. Fukuhara, H. Awano, T. Isomura, K. Suzuki, M. Yasaka, Y. Okada, Y. Kiyohara, and T.

Kitazono. 2013. "Clinical significance of plasma VEGF value in ischemic stroke - research for biomarkers in ischemic stroke (REBIOS) study." *BMC Neurol* no. 13:32. doi: 10.1186/1471-2377-13-32.

Mohan, S., A. S. Ahmad, A. V. Glushakov, C. Chambers, and S. Dore. 2012. "Putative role of prostaglandin receptor in intracerebral hemorrhage." *Front Neurol* no. 3:145. doi: 10.3389/fneur.2012.00145.

Montaner, J., J. Alvarez-Sabin, C. A. Molina, A. Angles, S. Abilleira, J. Arenillas, and J. Monasterio. 2001. "Matrix metalloproteinase expression is related to hemorrhagic transformation after cardioembolic stroke." *Stroke* no. 32 (12):2762-7.

Mould, W. A., J. R. Carhuapoma, J. Muschelli, K. Lane, T. C. Morgan, N. A. McBee, A. J. Bistran-Hall, N. L. Ullman, P. Vespa, N. A. Martin, I. Awad, M. Zuccarello, D. F. Hanley, and Mistie Investigators. 2013. "Minimally invasive surgery plus recombinant tissue-type plasminogen activator for intracerebral hemorrhage evacuation decreases perihaematomal edema." *Stroke* no. 44 (3):627-34. doi: 10.1161/STROKEAHA.111.000411.

Murphy, G. 2011. "Tissue inhibitors of metalloproteinases." *Genome Biol* no. 12 (11):233. doi: 10.1186/gb-2011-12-11-233.

Nakamura, T., G. Xi, R. F. Keep, M. Wang, S. Nagao, J. T. Hoff, and Y. Hua. 2006. "Effects of endogenous and exogenous estrogen on intracerebral hemorrhage-induced brain damage in rats." *Acta Neurochir Suppl* no. 96:218-21.

Nakase, T., S. Yoshioka, and A. Suzuki. 2011. "Free radical scavenger, edaravone, reduces the lesion size of lacunar infarction in human brain ischemic stroke." *BMC Neurol* no. 11:39. doi: 10.1186/1471-2377-11-39.

O'Donnell, M. J., D. Xavier, L. Liu, H. Zhang, S. L. Chin, P. Rao-Melacini, S. Rangarajan, S. Islam, P. Pais, M. J. McQueen, C. Mondo, A. Damasceno, P. Lopez-Jaramillo, G. J. Hankey, A. L. Dans, K. Yusoff, T. Truelsen, H. C. Diener, R. L. Sacco, D. Ryglewicz, A. Czlonkowska, C. Weimar, X. Wang, S. Yusuf, and Interstroke investigators. 2010. "Risk factors for ischaemic and intracerebral haemorrhagic stroke in 22 countries (the INTERSTROKE study): a case-control study." *Lancet* no. 376 (9735):112-23. doi: 10.1016/S0140-6736(10)60834-3.

Palta, S., R. Saroa, and A. Palta. 2014. "Overview of the coagulation system." *Indian J Anaesth* no. 58 (5):515-23. doi: 10.4103/0019-5049.144643.

Pober, J. S., and W. C. Sessa. 2007. "Evolving functions of endothelial cells in inflammation." *Nature Reviews Immunology* no. 7 (10):803-15. doi: 10.1038/nri2171.

Power, C., S. Henry, M. R. Del Bigio, P. H. Larsen, D. Corbett, Y. Imai, V. W. Yong, and J. Peeling. 2003. "Intracerebral hemorrhage induces macrophage activation and matrix metalloproteinases." *Ann Neurol* no. 53 (6):731-42. doi: 10.1002/ana.10553.

Powers, W. J., A. A. Rabinstein, T. Ackerson, O. M. Adeoye, N. C. Bambakidis, K. Becker, J. Biller, M. Brown, B. M. Demaerschalk, B. Hoh, E. C. Jauch, C. S. Kidwell, T. M. Leslie-Mazwi, B. Ovbiagele, P. A. Scott, K. N. Sheth, A. M. Southerland, D. V. Summers, D. L. Tirschwell, and Council American Heart Association Stroke. 2018. "2018 Guidelines for the Early Management of Patients With Acute Ischemic Stroke: A Guideline for Healthcare Professionals From the American Heart Association/American Stroke Association." *Stroke* no. 49 (3):e46-e110. doi: 10.1161/STR.0000000000000158.

Previtali, E., P. Bucciarelli, S. M. Passamonti, and I. Martinelli. 2011. "Risk factors for venous and arterial thrombosis." *Blood Transfus* no. 9 (2):120-38. doi: 10.2450/2010.0066-10.

Raya, A. K., and M. N. Diringer. 2014. "Treatment of subarachnoid hemorrhage." *Crit Care Clin* no. 30 (4):719-33. doi: 10.1016/j.ccc.2014.06.004.

Rego, A C, C. B. Duarte, and C R Oliveira. 2017. *Neurociências*. Edited by A C Rego, C. B. Duarte and C R Oliveira. 1st ed: Lidel - edições técnicas, lda.

Rinkel, G. J., and A. Algra. 2011. "Long-term outcomes of patients with aneurysmal subarachnoid haemorrhage." *Lancet Neurol* no. 10 (4):349-56. doi: 10.1016/S1474-4422(11)70017-5.

Rosenberg, G. A., and Y. Yang. 2007. "Vasogenic edema due to tight junction disruption by matrix metalloproteinases in cerebral ischemia." *Neurosurg Focus* no. 22 (5):E4.

Rothwell, P. M. 2007. "Lack of research funding for stroke." *Int J Stroke* no. 2 (2):73. doi: 10.1111/j.1747-4949.2007.00125.x.

Shaw, L., C. Price, S. McLure, D. Howel, E. McColl, P. Younger, and G. A. Ford. 2014. "Paramedic Initiated Lisinopril For Acute Stroke Treatment (PIL-FAST): results from the pilot randomised controlled trial." *Emerg Med J* no. 31 (12):994-9. doi: 10.1136/emermed-2013-202536.

Shirley, R., E. N. Ord, and L. M. Work. 2014. "Oxidative Stress and the Use of Antioxidants in Stroke." *Antioxidants (Basel)* no. 3 (3):472-501. doi: 10.3390/antiox3030472.

Shuvaev, V. V., J. S. Brenner, and V. R. Muzykantov. 2015. "Targeted endothelial nanomedicine for common acute pathological conditions." *Journal of Controlled Release* no. 219:576-95. doi: 10.1016/j.jconrel.2015.09.055.

Si-Tayeb, K., A. Monvoisin, C. Mazzocco, S. Lepreux, M. Decossas, G. Cubel, D. Taras, J. F. Blanc, D. R. Robinson, and J. Rosenbaum. 2006. "Matrix metalloproteinase 3 is present in the cell nucleus and is involved in apoptosis." *Am J Pathol* no. 169 (4):1390-401. doi: 10.2353/ajpath.2006.060005.

Speyer, C. L., and P. A. Ward. 2011. "Role of endothelial chemokines and their receptors during inflammation." *Journal of Investigative Surgery* no. 24 (1):18-27. doi: 10.3109/08941939.2010.521232.

Su, J. B. 2015. "Vascular endothelial dysfunction and pharmacological treatment." *World J Cardiol* no. 7 (11):719-41. doi: 10.4330/wjc.v7.i11.719.

Sun, J. H., L. Tan, and J. T. Yu. 2014. "Post-stroke cognitive impairment: epidemiology, mechanisms and management." *Ann Transl Med* no. 2 (8):80. doi: 10.3978/j.issn.2305-5839.2014.08.05.

Sussman, E. S., and E. S. Connolly, Jr. 2013. "Hemorrhagic transformation: a review of the rate of hemorrhage in the major clinical trials of acute ischemic stroke." *Front Neurol* no. 4:69. doi: 10.3389/fneur.2013.00069.

Terasaki, Y., Y. Liu, K. Hayakawa, L. D. Pham, E. H. Lo, X. Ji, and K. Arai. 2014. "Mechanisms of neurovascular dysfunction in acute ischemic brain." *Curr Med Chem* no. 21 (18):2035-42.

Terruso, V., M. D'Amelio, N. Di Benedetto, I. Lupo, V. Saia, G. Famoso, M. A. Mazzola, P. Aridon, C. Sarno, P. Ragonese, and G. Savettieri. 2009. "Frequency and determinants for hemorrhagic transformation of cerebral infarction." *Neuroepidemiology* no. 33 (3):261-5. doi: 10.1159/000229781.

Tikk, K., D. Sookthai, S. Monni, M. L. Gross, C. Lichy, M. Kloss, and R. Kaaks. 2014. "Primary preventive potential for stroke by avoidance of major lifestyle risk factors: the European Prospective Investigation into Cancer and Nutrition-Heidelberg cohort." *Stroke* no. 45 (7):2041-6. doi: 10.1161/STROKEAHA.114.005025.

Tran, N. D., J. Correale, S. S. Schreiber, and M. Fisher. 1999. "Transforming growth factor-beta mediates astrocyte-specific regulation of brain endothelial anticoagulant factors." *Stroke* no. 30 (8):1671-8.

van Asch, C. J., M. J. Luitse, G. J. Rinkel, I. van der Tweel, A. Algra, and C. J. Klijn. 2010. "Incidence, case fatality, and functional outcome of

intracerebral haemorrhage over time, according to age, sex, and ethnic origin: a systematic review and meta-analysis." *Lancet Neurol* no. 9 (2):167-76. doi: 10.1016/S1474-4422(09)70340-0.

Vestweber, D. 2015. "How leukocytes cross the vascular endothelium." *Nature Reviews Immunology* no. 15 (11):692-704. doi: 10.1038/nri3908.

Viscoli, C. M., L. M. Brass, W. N. Kernan, P. M. Sarrel, S. Suissa, and R. I. Horwitz. 2001. "A clinical trial of estrogen-replacement therapy after ischemic stroke." *N Engl J Med* no. 345 (17):1243-9. doi: 10.1056/NEJMoa010534.

von Kummer, R., and I. Dzialowski. 2017. "Imaging of cerebral ischemic edema and neuronal death." *Neuroradiology* no. 59 (6):545-553. doi: 10.1007/s00234-017-1847-6.

Wang, L., Z. G. Zhang, R. L. Zhang, S. R. Gregg, A. Hozeska-Solgot, Y. LeTourneau, Y. Wang, and M. Chopp. 2006. "Matrix metalloproteinase 2 (MMP2) and MMP9 secreted by erythropoietin-activated endothelial cells promote neural progenitor cell migration." *Journal of Neuroscience* no. 26 (22):5996-6003. doi: 10.1523/JNEUROSCI.5380-05.2006.

Wasserman, J. K., and L. C. Schlichter. 2007. "Minocycline protects the blood-brain barrier and reduces edema following intracerebral hemorrhage in the rat." *Exp Neurol* no. 207 (2):227-37. doi: 10.1016/j.expneurol.2007.06.025.

WHO, World Health Organization. 2018. *Global Health Estimates* 2012 [cited August 2018]. Available from http://www.who.int/healthinfo/global_burden_disease/en/.

Yanamoto, H., H. Kataoka, Y. Nakajo, and K. Iihara. 2012. "The role of the host defense system in the development of cerebral vasospasm: analogies between atherosclerosis and subarachnoid hemorrhage." *Eur Neurol* no. 68 (6):329-43. doi: 10.1159/000341336.

Yang, Y., E. Candelario-Jalil, J. F. Thompson, E. Cuadrado, E. Y. Estrada, A. Rosell, J. Montaner, and G. A. Rosenberg. 2010. "Increased intranuclear matrix metalloproteinase activity in neurons interferes with oxidative DNA repair in focal cerebral ischemia." *J Neurochem* no. 112 (1):134-49. doi: 10.1111/j.1471-4159.2009.06433.x.

Yang, Y., and G. A. Rosenberg. 2015. "Matrix metalloproteinases as therapeutic targets for stroke." *Brain Res* no. 1623:30-8. doi: 10.1016/j.brainres.2015.04.024.

Yilmaz, G., and D. N. Granger. 2008. "Cell adhesion molecules and ischemic stroke." *Neurol Res* no. 30 (8):783-93. doi: 10.1179/174313208X341085.

Zampetaki, A., J. P. Kirton, and Q. Xu. 2008. "Vascular repair by endothelial progenitor cells." *Cardiovascular Research* no. 78 (3):413-21. doi: 10.1093/cvr/cvn081.

Zhang, S., W. B. He, and N. H. Chen. 2014. "Causes of death among persons who survive an acute ischemic stroke." *Curr Neurol Neurosci Rep* no. 14 (8):467. doi: 10.1007/s11910-014-0467-3.

Zhao, B. Q., S. Wang, H. Y. Kim, H. Storrie, B. R. Rosen, D. J. Mooney, X. Wang, and E. H. Lo. 2006. "Role of matrix metalloproteinases in delayed cortical responses after stroke." *Nat Med* no. 12 (4):441-5. doi: 10.1038/nm1387.

Zheng, J., J. Sun, L. Yang, B. Zhao, and Z. Fan. 2017. "The potential role of vascular endothelial growth factor as a new biomarker in severe intracerebral hemorrhage." *J Clin Lab Anal* no. 31 (5). doi: 10.1002/jcla.22076.

CHAPTER SIX

MICROVASCULATURE DYSFUNCTION IN NEURODEGENERATIVE DISORDERS

ANA CLARA CRISTÓVÃO
AND FABIEN GOSSELET

Introduction

The brain is a very sensitive and complex organ which needs to have a highly controlled environment in order to function properly. Ionic and fluid movements between the blood and the central nervous system (CNS) are restricted to provide a brain interstitial fluid (ISF) that creates an optimal medium for neuronal function. Furthermore, nutrient and oxygen supply and waste products removal must be very efficient and adapted to brain activity. All these molecule exchanges between brain ISF and blood occur at brain microvasculature level, which tightly controls all these processes. In the adult brain, it is estimated that each neuron has a capillary so, as a consequence, cerebral microvasculature length represents almost 600 kilometers and a surface of about 20m² (Begley and Brightman 2003). Thus, microvasculature represents a blood-brain barrier (BBB) which is located at the endothelial cells lining these vessels. These cells possess tight junctions and demonstrate a lack of fenestrations as well as very low fluid phase activity, thus impeding molecules and cells from freely crossing between two adjacent cells and across endothelial cells (Cecchelli et al. 2007). Additionally, BBB endothelial cells express a large panel of efflux pumps and enzymes restricting the passage of molecules between blood and ISF. Therefore, the delivery of molecules, ions, and nutrients occurs with some active transport systems involving transporters and receptors (Zlokovic 2011).

The acquisition and maintenance of BBB properties are induced by different cell types surrounding the BBB endothelial cells. First, the brain pericytes for capillaries (or smooth muscle cells for arteries, arterioles, and

venules) and astrocytes which form some astrocytic end-foot processes interacting with blood vessels (Cecchelli et al. 2007, Abbott, Ronnback, and Hansson 2006). In addition, BBB endothelial cells receive signals from neurons, microglial cells, and oligodendrocytes to ensure the correct brain delivery of molecules every day. All these cell types and intercommunication systems form the neurovascular unit (NVU) (Neuwelt 2004).

There is growing evidence showing that microvascular dysfunction, usually linked with defective BBB functions and reduction in the cerebral blood flow (CBF), is closely linked to neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's (PD) diseases. On the other hand, it is suggested that the neurodegenerative process may also compromise BBB architecture and function. This chapter deals with the dysfunctions of the microvasculature in these neurodegenerative diseases.

Alzheimer's Disease

AD is mainly characterized by an accumulation of different forms of β amyloid peptide (Aβ peptides) within brain parenchyma and around brain microvessels but also by the intraneuronal accumulation of hyperphosphorylated Tau (Selkoe 2011). Aβ peptides are generated by the sequential cleavage of the Amyloid Precursor Protein (APP) and this process is finely regulated by the cholesterol content of the cellular membrane. Over the last decades, several transgenic mice models have been developed to investigate AD in vivo and to test different therapeutic approaches. These models consist of overexpressing the human forms of the APP and/or the enzymes responsible for the cleavage thereof. More recently, new models also include human mutations adding Tau component (Puzzo et al. 2015).

Brain microvascular dysfunction in AD

Morphological changes in microvessels are observed in the cerebral cortex of AD patients (Miyakawa et al. 1988). Some capillaries are terminated with tapered ends. The basement membrane is thickened and collagen content is increased (Claudio 1996). Brain pericytes and endothelial cells are atrophied and abnormal. Pinocytotic activity is increased and mitochondrial content is decreased (Claudio 1996). Further studies in AD mouse models have demonstrated that capillary density is reduced in this pathology (Bourasset et al. 2009). Altogether, these alterations have suggested that the CBF, as well as BBB integrity and

physiology, are altered in AD. Reduced or dysregulated CBF has also been reported in elderly individuals at high risk of developing AD, before the detection of cognitive decline, brain atrophy and Aβ peptide accumulation (Ruitenberg et al. 2005, Iadecola et al. 1999, Kisler et al. 2017).

BBB permeability in AD

The permeability of the BBB is a very important factor influencing the CBF, and therefore CNS functioning, but it is still debated whether it occurs in AD brains. The first studies investigating BBB breakdown in AD transgenic mice models reported a loss of BBB integrity before senile plaque formation (Ujiie et al. 2003). However, other works have not been able to observe any alterations in BBB permeability (Poduslo et al. 2001) or any changes in the transport of molecules across the BBB (Gustafsson et al. 2018). In humans, it is also difficult to determine whether BBB breakdown occurs in AD, mainly due to the diversity of the techniques used and the different stages of development shown in the studied patients. Importantly, recent neuroimaging studies using advanced dynamic contrast-enhanced MRI (DCE-MRI) in living human brains have elegantly shown BBB breakdown in the hippocampus of aged people that worsens in patients with mild cognitive impairment (MCI) and early AD (Montagne et al. 2015). To establish whether the accumulation of Aβ peptides might be responsible for this BBB opening, in vivo and in vitro studies have been performed to test the toxicity of these peptides at BBB level. They have demonstrated that Aβ peptides can increase BBB permeability by triggering brain pericyte death (Sagare et al. 2013, Bruinsma et al. 2010) and by altering tight junction protein expression and localization, in particular, Claudin-5 and Zonula Occludens-1(ZO-1) (Hartz et al. 2012, Carrano et al. 2011, Marco and Skaper 2006, Kook et al. 2012). Notably, however, the in vitro studies mainly focus on soluble forms of the Aβ peptides, whereas cerebral pools of Aβ are essentially coupled with apolipoproteins (ApoE, ApoJ, ApoA-I) and form some oligomers.

Amyloid transport across the BBB

Considering what is described above, it was rapidly demonstrated that the BBB actively transports Aβ peptides not only from the brain to the blood but also in the opposite direction (influx), allowing them to re-enter the brain. This bidirectional transport is mediated by receptors expressed by luminal (blood facing) and abluminal (brain facing) sides of the BBB

endothelial cells (Gosselet et al. 2013). The receptors termed low-density lipoprotein receptor-related protein 1 and 2 (LRP1, -2) and the receptor for advanced glycation end-products (RAGE) mediate the cerebral efflux and influx, respectively (Candela et al. 2010, Bell et al. 2007, Storck et al. 2016). In addition, some efflux pumps such as P-glycoprotein (ABCB1), BCRP (ABCG2) and MRP-1 (ABCC1) are involved in efflux but also in restricting the influx of these peptides (Candela et al. 2010, Storck et al. 2018, Xiong et al. 2009, Krohn et al. 2011). Interestingly, the expression of LRP1 and P-glycoprotein in brain microvessels is decreased in AD transgenic mice and AD brains (van Assema, Lubberink, Bauer, et al. 2012, Jeynes and Provias 2008). This decrease is also reported in ageing patients (van Assema, Lubberink, Boellaard, et al. 2012). On the contrary, the expression of RAGE, which mediates amyloid entry into the brain, is increased in AD brain microvessels (Donahue et al. 2006). All these dysregulations slowly contribute to reducing the brain's elimination of Aβ whereas its entry into CNS is increased, thus promoting accumulation and deposition. This hypothesis is supported by data obtained in humans demonstrating that there is no difference in Aβ peptide production in AD patients when compared with cognitively normal patients, but a significant decrease of CNS clearance of Aβ is measured (Mawuenyega et al. 2010). Given the recent demonstration that different isoforms of the Tau protein are also transported across the BBB (Banks et al. 2017), it is likely that this decrease in clearance might also be reported in the future for this protein, thus also contributing to AD onset and evolution. However, the origin of these dysregulations remains unknown but could involve lifestyle, ageing and vascular risk factors such as atherosclerosis, hypertension, diabetes, etc.

Therapeutic approaches in AD

From a therapeutic point of view, current approaches for AD only target the symptoms without directly preventing or treating the disease. Interestingly, several promising approaches or drugs have been developed to promote Aβ peptide efflux or decrease influx across the brain microvessels. For example, in vitro and in vivo, some works reported that caffeine increases LRP1 expression at the BBB and thus promotes the Aβ peptide elimination across the BBB (Qosa et al. 2012). Moreover, activating Retinoid or Liver X nuclear receptors (RXR/LXR) with agonists such as T0901317, bexarotene or oxysterols, increases P-glycoprotein expression at the BBB, thus restricting the entry of Aβ peptides into the brain and promoting efflux thereof (Saint-Pol et al. 2013, Kuntz et al.

2015). A similar approach has been done using Saint John's wort extracts, increasing MRP-1 expression and thus impeding the accumulation of amyloid within the brain parenchyma (Hofrichter et al. 2013).

Another approach consisting of increasing Aβ clearance from the brain is the use of passive immunotherapy strategies. Solanezumab, Gantenerumab, Aducanumab, BAN2401 are currently tested in clinical trials with the hope that they will significantly decrease the amyloid burden and improve the cognitive functions. The molecular mechanisms of these antibodies remain not clearly determined because it is not known if they need to cross the BBB to act or if this is a "sink effect" that decreases the Aβ burden in AD patients (Panza et al. 2019). In addition, if removal of amyloid is observed, the neurodegenerative process does not seem to be slowed (Holmes et al. 2008), suggesting that patients need to be treated earlier or in combination with other treatments such as passive immunotherapy anti-Tau.

Another innovative approach might be to transiently open the BBB with focused ultrasound (FUS). By introducing microbubbles (MB) into the bloodstream prior to FUS exposure, the BBB can be transiently opened at the ultrasound focus without acute neuronal damage (Hynynen et al. 2001). Amyloid burden decreases and improvement in cognitive functions have been reported in AD animal models treated only with FUS-MB (Leinenga and Gotz 2015, Raymond et al. 2008) or with FUS-MB and drugs/antibodies (Jordao et al. 2010). In humans, this approach seems to be very well tolerated in patients with glioblastoma (Carpentier et al. 2016) and AD (Lipsman et al. 2018), supporting the pursuit of research in this direction.

However, because lifestyle and vascular risk factors alter BBB functions and therefore may promote AD (Chakraborty et al. 2017), the best approach for the moment likely continues to be prevention. Several cross-sectional studies have clearly demonstrated that healthy people have a diet rich in fruit and vegetables rather than meat, processed carbohydrates and fats, compared to people that developed AD. Studies performed in AD transgenic mice suggest that antioxidants, vitamins, and polyunsaturated fatty acids can delay or reverse amyloid accumulation and slow down the pathological process. Of course, further studies are needed in this regard to better understand the impact of these nutrients and diets and lifestyle on BBB physiology, CBF and neurodegenerative diseases.

Parkinson's Disease

Parkinson's disease (PD) is a chronic neurodegenerative disorder that affects more than 6.1 million people worldwide. PD is characterized by the profound and progressive loss of dopaminergic (DA) neurons in the *substantia nigra pars compacta* (SNpc). The loss of these neurons leads to a concomitant decrease in dopamine level in the striatum (i.e. putamen and caudate nucleus), that results in a wide range of motor and non-motor deficits including tremor at rest, bradykinesia (slowness of movement), rigidity, loss of postural reflexes, freezing phenomenon, cardiovascular and gastrointestinal abnormalities, cognitive dysfunction and depression (Bernheimer et al. 1973, Dauer and Przedborski 2003). In addition to the loss of nigrostriatal DA neurons, intraneuronal proteinaceous cytoplasmic inclusions referred to as "Lewy Bodies" (LBs), rich in alpha-synuclein and ubiquitin, are observed as the pathological hallmark of PD (Spillantini et al. 1997, Matsuzaki et al. 2004). Selective degeneration of DA neurons in the SNpc causes the major PD symptoms, but there is often widespread neurodegeneration and LB formation in other regions of the brain depending on the progression stage of the disease (Braak et al. 2003, Braak and Del Tredici 2009).

The cause behind the pathogenesis of PD is still under extensive investigation, but several studies have suggested that mitochondrial dysfunction, oxidative stress, neuroinflammation, impairment of proteasomal function and protein aggregation could explain the common molecular basis for the loss of DA neurons in the SNpc (Thomas 2009). This incertitude reduces the possibility of developing an effective therapeutic intervention which could stop or slow down progression of the disease.

To investigate the mechanisms behind PD pathogenesis and develop new drugs or therapies for PD, the use of animal models representing the major symptoms and pathology hallmark observed in PD is crucial. Currently, several animal models for PD have been characterized, including both toxin-induced models and genetically modified models with knock-in and knock-out rodents based on known PD-associated genes (Vingill, Connor-Robson, and Wade-Martins 2018). The neurotoxins used to induce DA neurodegeneration include 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), Paraquat, and rotenone. Genetically modified mice with null mutations, an extra gene copy, or point mutations of genes comprise the recessively inherited Parkin, PINK1 and DJ-1, as well as the autosomal dominant SNCA, LRRK2, and VPS35.

The continued search for targets and elucidation of the molecular mechanism involved in DA degeneration will certainly advance our understanding of the complex pathogenesis of PD and help develop new therapies.

Brain microvascular dysfunction in PD

Cerebral vascular dysfunction plays a critical role in neurodegenerative disorders. As in AD, microvascular pathology has also been observed in human PD brains and animal models of the disease. Characterization of the vascular morphology of human brain tissue from both PD and control cases has shown the formation of endothelial cell "clusters" in PD brains, a possible cause of capillary fragmentation and thus vascular degeneration. This vessel degeneration associated with PD has been found in the *substantia nigra*, middle frontal cortex and brain stem nuclei (Guan et al. 2013). In human PD brains, at cellular and molecular levels, a reduction of pericytes, vascular cell proliferation and the loss of growth factors, such as Vascular endothelial growth factor (VEGF) and Platelet-derived growth factor (PDGF) and increases in Insulin-like growth factor-binding protein 2 (IGFBP-2) in the grey matter of the middle frontal gyrus support the idea that vascular remodeling is impaired in PD (Yang P, Waldvogel H, et al. 2017). Moreover, alpha-synuclein aggregation, a key biochemical feature of PD, and autophagy impairment have been related to the endothelial degeneration observed in PD brains (Yang P, Min XL, et al. 2017).

Changes in brain endothelial proliferation, angiogenesis or BBB permeability may be the result of a healthy adaptative mechanism or a response to injury. In PD, it has been demonstrated that the number of endothelial cells increases as a result of the ongoing neurodegenerative process together with neuroinflammation contributing to increased angiogenesis in the SNpc (Barcia et al. 2005). Importantly, this increment in the angiogenic process affecting PD brain microvasculature may also be attributed to the pharmacotherapy used for this pathology, namely levodopa. For instance, animals lesioned with 6-OHDA and concomitantly treated with levodopa showed increased angiogenic activity and microvasculature remodeling (Cenci, Ohlin, and Rylander 2009, Lindgren, Ohlin, and Cenci 2009). The impact of L-Dopa on microvasculature has also been detected in post-mortem tissue from dyskinetic patients with PD, where enhanced microvascular density along with indices of active angiogenesis have been observed (Ohlin et al. 2011), suggesting that vascular integrity and therapeutic approaches may have an implication for PD symptoms. Thus, treatments that prevent vascular degeneration and/or

improve vascular remodeling may be a new target for the treatment of PD. For instance, deep-brain stimulation, a surgical alternative used to tackle PD symptoms, which involves the implantation of a medical device to send electrical impulses to specific parts of the brain, has been associated with improved integrity of the vasculature in PD brains, with increased levels of microvessel endothelial cell thickness, length and density and upregulation of adherents junction and tight junction-associated proteins (Pienaar et al. 2015).

BBB disruption in PD

As detailed above, disruption of the BBB may strongly contribute to an imbalance in the normal transport routes between the blood and the brain parenchyma, allowing unwanted molecules to enter and also influencing the normal flux of critical nutrients to the CNS. Despite the evidence of BBB disruption as a contributor to PD pathology, its characterization in PD patients and animal models of the disease is highly unexplored (Weiss et al. 2009, Lee and Pienaar 2014). Nevertheless, studies have identified BBB dysfunction as occurring in the brain of patients with PD (Kortekaas et al. 2005, Bartels et al. 2008, Lee and Pienaar 2014). Idiopathic PD may represent a long and cumulative process, where the outcome is the result of a complex set of interactions between genetic predisposition, the innate vulnerabilities of the nigrostriatal DA system and exposure to environmental toxins (Brown et al. 2006, Baldereschi et al. 2003, Kamel et al. 2007, Di Monte, Lavasani, and Manning-Bog 2002). Inline, BBB disruption may be a critical mechanism by which these unwanted toxins reach the brain. Evidence of increased permeability of the BBB in PD patients was demonstrated when the presence of serum proteins, iron, and erythrocytes extravasation was found in the post-commissural putamen (Gray and Woulfe 2015). Moreover, expression levels and function of the efflux pump P-glycoprotein is decreased in the BBB and may contribute to the progression of PD (Abbott, Ronnback, and Hansson 2006, Bartels et al. 2008). Altogether, these events could facilitate the accumulation of toxic compounds in the brain, boosting neurodegeneration. It is known that PD is a multisystem disorder, in which several cellular mechanisms, like mitochondrial dysfunction, oxidative stress, neuroinflammation, and protein aggregation contribute to the neurodegenerative process. In the CNS, oxidative stress is a major contributor to disease and ageing and it has been largely mentioned as an extensive contributor to the development of PD (Dexter et al. 1994, Alam et al. 1997, Zhang et al. 1999, Choi et al. 2012, Cristovao et al. 2012). Cerebral endothelial cells are equipped with

an antioxidant defense system including increased GSH, glutathione peroxidase, glutathione reductase and catalase (Tayarani et al. 1987). However, these cells are also rich in mitochondria content, which increases the risk of oxidative stress deregulation. Mitochondria are vital to ATP generation, pyrimidine biosynthesis, fatty acid metabolism, calcium homeostasis, and apoptosis, and are the predominant source of reactive oxygen species (ROS) in the cell. Mitochondria generate most of the cellular energy by oxidative phosphorylation and produce ROS as a by-product. However, mitochondria dysfunction may lead to the inhibition of oxidative phosphorylation, causing a redirection of oxidative phosphorylation electrons into ROS production, thus increasing oxidative stress. A decline in mitochondrial energy production and an increase in oxidative stress can interfere with the mitochondrial permeability transition pore to initiate programmed cell death (Wallace 2005). Increasing evidence demonstrates that mitochondrial dysfunction is implicated in the ageing process and neurodegenerative diseases (Andersen 2004, Beal 2002, 2003, Melov 2004), and multiple lines of evidence suggest that mitochondrial dysfunction could represent a critical event in the pathogenesis of PD. It has been demonstrated that high content of mitochondria in endothelial cells increases the chances for oxidative stress generation affecting BBB function (Grammas, Martinez, and Miller 2011) and that mitochondria dysfunction is responsible for BBB permeabilization (Doll et al. 2015). It has been shown that endothelial cell dysfunction induced by exposure to lipopolysaccharide (LPS) is associated with increased ROS levels leading to BBB disruption (Gaillard, de Boer, and Breimer 2003). In light of this, targeting oxidative stress and mitochondrial dysfunction of cerebrovascular endothelial cells may be an effective therapeutic approach to target both neurovascular and neurodegenerative disorders.

The impact of neuroinflammation on BBB function is also described. Increased release of cytokines due to the activation of microglia has been shown to be pro-angiogenic and can compromise BBB integrity which in turn contributes to ongoing neuroinflammation by allowing peripheral molecules and immune cells to access the brain parenchyma (Lee and Pienaar 2014).

Alpha-synuclein overexpression and aggregation represent one of the most important pathologic contributors to PD developments (Beyer et al., 2009; Cookson, 2009), and is also associated with the mechanisms responsible for BBB dysfunction. It was recently reported that monomeric alpha-synuclein by targeting pericytes is involved in BBB dysfunction through the increased release of pro-inflammatory molecules and

enhancing of endothelial permeability (Dohgu et al. 2019). Moreover, it was recently reported that alpha-synuclein crosses the BBB bidirectionally, which could signify an important contributor event in PD pathogenesis (Peelaerts et al. 2015, Sui et al. 2014).

Therapeutic approaches targeting the BBB in PD

Current therapeutic approaches for PD, such as levodopa and Catechol-O-methyltransferase (COMT) and MAO-B inhibitors, are only able to target the symptoms. As symptomatic therapies lose their effectiveness over time, patients end up with few therapeutic options. Although no therapies have yet been developed in this direction, halting PD progression has revealed itself to be a promising solution and the interest for developing it has increased in recent years.

The incertitude of whether compromised integrity of the BBB is a component of the etiology of PD or a consequence thereof has contributed to a lack of therapeutic approaches to target BBB dysfunction in this pathology. Deep brain stimulation is the most widely used surgical alternative with benefits for PD patients that involve the implantation of a medical device, which sends electrical impulses to specific parts of the brain (Fang and Tolleson 2017). Besides the beneficial effect on motor symptoms, as mentioned above, deep-brain stimulation has also been found to improve the integrity of the vasculature in PD brains (Pienaar et al. 2015), and so can be taken into consideration as a candidate for the development of a new therapeutic approach to target the BBB in PD. Although studies need to be extended with a higher number of participating patients, it was recently shown that spinal cord stimulation might improve walking and decreased movement symptoms and freezing (de Lima-Pardini et al. 2018, Samotus, Parrent, and Jog 2018). Currently, the major focus of BBB research in PD is dedicated to developing new technologies to improve drugs' ability to cross it and concentrate in the brain parenchyma. An example of this is the development of nanoparticles as delivery systems of molecules with neuroprotective or neuro-regenerative potential, like retinoic-acid or micro-RNAs (Esteves et al. 2015, Saraiva, Ferreira, and Bernardino 2016, Saraiva et al. 2016). To boost the development of therapeutic approaches that specifically target BBB function, ameliorating its performance in PD, efforts should be made to clearly characterize the role of microvasculature in the pathologic context of PD.

Conclusion

The present chapter has recapitulated the knowledge available on the contribution of dysfunctional microvasculature, or its altered status, to the development and progression of neurodegenerative disorders. Despite the growing body of evidence that suggests the contribution of microvasculature pathology to the development and progression of neurodegenerative disorder, many are still unknown and under extensive investigation. A better understanding of the mechanisms underlying the neurodegenerative process and BBB disruption and its crosstalk will certainly boost the development of better therapeutic approaches to address both AD and PD.

References

Abbott, N. J., L. Ronnback, and E. Hansson. 2006. "Astrocyte-endothelial interactions at the blood-brain barrier." *Nat Rev Neurosci* 7 (1):41-53.

Alam, Z. I., S. E. Daniel, A. J. Lees, D. C. Marsden, P. Jenner, and B. Halliwell. 1997. "A generalised increase in protein carbonyls in the brain in Parkinson's but not incidental Lewy body disease." *J Neurochem* 69 (3):1326-9.

Andersen, J. K. 2004. "Oxidative stress in neurodegeneration: cause or consequence?" *Nat Med* 10 Suppl:S18-25. doi: 10.1038/nrn1434nrn1434 [pii].

Baldereschi, M., A. Di Carlo, P. Vanni, A. Ghetti, P. Carbonin, L. Amaducci, and D. Inzitari. 2003. "Lifestyle-related risk factors for Parkinson's disease: a population-based study." *Acta Neurol Scand* 108 (4):239-44.

Banks, W. A., A. Kovac, P. Majerova, K. M. Bullock, M. Shi, and J. Zhang. 2017. "Tau Proteins Cross the Blood-Brain Barrier." *J Alzheimers Dis* 55 (1):411-419. doi: 10.3233/jad-160542.

Barcia, C., V. Bautista, A. Sanchez-Bahillo, E. Fernandez-Villalba, B. Faucheux, M. Poza y Poza, A. Fernandez Barreiro, E. C. Hirsch, and M. T. Herrero. 2005. "Changes in vascularization in substantia nigra pars compacta of monkeys rendered parkinsonian." *J Neural Transm (Vienna)* 112 (9):1237-48. doi: 10.1007/s00702-004-0256-2.

Bartels, A. L., A. T. Willemsen, R. Kortekaas, B. M. de Jong, R. de Vries, O. de Klerk, J. C. van Oostrom, A. Portman, and K. L. Leenders. 2008. "Decreased blood-brain barrier P-glycoprotein function in the progression of Parkinson's disease, PSP and MSA." *J Neural Transm* 115 (7):1001-9. doi: 10.1007/s00702-008-0030-y.

Beal, M. F. 2002. "Oxidatively modified proteins in aging and disease." *Free Radic Biol Med* 32 (9):797-803. doi: S0891584902007803 [pii].

Beal, M. F. 2003. "Mitochondria, oxidative damage, and inflammation in Parkinson's disease." *Ann N Y Acad Sci* 991:120-31.

Begley, D. J., and M. W. Brightman. 2003. "Structural and functional aspects of the blood-brain barrier." *Prog Drug Res* 61:39-78.

Bell, R. D., A. P. Sagare, A. E. Friedman, G. S. Bedi, D. M. Holtzman, R. Deane, and B. V. Zlokovic. 2007. "Transport pathways for clearance of human Alzheimer's amyloid beta-peptide and apolipoproteins E and J in the mouse central nervous system." *J Cereb Blood Flow Metab* 27 (5):909-18.

Bernheimer, H., W. Birkmayer, O. Hornykiewicz, K. Jellinger, and F. Seitelberger. 1973. "Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations." *J Neurol Sci* 20 (4):415-55.

Bourasset, F., M. Ouellet, C. Tremblay, C. Julien, T. M. Do, S. Oddo, F. LaFerla, and F. Calon. 2009. "Reduction of the cerebrovascular volume in a transgenic mouse model of Alzheimer's disease." *Neuropharmacology* 56 (4):808-13.

Braak, H., and K. Del Tredici. 2009. "Neuroanatomy and pathology of sporadic Parkinson's disease." *Adv Anat Embryol Cell Biol* 201:1-119.

Braak, H., K. Del Tredici, U. Rub, R. A. de Vos, E. N. Jansen Steur, and E. Braak. 2003. "Staging of brain pathology related to sporadic Parkinson's disease." *Neurobiol Aging* 24 (2):197-211. doi: S0197458002000659 [pii].

Brown, T. P., P. C. Rumsby, A. C. Capleton, L. Rushton, and L. S. Levy. 2006. "Pesticides and Parkinson's disease--is there a link?" *Environ Health Perspect* 114 (2):156-64.

Bruinsma, I. B., M. M. Wilhelmus, M. Kox, R. Veerhuis, R. M. de Waal, and M. M. Verbeek. 2010. "Apolipoprotein E protects cultured pericytes and astrocytes from D-Abeta(1-40)-mediated cell death." *Brain Res* 1315:169-80.

Candela, P., F. Gosselet, J. Saint-Pol, E. Sevin, M. C. Boucau, E. Boulanger, R. Cecchelli, and L. Fenart. 2010. "Apical-to-basolateral transport of amyloid-beta peptides through blood-brain barrier cells is mediated by the receptor for advanced glycation end-products and is restricted by P-glycoprotein." *J Alzheimers Dis* 22 (3):849-59.

Carpentier, A., M. Canney, A. Vignot, V. Reina, K. Beccaria, C. Horodyckid, C. Karachi, D. Leclercq, C. Lafon, J. Y. Chapelon, L. Capelle, P. Cornu, M. Sanson, K. Hoang-Xuan, J. Y. Delattre, and A.

Idbaih. 2016. "Clinical trial of blood-brain barrier disruption by pulsed ultrasound." *Sci Transl Med* 8 (343):343re2. doi: 10.1126/scitranslmed.aaf6086.

Carrano, A., J. J. Hoozemans, S. M. van der Vies, A. J. Rozemuller, J. van Horssen, and H. E. de Vries. 2011. "Amyloid beta Induces Oxidative Stress-Mediated Blood-Brain Barrier Changes in Capillary Amyloid Angiopathy." *Antioxid Redox Signal* 15 (5):1167-78.

Cecchelli, R., V. Berezowski, S. Lundquist, M. Culot, M. Renftel, M. P. Dehouck, and L. Fenart. 2007. "Modelling of the blood-brain barrier in drug discovery and development." *Nat Rev Drug Discov* 6 (8):650-61.

Cenci, M. A., K. E. Ohlin, and D. Rylander. 2009. "Plastic effects of L-DOPA treatment in the basal ganglia and their relevance to the development of dyskinesia." *Parkinsonism Relat Disord* 15 Suppl 3:S59-63. doi: 10.1016/S1353-8020(09)70782-5.

Chakraborty, A., N. M. de Wit, W. M. van der Flier, and H. E. de Vries. 2017. "The blood brain barrier in Alzheimer's disease." *Vascul Pharmacol* 89:12-18. doi: 10.1016/j.vph.2016.11.008.

Choi, D. H., A. C. Cristovao, S. Guhathakurta, J. Lee, T. H. Joh, M. F. Beal, and Y. S. Kim. 2012. "NADPH oxidase 1-mediated oxidative stress leads to dopamine neuron death in Parkinson's disease." *Antioxid Redox Signal* 16 (10):1033-45. doi: 10.1089/ars.2011.3960.

Claudio, L. 1996. "Ultrastructural features of the blood-brain barrier in biopsy tissue from Alzheimer's disease patients." *Acta Neuropathol* 91 (1):6-14.

Cristovao, A. C., S. Guhathakurta, E. Bok, G. Je, S. D. Yoo, D. H. Choi, and Y. S. Kim. 2012. "NADPH oxidase 1 mediates alpha-synucleinopathy in Parkinson's disease." *J Neurosci* 32 (42):14465-77. doi: 10.1523/JNEUROSCI.2246-12.2012.

Dauer, W., and S. Przedborski. 2003. "Parkinson's disease: mechanisms and models." *Neuron* 39 (6):889-909. doi: S0896627303005683 [pii].

de Lima-Pardini, A. C., D. B. Coelho, C. P. Souza, C. O. Souza, Mgds Ghilardi, T. Garcia, M. Voos, M. Milosevic, C. Hamani, L. A. Teixeira, and E. T. Fonoff. 2018. "Effects of spinal cord stimulation on postural control in Parkinson's disease patients with freezing of gait." *Elife* 7. doi: 10.7554/eLife.37727.

Dexter, D. T., A. E. Holley, W. D. Flitter, T. F. Slater, F. R. Wells, S. E. Daniel, A. J. Lees, P. Jenner, and C. D. Marsden. 1994. "Increased levels of lipid hydroperoxides in the parkinsonian substantia nigra: an HPLC and ESR study." *Mov Disord* 9 (1):92-7. doi: 10.1002/mds.870090115.

Di Monte, D. A., M. Lavasani, and A. B. Manning-Bog. 2002. "Environmental factors in Parkinson's disease." *Neurotoxicology* 23 (4-5):487-502. doi: S0161-813X(02)00099-2 [pii].

Dohgu, S., F. Takata, J. Matsumoto, I. Kimura, A. Yamauchi, and Y. Kataoka. 2019. "Monomeric alpha-synuclein induces blood-brain barrier dysfunction through activated brain pericytes releasing inflammatory mediators in vitro." *Microvasc Res* 124:61-66. doi: 10.1016/j.mvr.2019.03.005.

Doll, D. N., H. Hu, J. Sun, S. E. Lewis, J. W. Simpkins, and X. Ren. 2015. "Mitochondrial crisis in cerebrovascular endothelial cells opens the blood-brain barrier." *Stroke* 46 (6):1681-9. doi: 10.1161/STROKEAHA.115.009099.

Donahue, J. E., S. L. Flaherty, C. E. Johanson, J. A. Duncan, 3rd, G. D. Silverberg, M. C. Miller, R. Tavares, W. Yang, Q. Wu, E. Sabo, V. Hovanesian, and E. G. Stopa. 2006. "RAGE, LRP-1, and amyloid-beta protein in Alzheimer's disease." *Acta Neuropathol* 112 (4):405-15.

Esteves, M., A. C. Cristovao, T. Saraiva, S. M. Rocha, G. Baltazar, L. Ferreira, and L. Bernardino. 2015. "Retinoic acid-loaded polymeric nanoparticles induce neuroprotection in a mouse model for Parkinson's disease." *Front Aging Neurosci* 7:20. doi: 10.3389/fnagi.2015.00020.

Fang, J. Y., and C. Tolleson. 2017. "The role of deep brain stimulation in Parkinson's disease: an overview and update on new developments." *Neuropsychiatr Dis Treat* 13:723-732. doi: 10.2147/NDT.S113998.

Gaillard, P. J., A. B. de Boer, and D. D. Breimer. 2003. "Pharmacological investigations on lipopolysaccharide-induced permeability changes in the blood-brain barrier in vitro." *Microvasc Res* 65 (1):24-31.

Gosselet, F., J. Saint-Pol, P. Candela, and L. Fenart. 2013. "Amyloid-beta Peptides, Alzheimer's Disease and the Blood-brain Barrier." *Curr Alzheimer Res* 10 (10):1015-33.

Grammas, P., J. Martinez, and B. Miller. 2011. "Cerebral microvascular endothelium and the pathogenesis of neurodegenerative diseases." *Expert Rev Mol Med* 13:e19. doi: 10.1017/S1462399411001918.

Gray, M. T., and J. M. Woulfe. 2015. "Striatal blood-brain barrier permeability in Parkinson's disease." *J Cereb Blood Flow Metab*. doi: 10.1038/jcbfm.2015.32.

Guan, J., D. Pavlovic, N. Dalkie, H. J. Waldvogel, S. J. O'Carroll, C. R. Green, and L. F. Nicholson. 2013. "Vascular degeneration in Parkinson's disease." *Brain Pathol* 23 (2):154-64. doi: 10.1111/j.1750-3639.2012.00628.x.

Gustafsson, S., V. Lindstrom, M. Ingelsson, M. Hammarlund-Udenaes, and S. Syvanen. 2018. "Intact blood-brain barrier transport of small

molecular drugs in animal models of amyloid beta and alpha-synuclein pathology." *Neuropharmacology* 128:482-491. doi: 10.1016/j.neuropharm.2017.08.002.

Hartz, A. M., B. Bauer, E. L. Soldner, A. Wolf, S. Boy, R. Backhaus, I. Mihaljevic, U. Bogdahn, H. H. Klunemann, G. Schuierer, and F. Schlachetzki. 2012. "Amyloid-beta contributes to blood-brain barrier leakage in transgenic human amyloid precursor protein mice and in humans with cerebral amyloid angiopathy." *Stroke* 43 (2):514-23.

Hofrichter, J., M. Krohn, T. Schumacher, C. Lange, B. Feistel, B. Walbroel, H. J. Heinze, S. Crockett, T. F. Sharbel, and J. Pahnke. 2013. "Reduced Alzheimer's disease pathology by St. John's Wort treatment is independent of hyperforin and facilitated by ABCC1 and microglia activation in mice." *Curr Alzheimer Res* 10 (10):1057-69.

Holmes, C., D. Boche, D. Wilkinson, G. Yadegarfar, V. Hopkins, A. Bayer, R. W. Jones, R. Bullock, S. Love, J. W. Neal, E. Zotova, and J. A. Nicoll. 2008. "Long-term effects of Abeta42 immunisation in Alzheimer's disease: follow-up of a randomised, placebo-controlled phase I trial." *Lancet* 372 (9634):216-23. doi: 10.1016/s0140-6736(08)61075-2.

Hynynen, K., N. McDannold, N. Vykhodtseva, and F. A. Jolesz. 2001. "Noninvasive MR imaging-guided focal opening of the blood-brain barrier in rabbits." *Radiology* 220 (3):640-6. doi: 10.1148/radiol.2202001804.

Iadecola, C., F. Zhang, K. Niwa, C. Eckman, S. K. Turner, E. Fischer, S. Younkin, D. R. Borchelt, K. K. Hsiao, and G. A. Carlson. 1999. "SOD1 rescues cerebral endothelial dysfunction in mice overexpressing amyloid precursor protein." *Nat Neurosci* 2 (2):157-61. doi: 10.1038/5715.

Jeynes, B., and J. Provias. 2008. "Evidence for altered LRP/RAGE expression in Alzheimer lesion pathogenesis." *Curr Alzheimer Res* 5 (5):432-7.

Jordao, J. F., C. A. Ayala-Grosso, K. Markham, Y. Huang, R. Chopra, J. McLaurin, K. Hynynen, and I. Aubert. 2010. "Antibodies targeted to the brain with image-guided focused ultrasound reduces amyloid-beta plaque load in the TgCRND8 mouse model of Alzheimer's disease." *PLoS One* 5 (5):e10549. doi: 10.1371/journal.pone.0010549.

Kamel, F., C. Tanner, D. Umbach, J. Hoppin, M. Alavanja, A. Blair, K. Comyns, S. Goldman, M. Korell, J. Langston, G. Ross, and D. Sandler. 2007. "Pesticide exposure and self-reported Parkinson's disease in the agricultural health study." *Am J Epidemiol* 165 (4):364-74. doi: kwk024 [pii]10.1093/aje/kwk024.

Kisler, K., A. R. Nelson, A. Montagne, and B. V. Zlokovic. 2017. "Cerebral blood flow regulation and neurovascular dysfunction in Alzheimer disease." *Nat Rev Neurosci* 18 (7):419-434. doi: 10.1038/nrn.2017.48.

Kook, S. Y., H. S. Hong, M. Moon, C. M. Ha, S. Chang, and I. Mook-Jung. 2012. "Abeta(1)(-)(4)(2)-RAGE interaction disrupts tight junctions of the blood-brain barrier via Ca(2)(+)-calcineurin signaling." *J Neurosci* 32 (26):8845-54.

Kortekaas, R., K. L. Leenders, J. C. van Oostrom, W. Vaalburg, J. Bart, A. T. Willemsen, and N. H. Hendrikse. 2005. "Blood-brain barrier dysfunction in parkinsonian midbrain in vivo." *Ann Neurol* 57 (2):176-9. doi: 10.1002/ana.20369.

Krohn, M., C. Lange, J. Hofrichter, K. Scheffler, J. Stenzel, J. Steffen, T. Schumacher, T. Bruning, A. S. Plath, F. Alfen, A. Schmidt, F. Winter, K. Rateitschak, A. Wree, J. Gsponer, L. C. Walker, and J. Pahnke. 2011. "Cerebral amyloid-beta proteostasis is regulated by the membrane transport protein ABCC1 in mice." *J Clin Invest* 121 (10):3924-31.

Kuntz, M., P. Candela, J. Saint-Pol, Y. Lamartiniere, M. C. Boucau, E. Sevin, L. Fenart, and F. Gosselet. 2015. "Bexarotene Promotes Cholesterol Efflux and Restricts Apical-to-Basolateral Transport of Amyloid-beta Peptides in an In Vitro Model of the Human Blood-Brain Barrier." *J Alzheimers Dis* 48 (3):849-62. doi: 10.3233/jad-150469.

Lee, H., and I. S. Pienaar. 2014. "Disruption of the blood-brain barrier in Parkinson's disease: curse or route to a cure?" *Front Biosci (Landmark Ed)* 19:272-80.

Leinenga, G., and J. Gotz. 2015. "Scanning ultrasound removes amyloid-beta and restores memory in an Alzheimer's disease mouse model." *Sci Transl Med* 7 (278):278ra33. doi: 10.1126/scitranslmed.aaa2512.

Lindgren, H. S., K. E. Ohlin, and M. A. Cenci. 2009. "Differential involvement of D1 and D2 dopamine receptors in L-DOPA-induced angiogenic activity in a rat model of Parkinson's disease." *Neuropsychopharmacology* 34 (12):2477-88. doi: 10.1038/npp.2009.74.

Lipsman, N., Y. Meng, A. J. Bethune, Y. Huang, B. Lam, M. Masellis, N. Herrmann, C. Heyn, I. Aubert, A. Boutet, G. S. Smith, K. Hynynen, and S. E. Black. 2018. "Blood-brain barrier opening in Alzheimer's disease using MR-guided focused ultrasound." *Nat Commun* 9 (1):2336. doi: 10.1038/s41467-018-04529-6.

Marco, S., and S. D. Skaper. 2006. "Amyloid beta-peptide1-42 alters tight junction protein distribution and expression in brain microvessel endothelial cells." *Neurosci Lett* 401 (3):219-24.

Matsuzaki, M., T. Hasegawa, A. Takeda, A. Kikuchi, K. Furukawa, Y. Kato, and Y. Itoyama. 2004. "Histochemical features of stress-induced aggregates in alpha-synuclein overexpressing cells." *Brain Res* 1004 (1-2):83-90. doi: 10.1016/j.brainres.2004.01.017
S0006899304000885 [pii].

Mawuenyega, K. G., W. Sigurdson, V. Ovod, L. Munsell, T. Kasten, J. C. Morris, K. E. Yarasheski, and R. J. Bateman. 2010. "Decreased clearance of CNS beta-amyloid in Alzheimer's disease." *Science* 330 (6012):1774.

Melov, S. 2004. "Modeling mitochondrial function in aging neurons." *Trends Neurosci* 27 (10):601-6. doi: 10.1016/j.tins.2004.08.004S0166-2236(04)00261-9 [pii].

Miyakawa, T., Y. Uehara, J. Desaki, T. Kimura, and R. Kuramoto. 1988. "Morphological changes of microvessels in the brain with Alzheimer's disease." *Jpn J Psychiatry Neurol* 42 (4):819-24.

Montagne, A., S. R. Barnes, M. D. Sweeney, M. R. Halliday, A. P. Sagare, Z. Zhao, A. W. Toga, R. E. Jacobs, C. Y. Liu, L. Amezcua, M. G. Harrington, H. C. Chui, M. Law, and B. V. Zlokovic. 2015. "Blood-brain barrier breakdown in the aging human hippocampus." *Neuron* 85 (2):296-302. doi: 10.1016/j.neuron.2014.12.032.

Neuwelt, E. A. 2004. "Mechanisms of disease: the blood-brain barrier." *Neurosurgery* 54 (1):131-40; discussion 141-2.

Ohlin, K. E., V. Francardo, H. S. Lindgren, S. E. Sillivan, S. S. O'Sullivan, A. S. Luksik, F. M. Vassoler, A. J. Lees, C. Konradi, and M. A. Cenci. 2011. "Vascular endothelial growth factor is upregulated by L-dopa in the parkinsonian brain: implications for the development of dyskinesia." *Brain* 134 (Pt 8):2339-57. doi: 10.1093/brain/awr165.

Panza, F., M. Lozupone, D. Seripa, and B. P. Imbimbo. 2019. "Amyloid-beta immunotherapy for alzheimer disease: Is it now a long shot?" *Ann Neurol* 85 (3):303-315. doi: 10.1002/ana.25410.

Peelaerts, W., L. Bousset, A. Van der Perren, A. Moskalyuk, R. Pulizzi, M. Giugliano, C. Van den Haute, R. Melki, and V. Baekelandt. 2015. "alpha-Synuclein strains cause distinct synucleinopathies after local and systemic administration." *Nature* 522 (7556):340-4. doi: 10.1038/nature14547.

Pienaar, I. S., C. H. Lee, J. L. Elson, L. McGuinness, S. M. Gentleman, R. N. Kalaria, and D. T. Dexter. 2015. "Deep-brain stimulation associates

with improved microvascular integrity in the subthalamic nucleus in Parkinson's disease." *Neurobiol Dis* 74:392-405. doi: 10.1016/j.nbd.2014.12.006.

Poduslo, J. F., G. L. Curran, T. M. Wengenack, B. Malester, and K. Duff. 2001. "Permeability of proteins at the blood-brain barrier in the normal adult mouse and double transgenic mouse model of Alzheimer's disease." *Neurobiol Dis* 8 (4):555-67.

Puzzo, D., W. Gulisano, A. Palmeri, and O. Arancio. 2015. "Rodent models for Alzheimer's disease drug discovery." *Expert Opin Drug Discov* 10 (7):703-11. doi: 10.1517/17460441.2015.1041913.

Qosa, H., A. H. Abuznait, R. A. Hill, and A. Kaddoumi. 2012. "Enhanced Brain Amyloid-beta Clearance by Rifampicin and Caffeine as a Possible Protective Mechanism Against Alzheimer's Disease." *J Alzheimers Dis* 31 (1):151-65.

Raymond, S. B., L. H. Treat, J. D. Dewey, N. J. McDannold, K. Hynynen, and B. J. Bacskai. 2008. "Ultrasound enhanced delivery of molecular imaging and therapeutic agents in Alzheimer's disease mouse models." *PLoS One* 3 (5):e2175. doi: 10.1371/journal.pone.0002175.

Ruitenberg, A., T. den Heijer, S. L. Bakker, J. C. van Swieten, P. J. Koudstaal, A. Hofman, and M. M. Breteler. 2005. "Cerebral hypoperfusion and clinical onset of dementia: the Rotterdam Study." *Ann Neurol* 57 (6):789-94. doi: 10.1002/ana.20493.

Sagare, A. P., R. D. Bell, Z. Zhao, Q. Ma, E. A. Winkler, A. Ramanathan, and B. V. Zlokovic. 2013. "Pericyte loss influences Alzheimer-like neurodegeneration in mice." *Nat Commun* 4:2932. doi: 10.1038/ncomms3932.

Saint-Pol, J., P. Candela, M. C. Boucau, L. Fenart, and F. Gosselet. 2013. "Oxysterols decrease apical-to-basolateral transport of Abeta peptides via an ABCB1-mediated process in an in vitro Blood-brain barrier model constituted of bovine brain capillary endothelial cells." *Brain Res* 1517:1-15.

Samotus, O., A. Parrent, and M. Jog. 2018. "Spinal Cord Stimulation Therapy for Gait Dysfunction in Advanced Parkinson's Disease Patients." *Mov Disord* 33 (5):783-792. doi: 10.1002/mds.27299.

Saraiva, C., L. Ferreira, and L. Bernardino. 2016. "Traceable microRNA-124 loaded nanoparticles as a new promising therapeutic tool for Parkinson's disease." *Neurogenesis (Austin)* 3 (1):e1256855. doi: 10.1080/23262133.2016.1256855.

Saraiva, C., J. Paiva, T. Santos, L. Ferreira, and L. Bernardino. 2016. "MicroRNA-124 loaded nanoparticles enhance brain repair in Parkinson's disease." *J Control Release* 235:291-305.

doi: 10.1016/j.jconrel.2016.06.005.
Selkoe, D. J. 2011. "Alzheimer's disease." *Cold Spring Harb Perspect Biol* 3 (7). doi: 10.1101/cshperspect.a004457.
Spillantini, M. G., M. L. Schmidt, V. M. Lee, J. Q. Trojanowski, R. Jakes, and M. Goedert. 1997. "Alpha-synuclein in Lewy bodies." *Nature* 388 (6645):839-40. doi: 10.1038/42166.
Storck, S. E., A. M. S. Hartz, J. Bernard, A. Wolf, A. Kachlmeier, A. Mahringer, S. Weggen, J. Pahnke, and C. U. Pietrzik. 2018. "The concerted amyloid-beta clearance of LRP1 and ABCB1/P-gp across the blood-brain barrier is linked by PICALM." *Brain Behav Immun* 73:21-33. doi: 10.1016/j.bbi.2018.07.017.
Storck, S. E., S. Meister, J. Nahrath, J. N. Meissner, N. Schubert, A. Di Spiezio, S. Baches, R. E. Vandenbroucke, Y. Bouter, I. Prikulis, C. Korth, S. Weggen, A. Heimann, M. Schwaninger, T. A. Bayer, and C. U. Pietrzik. 2016. "Endothelial LRP1 transports amyloid-beta(1-42) across the blood-brain barrier." *J Clin Invest* 126 (1):123-36. doi: 10.1172/jci81108.
Sui, Y. T., K. M. Bullock, M. A. Erickson, J. Zhang, and W. A. Banks. 2014. "Alpha synuclein is transported into and out of the brain by the blood-brain barrier." *Peptides* 62:197-202. doi: 10.1016/j.peptides.2014.09.018.
Tayarani, I., J. Chaudiere, J. M. Lefauconnier, and J. M. Bourre. 1987. "Enzymatic protection against peroxidative damage in isolated brain capillaries." *J Neurochem* 48 (5):1399-402.
Thomas, B. 2009. "Parkinson's disease: from molecular pathways in disease to therapeutic approaches." *Antioxid Redox Signal* 11 (9):2077-82. doi: 10.1089/ars.2009.2697.
Ujiie, M., D. L. Dickstein, D. A. Carlow, and W. A. Jefferies. 2003. "Blood-brain barrier permeability precedes senile plaque formation in an Alzheimer disease model." *Microcirculation* 10 (6):463-70.
van Assema, D. M., M. Lubberink, M. Bauer, W. M. van der Flier, R. C. Schuit, A. D. Windhorst, E. F. Comans, N. J. Hoetjes, N. Tolboom, O. Langer, M. Muller, P. Scheltens, A. A. Lammertsma, and B. N. van Berckel. 2012. "Blood-brain barrier P-glycoprotein function in Alzheimer's disease." *Brain* 135 (Pt 1):181-9.
van Assema, D. M., M. Lubberink, R. Boellaard, R. C. Schuit, A. D. Windhorst, P. Scheltens, A. A. Lammertsma, and B. N. van Berckel. 2012. "P-Glycoprotein Function at the Blood-Brain Barrier: Effects of Age and Gender." *Mol Imaging Biol* 14 (6):771-776.

Vingill, S., N. Connor-Robson, and R. Wade-Martins. 2018. "Are rodent models of Parkinson's disease behaving as they should?" *Behav Brain Res* 352:133-141. doi: 10.1016/j.bbr.2017.10.021.

Wallace, Douglas C. 2005. "A MITOCHONDRIAL PARADIGM OF METABOLIC AND DEGENERATIVE DISEASES, AGING, AND CANCER: A Dawn for Evolutionary Medicine." *Annual Review of Genetics* 39 (1):359-407. doi: doi:10.1146/annurev.genet.39.110304.095751.

Weiss, N., F. Miller, S. Cazaubon, and P. O. Couraud. 2009. "The blood-brain barrier in brain homeostasis and neurological diseases." *Biochim Biophys Acta* 1788 (4):842-57. doi: 10.1016/j.bbamem.2008.10.022.

Xiong, H., D. Callaghan, A. Jones, J. Bai, I. Rasquinha, C. Smith, K. Pei, D. Walker, L. F. Lue, D. Stanimirovic, and W. Zhang. 2009. "ABCG2 is upregulated in Alzheimer's brain with cerebral amyloid angiopathy and may act as a gatekeeper at the blood-brain barrier for Abeta(1-40) peptides." *J Neurosci* 29 (17):5463-75.

Yang P, Min XL, Mohammadi M, Turner C, Faull R, Waldvogel H, Dragunow M, and Guan J. 2017. "Endothelial Degeneration of Parkinson's Disease is Related to Alpha-Synuclein Aggregation." *J Alzheimers Dis Parkinsonism* 7: 370.

Yang P, Waldvogel H, Turner C, Faull R, Dragunow M, and Guan J. 2017. "Vascular Remodelling is Impaired in Parkinson Disease." *J Alzheimers Dis Parkinsonism* 7: 313.

Zhang, J., G. Perry, M. A. Smith, D. Robertson, S. J. Olson, D. G. Graham, and T. J. Montine. 1999. "Parkinson's disease is associated with oxidative damage to cytoplasmic DNA and RNA in substantia nigra neurons." *Am J Pathol* 154 (5):1423-9.

Zlokovic, B. V. 2011. "Neurovascular pathways to neurodegeneration in Alzheimer's disease and other disorders." *Nat Rev Neurosci* 12 (12):723-38.

CHAPTER SEVEN

THE IMPACT OF DRUGS OF ABUSE ON BLOOD-BRAIN BARRIER

RICARDO ALEXANDRE LEITÃO AND ANA PAULA SILVA

Introduction

According to the World Drug Report 2019 (WDR, United Nations Office on Drugs and Crime), in 2017, an estimated 271 million people aged 15-64 had used drugs in the previous year. The most widely used drug worldwide is cannabis, with an estimated 188 million people having used this drug in 2017. Additionally, around 53, 29, 21 and 18 million people used opioids, amphetamines, "ecstasy" and cocaine, respectively, in that period. The adverse health consequences associated with the use of illicit drugs are significant and may include mental disorders, infections and associated cancer and cirrhosis, overdose and premature death. This phenomenon also has significant economic and criminal justice implications (WDR 2019). Thus, understanding drug use as a complex and relapsing chronic condition, drawing on different disciplines including scientific evidence from both pre-clinical and clinical research will contribute to better clarifying this condition and achieving effective treatment interventions.

Addiction is characterized by compulsive behavior, continued abuse of substances despite negative consequences, craving when drugs are not available, and high risk of relapse. However, not everyone who uses these substances becomes addicted because a combination of psychobiological and environmental factors, as well as neuroadaptations induced by drugs themselves, influence the risk of addiction (Marie et al., 2019). It is also known that environmental and genetic factors influence the propensity to become addicted. Interestingly, changes in epigenetic modulation of gene expression have emerged as an important contributor to the development

of addiction and some of these epigenetic modifications are heritable, leading to alterations in descendent physiology and behavior (Pierce et al., 2018). Moreover, brain changes induced by drugs of abuse have been extensively studied, with particular emphasis on neurobehavioral deficits, neurodevelopmental processes and synaptic perturbations (Higuera-Matas et al., 2011; Cadet et al., 2014; Alexandre et al., 2019; Bisagno and Cadet 2019; Cohen et al., 2019; Krebs et al., 2019). Aberrant patterns of brain functional connectivity have also been observed in different groups of substance use disorders, and are associated with craving and relapse (Zhang and Volkow 2019). Also, it is now well accepted that glial cells are involved in drug reward and contribute to drug abuse liability since these substances have a direct action on glial function and glia-neural communication with an impact on neuronal structure and function (Linker et al., 2019). Decades of research suggest that recreational substance use confers risk for cognitive impairment and psychiatric disorders, and indeed structural and functional differences in the brain have been associated with early and heavy substance use (Krebs et al., 2019; Lapin and Sara, 2019). Nevertheless, the underlying mechanisms that can explain the impact of drugs of abuse on the brain remain to be fully characterized.

The classical molecular mechanisms by which drugs of abuse produce their effects in the brain involve alteration on the monoaminergic system, oxidative stress, mitochondrial dysfunction, excitotoxicity, and inflammatory processes, among others (Silva et al., 2010). More recently, the impact of drugs of abuse on brain vascular function has received significant attention and has emerged as a critical phenomenon of drug-related neuropathology (Egleton and Abbruscato 2014; Gonçalves et al., 2014). In fact, one of the most important mechanisms by which these substances affect the central nervous system (CNS) involves blood-brain barrier (BBB) dysfunction. Moreover, several parameters are involved in the effects induced by a drug of abuse, and consequently in the neuroadaptations that may occur following administration, including transport across the barrier and the rate of delivery to the brain (Marie et al., 2019). The importance of the BBB is clearly demonstrated by its role in brain homeostasis and protection, but also by simultaneously providing nutrients essential for normal brain function. Thus, given the importance of the BBB, the growing interest in this structure as a therapeutic target in several neurological conditions is not surprising.

For a more comprehensive understanding of this chapter, readers are referred to other chapters in this book and reviews on BBB structure and function (Zlokovic 2008; Abbot et al., 2010; Cardoso et al., 2010; Liebner et al., 2018; Langen et al., 2019).

Cannabinoids

Cannabinoids are a class of chemical compounds that act on cannabinoid receptors, which regulate both the release of neurotransmitters in the CNS and the activity of immune cells in the periphery. Until now, two receptor subtypes have been described, CB1 and CB2 receptors. The CB1 receptor is mainly present in the brain tissue, particularly in basal ganglia, cerebellum, and the limbic system, including the striatum and hippocampus. However, it can also be found in the anterior eye and retina, as well as in the reproductive system (Pacher et al., 2006). CB2 receptors are essentially present in the immune system and immune-derived cells, but can also be detected in the peripheral nervous system. Moreover, it has been demonstrated that the CB2 receptor is more prone to having an anti-inflammatory role, thus being responsible for some of the therapeutic effects of cannabis (Pacher and Mechoulam, 2011).

The principal and more potent psychoactive component of the cannabis plant is Δ9-tetrahydrocannabinol (THC). This compound is responsible for almost all the effects associated with cannabis use, such as relaxation, euphoria (Osborne and Fogel, 2008), and alteration of conscious perception (Johnson, 1990). The second most relevant component of cannabis, although non-psychotropic, is cannabidiol (CBD). Interestingly, CBD has been associated with the prevention of short-term memory loss due to THC exposure (Iseger and Bossong, 2015). Additionally, cannabinol (CBN) is the main product of THC degradation, with mild psychoactive effects and present at low levels in the fresh cannabis plant. The CBN has greater affinity to the CB2 receptor. Importantly, cannabis has more than 100 cannabinoid compounds but most of them are present at very low levels and are non-psychoactive. Nevertheless, some of these compounds, such as cannabigerol, contribute to the overall effects observed after cannabis consumption.

Despite being the most relevant constituent of cannabis and the main element responsible for its effects, the impact of THC on BBB function and structure has largely been overlooked. Nevertheless, an in vitro study with a human endothelial cell line (hCMEC/D3) has demonstrated that THC increases the CYP1A1 isoform of cytochrome P450, which is part of the metabolic barrier and is involved in the cleavage of pro-form onto active form of proteins responsible for vessel contraction and dilation (Ghosh et al., 2016). However, THC did not interfere with ATP-binding cassette (ABC) transporters or other cytochrome P450 isoforms (Dauchy et al., 2009). ABC transporters are energy-dependent carriers that uptake nutrients, vitamins and other molecules important for brain function, or

can export drugs and primary/secondary metabolites of brain activity (Jones and George, 2004). These transporters therefore have a crucial role not only in maintaining the normal function of endothelial cells but of all brain tissue. Furthermore, the cytochrome P450 is a large family of enzymes responsible for the clearance of several exogenous compounds and for the synthesis of hormones or cell function regulatory proteins (Nebert and Gonzalez, 1987). More recently, it was shown that THC can decrease the adhesion capability of human monocytes to the extracellular matrix (ECM) similar to that observed in isolated human brain vessels (Raborn et al., 2014). Specifically, monocytes treated with both human immunodeficiency virus (HIV-1) protein Tat and THC showed lower levels of adhesion to the ECM gel than the monocytes treated with Tat protein alone (Raborn et al., 2014). These observations suggest that, at least under conditions of viral infection, THC could exert a protective effect by preventing the transmigration of peripheral immune cells.

Regarding the impact of CBD on the BBB, the first study to explore this issue demonstrated a decrease in leukocyte adhesion to pial vessels when mice were simultaneously administrated with bacterial lipopolysaccharide (LPS; Ruiz-Valdepenas et al., 2011). Also, CBD was able to prevent BBB hyperpermeability to 70-kDa-dextran, and the increase in mRNA levels of cyclooxygenase-2 (COX-2), tumor necrosis factor-alpha (TNF-α) and inducible nitric oxide synthase (iNOS) (Ruiz-Valdepenas et al., 2011). Moreover, an animal model of viral multiple sclerosis (MS) injected with CBD presented a lower degree of leukocyte transmigration in both prefrontal cortex and striatum (Mecha et al., 2013). Accordingly, CBD, by activating adenosine receptor A2A, prevented the increase of both leukocyte adhesion and soluble vascular cell adhesion molecule 1 (VCAM-1) levels in a murine endothelial cell line (b.END3) exposed to the same virus used to induce MS (Mecha et al., 2013). The same authors also showed that CBD inhibited an increase in mRNA levels of C-C motif chemokine ligand-2 (CCL2) and CCL5 chemokines and pro-inflammatory cytokines, TNF-α and interleukin (IL)-1β (Mecha et al., 2013). Using human brain microvascular endothelial cells (HBMEC) and co-cultures with human astrocytes, it was also demonstrated that exposure to CBD counteracted the decrease in transendothelial electrical resistance (TEER) induced by oxygen-glucose deprivation (OGD), an in vitro model of ischemia with increased BBB permeability (Hind et al., 2016). However, the beneficial effects of CBD were not mediated by activation of CB1 or CB2 receptors but by the activation of either PPARγ (peroxisome proliferator-activated receptor-gamma) or serotonin receptor 5-HT_{1A}. Moreover, in the co-culture model, CBD decreased the release of lactate

dehydrogenase (LDH; a marker of cell death or membrane damage), and levels of VCAM-1, IL-6 and vascular endothelial growth factor (VEGF), showing that CBD has an anti-inflammatory effect in conditions of ischemia (Hind et al., 2016).

The impact of three different doses of CBD (50, 100, 200 ng/rat, intracerebroventricular administration) were also tested in an animal model of ischemia (middle cerebral artery occlusion model, MCAO), in which BBB disruption is a hallmark. The lower dose (50 ng/rat) had no effect, whereas 100 ng/rat of CBD had a significant protective effect regarding brain edema and BBB disruption in core and penumbra areas of ischemic focus, specifically in the cortex and striatum (Khaksar and Bigdeli, 2017a, 2017b). This same dose induced a downregulation of TNF-α, TNF-receptor 1 and nuclear factor kappaB (NF-κB) expression, and also prevented the neurological deficits of MCAO (Khaksar and Bigdeli, 2017b). Interestingly, the dose of 200 ng/rat had a protective effect in all parameters only in the penumbra area (Khaksar and Bigdeli, 2017b) and in the striatum (Khaksar and Bigdeli, 2017a). Curiously, a very recent study tested whether lipid nanocapsules coated with CBD could have a higher capacity of overpassing the BBB in physiological conditions (Aparicio-Blanco et al., 2019), and therefore be used to carry drugs into the brain tissue. The authors showed a higher permeability of CBD-coated nanocapsules through an in vitro BBB model (hCMED/D3 cell line) compared with non-coated particles. It is worth noting that TEER values were unchanged, indicating that the paracellular route was not affected and therefore the transport of CBD-particles may occur through the transcellular pathway (Aparicio-Blanco et al., 2019). Thus, this study suggests that CBD can be used as a tool to facilitate the transcytosis of lipid nanocapsules but, to date, it has only been tested in control conditions.

The available literature supports a beneficial effect of CBD in several disease settings, which is summarized in Figure 7-1. Nevertheless, the diversity of administration and dose paradigms found in published papers makes it difficult to draw a conclusion with a great degree of confidence.

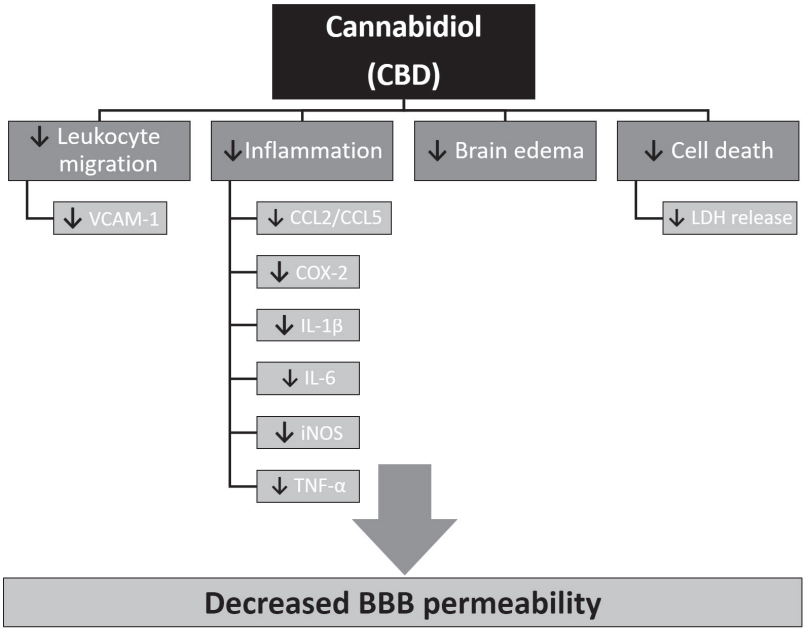

Figure 7-1: Schematic summary of the cannabidiol effects on the blood-brain barrier. The scheme compiles the main protective effects triggered by cannabidiol in blood-brain barrier properties and function, such as a decrease in leukocyte transmigration, inflammatory processes, brain edema and cell death. BBB: Blood-brain barrier; CCL2: C-C motif chemokine ligand-2; CCL5: C-C motif chemokine ligand-5; COX-2: Cyclooxygenase-2; IL-1β: Interleukin-1 beta; IL-6: Interleukin-6; iNOS: Inducible nitric oxide synthase; LDH: Lactate dehydrogenase; TNF-α: Tumor necrosis factor-alpha; VCAM-1: Vascular cell adhesion molecule-1.

Opioids

Heroin is the most consumed opioid due to its euphoric and sedative effects that occur rapidly and can last for a few hours. This drug is commonly administered by intravenous injection, but can also be smoked, snorted or inhaled. When taken orally, heroin is quickly metabolized into morphine, but after being injected intravenously, heroin bypasses the metabolization process and rapidly crosses the BBB. Once in the brain, it is processed to originate a non-active form and the active form 6-monoacetylmorphine (6-MAM), which in turn is converted into morphine. It is morphine that will act on the μ-opioid receptors, since heroin has a very low affinity to these receptors (Inturrisi et al., 1983).

To date, the only study that has evaluated the impact of heroin on the BBB aimed to measure levels of both heroin and morphine in the brain tissue under inhibition of P-glycoprotein. In fact, by blocking P-glycoprotein with PSC833, the authors showed an increase in morphine brain levels and consequently of its nociceptive effect, but with no alterations to the central levels of heroin or 6-MAM (Seleman et al., 2014). Therefore, it is possible to conclude that morphine is a substrate of P-glycoprotein, and its presence in the brain tissue is dependent on the activity of this efflux pump, in opposition to heroin, which is not a substrate of P-glycoprotein (Seleman et al., 2014).

Regarding the impact of morphine on the BBB, the first study investigating this issue showed that chronic exposure to the drug caused only a slight decrease in BBB permeability, observed by Evans Blue and ^{131}Iodine extravasation in the rat hippocampus, thalamus, hypothalamus, brain stem and spinal cord (Sharma and Ali, 2006; Sharma et al., 2010). However, after 1 or 2 days of withdrawal, BBB hyperpermeability in all brain regions was identified, as well as brain edema (Sharma et al., 2010) and hippocampal astrocytic perivascular end-feet swelling (Sharma and Ali, 2006). Curiously, an analysis of microvessels also revealed an increase in the presence of endothelial lanthanum (used as a marker of transcellular transport) but without alterations of the tight junctions (TJ) (Sharma and Ali, 2006), indicating that morphine promotes endothelial transcellular transport. Additionally, an antioxidant and potent inhibitor of lipid peroxidation, H-290/51, was able to prevent the withdrawal effects of morphine (Sharma et al., 2010), suggesting the involvement of oxidative stress in the deleterious effects induced by this drug. Moreover, Lynch and Banks (2008) demonstrated that an acute administration of morphine did not promote the transport of cytokines across the BBB since the presence of IL-2, IL-1α and TNF-α in the brain parenchyma was not detected after 24h (Lynch and Banks, 2008). On the contrary, 48h later it was possible to identify the presence of IL-2, which was potentiated by morphine withdrawal (Lynch and Banks, 2008). Importantly, the pro-inflammatory cytokine IL-2 is one of the key players in lymphocyte maturation (Liao et al., 2011) and is also involved in autoimmune responses (Reich and Szepietowski, 2007). Moreover, it can lead to a decrease in adherens junction proteins (Kim et al., 2014), activation of pro-inflammatory signaling pathways and cytoskeletal reorganization (Wylezinski and Hawiger, 2016). Also, IL-2 can suppress long-term potentiation in the hippocampus (Trancredi et al., 1990) causing memory impairment (Petitto et al., 1999). Ultimately, IL-2 seems to be implicated in neurological

disorders, such as schizophrenic-like behavior, multiple sclerosis and HIV-related dementia (Konsman et al., 2007).

The impact of different morphine paradigm administrations on brain vessels has also been investigated in detail. An escalating protocol was shown to increase gene expression of several ABC transporters (Mdr1a, Mrp1, and Bcrp), glucose transporter 1 (Glut1), and occludin, as well as to downregulate Flk-1 gene (which codifies for one VEGF receptor) in isolated cortical microvessels (Yousif et al., 2008). The same authors further demonstrated that acute morphine administration caused an upregulation of gene expression of two ABC transporters (Mdr1a and Bcrp) in cortical macro and microvessels (Yousif et al., 2012). Additionally, protein levels were also analyzed on the escalating morphine administration protocol and an upregulation of P-glycoprotein was observed in the cerebral cortex and hippocampus (Yousif et al., 2008), as well as in cortical macrovessels (Yousif et al., 2008; Yousif et al., 2012). Intriguingly, this upregulation of P-glycoprotein was dependent on NMDA receptor or COX-2 enzyme activation (Yousif et al., 2012). However, another study evaluated the impact of three different subchronic morphine administrations and concluded that none of the protocols changed P-glycoprotein or BCRP protein levels in cortical vessels (Chaves et al., 2016). Recently, in female mice exposed subchronically to morphine (ALZET osmotic pumps, for 5-7 days), a significant hyperpermeability of BBB to three different size fluorescent probes (10, 40 and 70 kDa) was observed, as well as a diffuse zonula occludens (ZO)-1 immunoreactivity in the striatum (Leibrand et al., 2019). Moreover, an increase in horseradish peroxidase (HRP) transport in the striatum was identified, proving once again that morphine is also able to promote vesicular transport (Leibrand et al., 2019).

To date, not much is known about the cellular players responsible for morphine outcomes at the BBB. Nevertheless, some authors have used in vitro approaches to better clarify such an effect. The first study reporting the impact of morphine on endothelial cells showed an increase in permeability of [^{14}C]-inulin (~5.5 kDa size) in both human and rat endothelial cell lines (Liu et al., 2004). Moreover, a significant decrease in cell viability was observed in both cell lines (Liu et al., 2004). Interestingly, the co-incubation of morphine and LPS did not cause any alteration compared to morphine alone (Liu et al., 2004). Additionally, Strazza and collaborators (2016) demonstrated that morphine alters barrier permeability by using an endothelial cell line (hCMEC/D3 cells), with a significant increase in gene expression and protein levels of intercellular adhesion molecule 1 (ICAM-1) at early time-points, as well as an

upregulation of VCAM-1 and activated leukocyte cell adhesion molecule (ALCAM) after longer exposure to morphine (Strazza et al., 2016). Also, an increase in the adhesion of peripheral blood mononuclear cells (PBMCs; CD3+ and CD14+) to endothelial cell monolayers was observed between 2h and 72h of morphine exposure (Strazza et al., 2016). Wen and collaborators (2011) further showed that morphine enhanced the protein levels of platelet-derived growth factor (PDGF-BB) in brain endothelial cells, which was correlated with ZO-1 downregulation and increased cell permeability. Interestingly, a co-culture model of the BBB composed of human primary cultures of endothelial cells and astrocytes treated with morphine showed a decrease in TEER values together with an increase in PBMC transmigration (Mahajan et al., 2008b). Additionally, upregulation of TNF-α, IL-8, junctional adhesion molecule 2 (JAM-2) and P-glycoprotein, together with a decrease in ZO-1 and occludin protein levels, was observed in monocultures of endothelial cells (Mahajan et al., 2008b). These observations suggest that morphine can impair BBB function, and a schematic representation of such effects is shown in Figure 7-2.

Psychostimulants
Focus on cocaine, methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA, “ecstasy”)

Cocaine

Although the mechanisms associated with cocaine-induced CNS disorders remain largely unknown, several studies have implicated neuroinflammation, oxidative stress and neurotoxicity as important factors in such conditions (Yamamoto and Raudensky, 2008; Silva et al., 2010; Clark et al., 2013; Gonçalves et al., 2014). It has also become unquestionable that cocaine compromises the integrity of the BBB (Kumar 2011; Sajja et al., 2016). In fact, cocaine has a detrimental effect on most cells of the CNS, including the endothelium (Brailoiu et al., 2016; Sajja et al., 2016). The impact of cocaine on BBB structure and function involves both a direct effect on brain endothelium and indirect responses through increased inflammatory mediators. By interfering with other components of the neurovascular unit, such as astrocytes, pericytes and microglia, cocaine can also lead to significant BBB alterations. Moreover, it has been demonstrated that inflammation and oxidative stress, as well as endothelium activation, are the main processes implicated in cocaine-induced BBB dysfunction (Gan et al., 1999; Fiala et al., 2005; Sajja et al., 2016).

Animal studies clearly showed that cocaine can induce BBB breakdown involving a decline in TJ proteins, as well as triggering edema formation that culminates in brain dysfunction and neuropathology (Yao et al., 2011a). Intense hyperthermia has also been associated with effects induced by cocaine administration (Sharma et al., 2009). For a better understanding of the underlying mechanisms involved in cocaine-induced BBB disruption, Kolodgie and collaborators (1999) started by demonstrating that endothelial cells treated with cocaine presented a disruption of F-actin and the formation of intercellular gaps, without evidence of cell lysis and/or detachment. These results indicate that cocaine directly leads to structural alterations of the endothelial cell barrier with an increase in the transport of macromolecular tracers. Accordingly, others have shown that cocaine can disrupt the endothelial TJ complexes to decrease TEER across endothelial monolayers and to reorganize the cytoskeleton resulting in stress fiber formation (Fiala et al., 2005; Dhillon et al., 2008; Gandhi et al., 2010). Some studies also suggested the existence of a cocaine binging site at the brain endothelium by activating sigma-1 (σ1) receptors and stimulating the Egr1 pathway-dependent release of endothelial PDGF (Kumar 2011; Yao et al., 2011b). Additionally, it is evident that cocaine originates abnormal astroglial responses and stimulates the release of proinflammatory mediators that can further compromise BBB integrity. Besides endothelial cells and glia, a recent study demonstrated that exposure of human brain vascular pericytes to cocaine can also trigger the secretion of a chemokine, CXCL10, and promoting monocyte transmigration across the BBB, both in vitro and in vivo (Niu et al., 2019).

A major consequence of drug-induced BBB dysfunction is the infiltration of circulating immune cells (Dhillon et al., 2008; Gandhi et al., 2010), which involves the upregulation of inflammatory mediators and endothelial adhesion molecules such as ICAM-1 and VCAM-1 (Fiala et al., 1998; Yao et al., 2011c; Kousik et al., 2012). In fact, ICAM-1 expression was shown to be elevated in cocaine abusers compared with controls (Moretti et al., 2019), as previously demonstrated in vitro (Fiala et al., 1998; Yao et al., 2011c). The activation of NF-κB and consequent transcription of inflammatory signaling molecules seems to be involved in cocaine-induced endothelial activation and monocyte transmigration (Fiala et al., 2005; Yao et al., 2011c) by activating matrix metalloproteinases (MMP) and consequent extracellular matrix degradation. Moreover, cocaine abuse was associated with CD3+ lymphocyte presence in the brain parenchyma, once again suggesting that cocaine promotes microvascular changes and significant neuroinflammatory response. Interestingly, glial

responses, neuroinflammation, and BBB alterations were also identified in post-mortem brain samples collected from drug abusers who died from cocaine acute intoxication (Moretti et al., 2019). In humans, chronic effects of repeated cocaine exposure must be also considered in addition to the effects of cocaine metabolites, which can provoke a direct endothelial injury.

The CNS is a major target of HIV-1, causing neuronal damage and several deficits termed as HIV-associated neurological disorder (HAND) (McArthur et al., 2010; Sanmarti et al., 2014). The entry of HIV-1 into the brain is facilitated by a "Trojan Horse" mechanism, where infected CD4+ cells and/or monocytes are trafficked into the CNS through the BBB (Ivey et al., 2009). Curiously, cocaine has been found to enhance HIV-1 infectivity of monocyte-derived dendritic cells and macrophages (Dhillon et al., 2007), and also to facilitate monocyte infiltration across the BBB, leading to enhanced disease progression and increased neuropathology (Yao et al., 2011b; Dahal et al., 2015; Dash et al., 2015). Although the exact mechanism remains unclear, it is unquestionable that drug exposure enhances HIV-1 neuro-invasion by disrupting the BBB (Zhang et al., 1998; Kousik et al., 2012; Chilunda et al., 2019). For example, alterations in endothelial TJ proteins are promoted by HIV-1 encoded proteins and cocaine resulting in a synergistic loss of the barrier function (Gandhi et al., 2010). Moreover, cocaine administration modulates microglial cells and upregulates inflammatory mediators (Cearley et al., 2011) that can facilitate HIV-infected monocyte transmigration across BBB (Dhillon et al., 2008; Dash et al., 2015). Others also demonstrated that cocaine induces chemokine monocyte chemotactic protein-1 (MCP-1/CCL2) in rodent microglia through sigma receptor and activation of Src, MAPK, PI3K/Akt and NF-κB, which accelerated monocyte extravasation across the endothelium (Yao et al., 2010).

In sum, cocaine triggers several molecular and cellular events leading to neuroinflammation and oxidative stress that ultimately can originate the recruitment and infiltration of circulating immune cells further aggravating BBB damage. A schematic representation of such effects is illustrated in Figure 7-2.

Methamphetamine

Methamphetamine (METH) consumption originates several central effects with significant acute and chronic consequences. It is well known that METH is highly neurotoxic compromising dopamine (DA) signaling and leading to oxidative/nitrosative stress, lipid peroxidation, as well as

impairment of energetic metabolism (Quinton and Yamamoto, 2006; Yamamoto and Raudensky, 2008; Cadet et al., 2014). Additionally, the glutamatergic system is involved in behavioral alterations due to METH use, and glial cells are also highly affected occurring a significant neuroinflammatory response (Silva et al., 2010; Gonçalves et al, 2014). In the last few years, BBB dysfunction induced by METH has been raised as an important feature of its neurotoxicity (Leitão et al., 2016). Several pathological consequences due to METH consumption are indeed strongly associated with brain vascular abnormalities. BBB alterations can occur as an indirect consequence of pathophysiological changes following METH use, such as hyperthermia (Kiyatkin and Sharma 2016), and the neurotoxicity and astrogliosis associated with METH also seem to highly contribute to BBB dysfunction (Dietrich, 2009). Nevertheless, several studies have clearly shown a direct effect of METH on barrier properties by affecting both paracellular and transcellular transport.

METH has been reported to promote BBB permeability, both in vitro and in vivo, as a result of endothelial junction proteins and cytoskeleton disarrangement. Actin cytoskeletal dynamics is detrimental to METH-induced BBB dysfunction by increasing the internalization of occludin (Park et al., 2013). METH also leads to α-tubulin deacetylation (Fernandes et al., 2015) and disruption of the actin filaments concomitant with claudin-5 translocation to the cytoplasm, which is mediated by MMP-9 activation in association with integrin-linked kinase (ILK) (Fernandes et al., 2016). Moreover, alterations of endothelial redox status have been demonstrated by a decreased total glutathione content and activation of oxidative stress-responsive transcription factors, like as NF-κB and AP-1, followed by upregulation of inflammatory genes, such as TNF-α (Lee et al., 2001). Interestingly, METH can directly interfere with endothelial cell properties or indirectly via astrocytes through the release of TNF-α by both cells and subsequent activation of the NF-κB pathway culminating in barrier dysfunction through the increase of transcellular and paracellular endothelial transport (Coelho-Santos et al., 2015). Specifically, this drug interferes with the expression and distribution of TJ proteins, such as occludin and claudin-5, and promotes monocyte transendothelial migration involving the production of reactive oxygen species (ROS) through increase of NOX activity (Park et al., 2012) and consequent activation of endothelial myosin light chain kinase (MLCK) (Ramirez et al., 2009). Endothelial transcytosis and enhanced lymphocyte migration may also occur via endothelial nitric oxide synthase (eNOS) activation (Martins et al., 2013). Moreover, METH significantly decreases the rate of glucose uptake and glucose transporter protein-1 (GLUT1) expression followed by

impairment of BBB integrity, which was observed both in vivo and in vitro (Abdul Muneer et al., 2011).

Animal studies have further demonstrated a region-specific BBB disruption. An acute high dose of METH was shown to elicit transient BBB permeability in the mouse hippocampus (Boyer and Ali, 2006; Martins et al., 2013), rat prefrontal cortex and nucleus accumbens (Kousik et al., 2011). Under such treatment, mice showed oxidative stress in brain capillaries with a prominent increase in superoxide radicals. Indeed, the role of oxidative stress in METH-induced toxicity is further supported by observations that the administration of antioxidants (Banerjee et al., 2010) or physical exercise (Toborek et al., 2013; Morais et al., 2018) can attenuate METH-induced neurotoxicity. Differential regulation of ABC transporters, namely an upregulation of the luminal endothelial transporter ABCB1 (P-glycoprotein) and downregulation of the abluminal transporter ABCC1 (MRP1), was also observed in cerebral microvessels (ElAli et al., 2012). Moreover, a binge administration of METH decreased collagen IV staining and increased TNF-α levels in the mouse striatum, simultaneously with BBB disruption observed by the presence of albumin in the brain parenchyma (Coelho-Santos et al., 2015). The same drug paradigm administration triggered brain edema formation in the striatum and hippocampus due to alterations in the water channel aquaporin 4 (AQP4), as well as BBB dysfunction and behavioral alterations (Leitão et al., 2018). METH-induced BBB alterations in the mouse hippocampus have further been shown to involve a decrease in TJ proteins ZO-1 and claudin-5, and an increase in both immunoreactivity and activity of MMP-9 (Martins et al., 2011). Accordingly, repeated low doses of METH have promoted striatal JNK1/2 phosphorylation and a transient increase in MMP-9 protein levels and activity, associated with an increase in both laminin degradation and BBB permeability (Urrutia et al., 2013). An escalating dosing regimen of METH has also induced BBB permeability in mice observed by detection of sodium fluorescein dye (Na-F; 376 Da) in the brain tissue (Ramirez et al., 2009).

Data have clearly demonstrated that METH induces BBB disruption, but most of these studies have used in vitro models or acute METH administration paradigm. Drug self-administration methodology provides the most direct match with human drug abuse features, and more recently METH-self administration was also shown to induce BBB breakdown. This effect has been identified in the rat striatum involving dopaminergic D2 receptors (Kousik et al., 2014) and in the hippocampus where a down-regulation of collagen IV staining has been observed, together with a decrease in intercellular junction proteins, namely claudin-5, occludin and

vascular endothelial-cadherin (Gonçalves et al., 2017). Additionally, an up-regulation of ICAM-1 and VCAM-1 has been demonstrated, simultaneously with the identification of T cell antigen CD4 and tissue macrophage marker CD169 in the brain parenchyma. Interestingly, these same animals also presented a neuroinflammatory profile characterized by microglial activation, astrogliosis, and increased pro-inflammatory mediators, like TNF-α, IL-1β, and MMP-9 (Gonçalves et al., 2017). Curiously, BBB changes can persist or even get worse following the withdrawal of METH (Sharma and Ali 2006; Kousik et al., 2011; Gonçalves et al., 2017). Clinical observation from abstinent METH abusers shows a long-lasting loss of BBB integrity and brain vascular functions (Rahman, 2008), and binge use of METH induced a sustained reduction in the cerebral flow that is present even after two years of abstinence (Chung et al., 2010).

As previously mentioned for cocaine, a consequence of HIV infection is HAND, with a high percentage of patients developing HAND even in the presence of antiretroviral therapy. Substance abuse is a major comorbidity in people with HIV and several studies have shown that substances of abuse increase HIV CNS pathogenesis. To characterize mechanisms that facilitate HAND in HIV-infected drug users, it is important to understand how substances of abuse lead to BBB dysfunction and the entry of uninfected and HIV-infected monocytes into the brain. Many studies have explored the relationship between METH use and HIV infection (Chilunda et al., 2019) and it is known that neuropathogenesis of HIV-1 is exacerbated by METH, which leads to BBB dysregulation. Since both METH and HIV viral proteins induce oxidative stress, drug abusers are at a greater risk. In fact, the incubation of brain microvascular endothelial cells (BMVEC) to METH and/or gp120, an HIV-1 viral protein, modulated TJ expression through Rho-A activation and led to BBB breakdown (Mahajan et al., 2008a). Mice exposed to HIV viral proteins (gp120 and Tat) also showed significant oxidative stress, alteration of TJ proteins and compromised BBB integrity, which was potentiated by METH (Banerjee et al., 2010). Moreover, it is known that advanced HIV disease is a major risk factor for cryptococcosis (Park et al., 2009). *Cryptococcus neoformans* is a neurotropic fungus that causes a significant number of meningoencephalitis cases globally (Park et al., 2009) and can cross the BBB either directly (Chang et al., 2004) or within macrophages (Charlier et al., 2009). Interestingly, Eugenin and collaborators (2013) demonstrated that METH facilitates the transmigration of *C. neoformans* through BBB due to alterations of TJ and adhesion molecules. Thus, substance abuse contributes to increased

peripheral inflammation and immune cell activation, as well as to BBB dysfunction, which may result in the consequent entry of immune cells into the CNS contributing to or aggravating brain toxicity. The impact of METH on the BBB is illustrated in Figure 7-2.

3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy")

MDMA induces hyperthermia, neuroinflammation and oxidative stress (Orio et al., 2004; Yamamoto and Bankson 2005; O'Shea et al., 2014), all of which are factors that mediate BBB disruption in diverse neuropathologies (O'Shea et al., 2014). Sharma and Ali (2008) were the first to show that acute administration of MDMA to rats or mice originated a prominent increase in brain water content, activation of astrocytes, and both leakage of Evans blue dye and increased albumin immunoreactivity, indicating BBB disruption, particularly in the cerebellum, hippocampus, cortex, thalamus, and hypothalamus. These effects were associated with MDMA-induced hyperthermia and cellular stress. However, it is also known that MDMA increases the expression of proinflammatory cytokines (Torres et al., 2011) that can lead to BBB breakdown. Additionally, it triggers an oxidative stress response (Yamamoto and Bankson, 2005) with excess release of DA and 5-hydroxytryptamine (5-HT, serotonin) that will result in the formation of ROS, DA-derived quinone, and toxic metabolites (Quinton and Yamamoto, 2006). The accumulation of toxic free radicals triggers a variety of signaling cascades leading to BBB alterations, brain edema, and neuroinflammation. Thus, both proinflammatory cytokines and ROS can modify BBB permeability through the induction of protease expression and activity (Candelario-Jalil et al., 2009). In fact, MMP are the principal proteases associated with BBB disruption in pathological conditions through basal lamina and TJ protein degradation (Rosenberg, 2009).

More recently, a single neurotoxic dose of MDMA was further demonstrated to promote brain edema and BBB permeability, evaluated by an increase of plasmatic immunoglobulin G and Evans blue extravasation, as well as a decrease in the expression of the basal lamina proteins laminin, collagen type IV, and claudin-5 in the rat hippocampus (Pérez-Hernández et al., 2017). These alterations occurred through microglial P2X7 receptor-mediated signaling, which in turn increases MMP-9 activity. Interestingly, the inhibition of MMP-9 was able to prevent these changes, proving its involvement in MDMA-induced BBB dysfunction (Pérez-Hernández et al., 2017). Another study using bovine brain microvessel endothelial cells and comparing different psychostimulants

showed that MDMA and METH can decrease cellular proliferation, increase LDH release and disrupt endothelial cell monolayer (Rosas-Hernandez et al., 2016). Although not so explored as much as cocaine and methamphetamine, it is clear that MDMA has also a significant impact on BBB structure and function, which is illustrated in Figure 7-2.

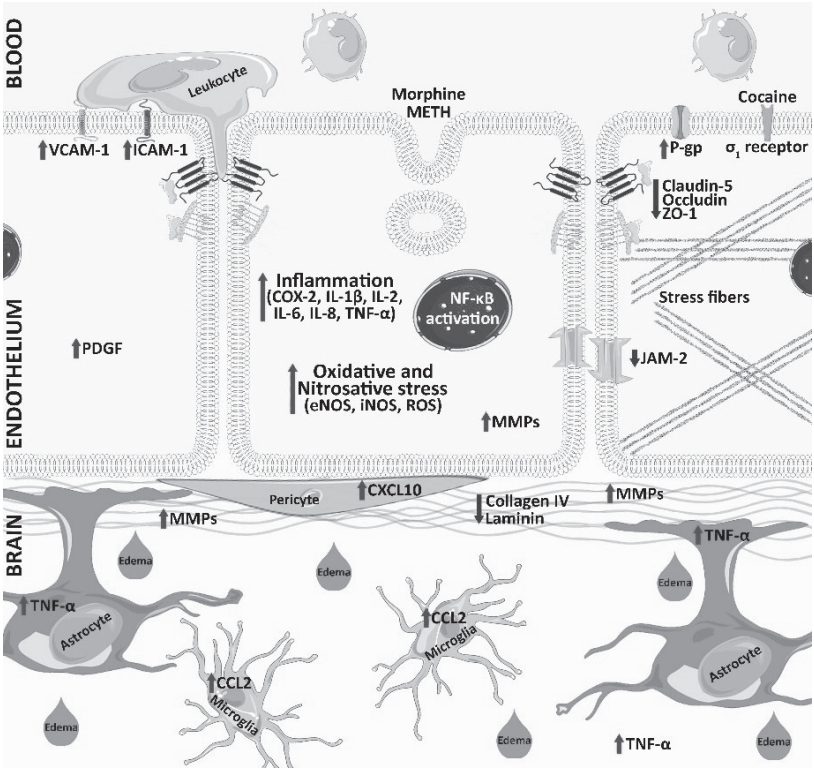

Figure 7-2: An overview of the most significant effects of opioids and psychostimulants on the blood-brain barrier. Summaries of the most important outcomes at the blood-brain barrier after exposure to opioids (heroin or morphine) and psychostimulants (cocaine, methamphetamine, MDMA). In short, drugs of abuse cause an increase in leukocyte adhesion and migration, as well as augmentation of inflammatory and oxidative processes. Moreover, there is a downregulation and disorganization of intercellular junctions and extracellular matrix proteins (collagen IV and laminin), as well as the formation of stress fibres. It is worth noting that other cells in the neurovascular unit, such as pericytes, astrocytes and microglia, can also respond to opioid and psychostimulant exposure by releasing several factors, such as CXCL10, TNF-α, CCL2 and MMPs, that will

lead to BBB dysfunction and brain edema. Besides interfering with paracellular transport, methamphetamine and morphine can also induce vesicular transport across the endothelium (transcytosis). CCL2: C-C Motif Chemokine Ligand 2; COX-2: Cyclooxygenase 2; CXCL10: C-X-C motif chemokine 10; eNOS: Endothelial nitric oxide synthase; ICAM-1: Intercellular adhesion molecule-1; IL-1β: Interleukin-1 beta; IL-2: Interleukin-2; IL-8: Interleukin-8; iNOS: Inducible nitric oxide synthase; JAM-2: Junctional adhesion molecule-2; METH: Methamphetamine; MMPs: Matrix metalloproteinases; NF-κB: Nuclear factor-kappa B; PDGF: Platelet-derived growth factor; P-gp: P-glycoprotein; ROS: Reactive oxygen species; TNF-α: Tumor necrosis factor-alpha; VCAM-1: Vascular cell adhesion molecule-1; ZO-1: Zonula occludens-1; σ1 receptor: sigma-1 receptor.

Concluding remarks

In the present chapter, we have gathered several studies that explored the effect of drugs of abuse on BBB function, with a focus on cannabinoids, opioids and psychostimulants. Despite cannabinoids and opioids being the two most consumed drugs worldwide, their impact on BBB properties and function has been poorly studied. Nevertheless, opioids seem to have a deleterious impact on the BBB (Figure 7-2), whereas CBD has a protective role (Figure 7-1). Regarding psychostimulants, the data available concerning cocaine, METH and MDMA are very consistent, pointing towards a similar impact on the BBB by triggering its dysfunction (Figure 7-2). Additionally, several pathways have already been identified which can be used as possible targets to counteract or at least diminish the impact of drug abuse on the BBB and, consequently, on brain function.

References

Abbott, N.J., A.A. Patabendige, D.E. Dolman, S.R. Yusof, and D.J. Begley. 2010. “Structure and function of the blood-brain barrier.” Neurobiol Dis. 37(1):13-25. doi: 10.1016/j.nbd.2009.07.030. Review.

Abdul Muneer, P.M., S. Alikunju, A.M. Szlachetka, L.C. Murrin, and J. Haorah. 2011. “Impairment of brain endothelial glucose transporter by methamphetamine causes blood-brain barrier dysfunction.” Mol Neurodegener. 6:23. doi: 10.1186/1750-1326-6-23.

Alexandre, J., H. Carmo, F. Carvalho, and J.P. Silva JP. 2019. “Synthetic cannabinoids and their impact on neurodevelopmental processes.” Addict Biol. Aug 23:e12824. doi: 10.1111/adb.12824.

Aparicio-Blanco, J., I.A. Romero, D.K. Male, K. Slowing, L. Garcia-Garcia, and A.I. Torres-Suarez. 2019. "Cannabidiol enhances the passage of lipid nanocapsules across the blood-brain barrier both in vitro and in vivo." Mol Pharm 16 (5):1999-2010. doi: 10.1021/acs.molpharmaceut.8b01344.

Banerjee, A., X. Zhang, K.R. Manda, W.A. Banks, and N. Ercal. 2010. "HIV proteins (gp120 and Tat) and methamphetamine in oxidative stress-induced damage in the brain: potential role of the thiol antioxidant N-acetylcysteine amide." Free Radic Biol Med. 48(10):1388-98. doi: 10.1016/j.freeradbiomed.2010.02.023.

Bisagno, V., and J.L. Cadet. 2019. "Expression of immediate early genes in brain reward circuitries: Differential regulation by psychostimulant and opioid drugs." Neurochem Int. 124:10-18. doi: 10.1016/j.neuint.2018.12.004. Review.

Bowyer, J.F., and S. Ali. 2006. "High doses of methamphetamine that cause disruption of the blood-brain barrier in limbic regions produce extensive neuronal degeneration in mouse hippocampus." Synapse. 60(7):521-32.

Brailoiu, G.C., E. Deliu, L.M. Console-Bram, J. Soboloff, M.E. Abood, E.M. Unterwald, and E. Brailoiu. 2016. "Cocaine inhibits store-operated Ca2+ entry in brain microvascular endothelial cells: critical role for sigma-1 receptors." Biochem J. 473(1):1-5. doi: 10.1042/BJ20150934.

Cadet, J.L., V. Bisagno, and C.M. Milroy. 2014. "Neuropathology of substance use disorders." Acta Neuropathol. 127(1):91-107. doi: 10.1007/s00401-013-1221-7.

Candelario-Jalil, E., Y. Yang, and G.A. Rosenberg. 2009. "Diverse roles of matrix metalloproteinases and tissue inhibitors of metalloproteinases in neuroinflammation and cerebral ischemia." Neuroscience. 158(3):983-94. doi: 10.1016/j.neuroscience.2008.06.025. Review.

Cardoso, F.L., D. Brites, and M.A. Brito. 2010. "Looking at the blood-brain barrier: molecular anatomy and possible investigation approaches." Brain Res Rev. 64(2):328-63. doi: 10.1016/j.brainresrev.2010.05.003. Review.

Cearley, C.N., K. Blindheim, B.A. Sorg, J.M. Krueger, and L. Churchill. 2011. "Acute cocaine increases interleukin-1β mRNA and immunoreactive cells in the cortex and nucleus accumbens." Neurochem Res. 36(4):686-92. doi: 10.1007/s11064-011-0410-9.

Chaves, C., D. Gomez-Zepeda, S. Auvity, M.C. Menet, D. Crete, L. Labat, F. Remiao, S. Cisternino, and X. Decleves. 2016. "Effect of Subchronic Intravenous Morphine infusion and naloxone-precipitated

morphine withdrawal on P-gp and Bcrp at the rat blood-brain barrier." J Pharm Sci 105 (1):350-8. doi: 10.1002/jps.24697.

Chang, Y.C., M.F. Stins, M.J. McCaffery, G.F. Miller, D.R. Pare, T. Dam, M. Paul-Satyaseela, K.S. Kim, and K.J. Kwon-Chung. 2004. "Cryptococcal yeast cells invade the central nervous system via transcellular penetration of the blood-brain barrier". Infect Immun. 72(9):4985–95. doi: 10.1128/IAI.72.9.4985-4995.2004.

Charlier C., K. Nielsen, S. Daou, M. Brigitte, F. Chretien, and F. Dromer. 2009. "Evidence of a role for monocytes in dissemination and brain invasion by Cryptococcus neoformans". Infect Immun. 77(1):120–7. doi: 10.1128/IAI.01065-08.

Chilunda, V., T.M. Calderon, P. Martinez-Aguado, and J.W. Berman. 2019. "The impact of substance abuse on HIV-mediated neuropathogenesis in the current ART era." Brain Res. 1724:146426. doi: 10.1016/j.brainres.2019.146426.

Chung, Y.A., B.S. Peterson, S.J. Yoon, S.N. Cho, S. Chai, J. Jeong, and D.J. Kim. 2010. "In vivo evidence for long-term CNS toxicity, associated with chronic binge use of methamphetamine." Drug Alcohol Depend. 111(1-2):155-60. doi: 10.1016/j.drugalcdep.2010.04.005.

Clark, K.H., C.A. Wiley, and C.W. Bradberry. 2013. "Psychostimulant abuse and neuroinflammation: emerging evidence of their interconnection." Neurotox Res. 23(2):174-88. doi: 10.1007/s12640-012-9334-7. Review.

Coelho-Santos, V., R.A. Leitão, F.L. Cardoso, I. Palmela, M. Rito, M. Barbosa, M.A. Brito, C.A. Fontes-Ribeiro, and A.P. Silva. 2015. "The TNF-α/NF-κB signaling pathway has a key role in methamphetamine-induced blood-brain barrier dysfunction." J Cereb Blood Flow Metab. 35(8):1260-71. doi: 10.1038/jcbfm.2015.59.

Cohen, K., A. Weizman, and A. Weinstein. 2019. "Modulatory effects of cannabinoids on brain neurotransmission." Eur J Neurosci. 50(3):2322-2345. doi: 10.1111/ejn.14407.

Dahal, S., S.V. Chitti, M.P. Nair, and S.K. Saxena. 2015. "Interactive effects of cocaine on HIV infection: implication in HIV-associated neurocognitive disorder and neuroAIDS." Front Microbiol. 6:931. doi: 10.3389/fmicb.2015.00931. Review.

Dash, S., M. Balasubramaniam, F. Villalta, C. Dash, and J. Pandhare. 2015. "Impact of cocaine abuse on HIV pathogenesis." Front Microbiol. 6:1111. doi: 10.3389/fmicb.2015.01111. Review.

Dauchy, S., F. Miller, P.O. Couraud, R.J. Weaver, B. Weksler, I.A. Romero, J.M. Scherrmann, I. De Waziers, and X. Decleves. 2009.

"Expression and transcriptional regulation of ABC transporters and cytochromes P450 in hCMEC/D3 human cerebral microvascular endothelial cells." Biochem Pharmacol 77 (5):897-909. doi: 10.1016/j.bcp.2008.11.001.

Dhillon, N.K., R. Williams, F. Peng, Y.J. Tsai, S. Dhillon, B. Nicolay, M. Gadgil, A. Kumar, and S.J. Buch. 2007. "Cocaine-mediated enhancement of virus replication in macrophages: implications for human immunodeficiency virus-associated dementia." J Neurovirol. 13(6):483-95.

Dhillon, N.K., F. Peng, S. Bokhari, S. Callen, S.H. Shin, X. Zhu, K.J. Kim, and S.J. Buch. 2008. "Cocaine-mediated alteration in tight junction protein expression and modulation of CCL2/CCR2 axis across the blood-brain barrier: implications for HIV-dementia." J Neuroimmune Pharmacol. 3(1):52-6.

Dietrich, J.B. 2009. "Alteration of blood-brain barrier function by methamphetamine and cocaine." Cell Tissue Res. 336(3):385-92. doi: 10.1007/s00441-009-0777-y. Review.

Egleton, R.D., and T. Abbruscato. 2014. "Drug abuse and the neurovascular unit." Adv Pharmacol. 71:451-80. doi: 10.1016/bs.apha.2014.06.019. Review.

ElAli, A., A. Urrutia, A. Rubio-Araiz, M. Hernandez-Jimenez, M.I. Colado, T.R. Doeppner, and D.M. Hermann. 2012. "Apolipoprotein-E controls adenosine triphosphate-binding cassette transporters ABCB1 and ABCC1 on cerebral microvessels after methamphetamine intoxication." Stroke. 43(6):1647-53. doi: 10.1161/STROKEAHA.111.648923.

Eugenin, E.A., J.M. Greco, S. Frases, J.D. Nosanchuk, and L.R. Martinez. 2013. "Methamphetamine alters blood brain barrier protein expression in mice, facilitating central nervous system infection by neurotropic Cryptococcus neoformans." J Infect Dis. 208(4):699-704. doi: 10.1093/infdis/jit117.

Fernandes, S., S. Salta, and T. Summavielle. 2015. "Methamphetamine promotes α-tubulin deacetylation in endothelial cells: the protective role of acetyl-l-carnitine." Toxicol Lett. 234(2):131-8. doi: 10.1016/j.toxlet.2015.02.011.

Fernandes, S., S. Salta, J. Bravo, A.P. Silva, and T. Summavielle. 2016. "Acetyl-L-Carnitine Prevents Methamphetamine-induced structural damage on endothelial cells via ILK-related MMP-9 activity." Mol Neurobiol. 53(1):408-422. doi: 10.1007/s12035-014-8973-5.

Fiala, M., X.H. Gan, L. Zhang, S.D. House, T. Newton, M.C. Graves, P. Shapshak, M. Stins, K.S. Kim, M. Witte, and S.L. Chang. 1998.

"Cocaine enhances monocyte migration across the blood-brain barrier. Cocaine's connection to AIDS dementia and vasculitis?" Adv Exp Med Biol. 437:199-205.

Fiala, M., A.J. Eshleman, J. Cashman, J. Lin, A.S. Lossinsky, V. Suarez, W. Yang, J. Zhang, W. Popik, E. Singer, F. Chiappelli, E. Carro, M. Weinand, M. Witte, and J. Arthos. 2005. "Cocaine increases human immunodeficiency virus type 1 neuroinvasion through remodeling brain microvascular endothelial cells." J Neurovirol. 11(3):281-91.

Gan, X., L. Zhang, O. Berger, M.F. Stins, D. Way, D.D. Taub, S.L. Chang, K.S. Kim, S.D. House, M. Weinand, M. Witte, M.C. Graves, and M. Fiala. 1999. "Cocaine enhances brain endothelial adhesion molecules and leukocyte migration." Clin Immunol. 91(1):68-76.

Gandhi, N., Z.M. Saiyed, J. Napuri, T. Samikkannu, P.V. Reddy, M. Agudelo, P. Khatavkar, S.K. Saxena, and M.P. Nair. 2010. "Interactive role of human immunodeficiency virus type 1 (HIV-1) clade-specific Tat protein and cocaine in blood-brain barrier dysfunction: implications for HIV-1-associated neurocognitive disorder." J Neurovirol. 16(4):294-305. doi: 10.3109/13550284.2010.499891.

Ghosh, C., M. Hossain, J. Solanki, A. Dadas, N. Marchi, and D. Janigro. 2016. "Pathophysiological implications of neurovascular P450 in brain disorders." Drug Discov Today. 21(10):1609-1619. doi: 10.1016/j.drudis.2016.06.004.

Gonçalves, J., S. Baptista, and A.P. Silva. 2014. "Psychostimulants and brain dysfunction: a review of the relevant neurotoxic effects." Neuropharmacology. 87:135-49. doi: 10.1016/j.neuropharm.2014.01.006. Review.

Gonçalves, J., R.A. Leitão, A. Higuera-Matas, M.A. Assis, S.M. Coria, C. Fontes-Ribeiro, E. Ambrosio, and A.P. Silva. 2017. "Extended-access methamphetamine self-administration elicits neuroinflammatory response along with blood-brain barrier breakdown." Brain Behav Immun. 62:306-317. doi: 10.1016/j.bbi.2017.02.017.

Higuera-Matas, A., M. Miguens, N.D. Olmo, C. García-Lecumberri, and E. Ambrosio. 2011. "Neural changes developed during the extinction of cocaine self-administration behavior." Pharmaceuticals (Basel). 4(10):1315-27. doi: 10.3390/ph4101315.

Hind, W.H., T.J. England, and S.E. O'Sullivan. 2016. "Cannabidiol protects an in vitro model of the blood-brain barrier from oxygen-glucose deprivation via PPARgamma and 5-HT1A receptors." Br J Pharmacol 173 (5):815-25. doi: 10.1111/bph.13368.

Inturrisi, C.E., M. Schultz, S. Shin, J.G. Umans, L. Angel, and E.J. Simon. 1983. “Evidence from opiate binding studies that heroin acts through its metabolites.” Life Sci. 33 Suppl 1:773-6.

Iseger, T.A., and M.G. Bossong. 2015. “A systematic review of the antipsychotic properties of cannabidiol in humans.” Schizophr Res. 162(1-3):153-61. doi: 10.1016/j.schres.2015.01.033. Review.

Ivey, N.S., A.G. MacLean, and A.A. Lackner. 2009. “AIDS and the blood-brain barrier”. J Neurovirol. 15(2): 111–122. doi: 10.1080/13550280902769764.

Johnson, B.A. 1990. “Psychopharmacological effects of cannabis.” Br J Hosp Med. 43(2):114-6, 118-20, 122.

Jones, P.M., and A.M. George. 2004. “The ABC transporter structure and mechanism: perspectives on recent research.” Cell Mol Life Sci. 61(6):682-99.

Khaksar, S., and M.R. Bigdeli. 2017a. “Anti-excitotoxic effects of cannabidiol are partly mediated by enhancement of NCX2 and NCX3 expression in animal model of cerebral ischemia.” Eur J Pharmacol 794:270-279. doi: 10.1016/j.ejphar.2016.11.011.

Khaksar, S., and M.R. Bigdeli. 2017b. “Intra-cerebral cannabidiol infusion-induced neuroprotection is partly associated with the TNF-alpha/TNFR1/NF-κB pathway in transient focal cerebral ischaemia.” Brain Inj 31 (13-14):1932-1943. doi: 10.1080/02699052.2017.1358397.

Kim, D.W., A. Zloza, J. Broucek, J.M. Schenkel, C. Ruby, G. Samaha, H.L. Kaufman. 2014. "Interleukin-2 alters distribution of CD144 (VE-cadherin) in endothelial cells." J Transl Med. 12:113. doi: 10.1186/1479-5876-12-113.

Kiyatkin, E.A., and H.S. Sharma. 2016. “Breakdown of blood-brain and blood-spinal cord barriers during acute methamphetamine intoxication: role of brain temperature.” CNS Neurol Disord Drug Targets. 15(9):1129-1138. Review.

Kolodgie, F.D., P.S. Wilson, W.J. Mergner, and R. Virmani. 1999. “Cocaine-induced increase in the permeability function of human vascular endothelial cell monolayers.” Exp Mol Pathol. 66(2):109-22.

Konsman, J.P., B. Drukarch, and A.M. van Dam. 2007. “(Peri)vascular production and action of proinflammatory cytokines in brain pathology.” Annals of the New York Academy of Sciences, 1–25.

Kousik, S.M., S.M. Graves, T.C. Napier, C. Zhao, and P.M. Carvey. 2011. “Methamphetamine-induced vascular changes lead to striatal hypoxia and dopamine reduction.” Neuroreport. 22(17):923-8. doi: 10.1097/WNR.0b013e32834d0bc8.

Kousik, S.M., T.C. Napier, and P.M. Carvey. 2012. "The effects of psychostimulant drugs on blood brain barrier function and neuroinflammation." Front Pharmacol. 29;3:121. doi: 10.3389/fphar.2012.00121.

Kousik, S.M., T.C. Napier, R.D. Ross, D.R. Sumner, and P.M. Carvey. 2014. "Dopamine receptors and the persistent neurovascular dysregulation induced by methamphetamine self-administration in rats." J Pharmacol Exp Ther. 351(2):432-9. doi: 10.1124/jpet.114.217802.

Krebs, M.O., O. Kebir, and T.M. Jay. 2019. "Exposure to cannabinoids can lead to persistent cognitive and psychiatric disorders." Eur J Pain. 23(7):1225-1233. doi: 10.1002/ejp.1377.

Kumar, M. 2011. "Cocaine and the blood-brain-barrier." Blood. 117(8):2303. doi: 10.1182/blood-2010-12-324939.

Langen, U.H., S. Ayloo, and C. Gu. 2019. "Development and cell biology of the blood-brain barrier." Annu Rev Cell Dev Biol. 35:591-613. doi: 10.1146/annurev-cellbio-100617-062608.

Lappin, J.M., and G.E. Sara. 2019. "Psychostimulant use and the brain." Addiction. 2019 Jul 19. doi: 10.1111/add.14708.

Lee, Y.W., B. Hennig, J. Yao, and M. Toborek. 2001. "Methamphetamine induces AP-1 and NF-kappaB binding and transactivation in human brain endothelial cells." J Neurosci Res. 66(4):583-91.

Leibrand, C.R., J.J. Paris, A.M. Jones, Q.N. Masuda, M.S. Halquist, W.K. Kim, P.E. Knapp, A.D.M. Kashuba, K.F. Hauser, and M. McRae. 2019. "HIV-1 Tat and opioids act independently to limit antiretroviral brain concentrations and reduce blood-brain barrier integrity." J Neurovirol 25 (4):560-577. doi: 10.1007/s13365-019-00757-8.

Leitão, R.A., V. Coelho-Santos, A.P. Silva. 2016. "Methamphetamine and the blood-brain barrier." In Victor R Preedy (ed) Neuropathology of Drug Addictions and Substance Misuse Volume 2: Part I Stimulants, 1st edn Academic Press-Elsevier, London, pp 155-168. Review.

Leitão, R.A., J. Sereno, J.M. Castelhano, S.I. Gonçalves, V. Coelho-Santos, C. Fontes-Ribeiro, M. Castelo-Branco, and A.P. Silva. 2018. "Aquaporin-4 as a new target against methamphetamine-induced brain alterations: focus on the neurogliovascular unit and motivational behavior." Mol Neurobiol. 55(3):2056-2069. doi: 10.1007/s12035-017-0439-0.

Liao, W., J.X. Lin, and W.J. Leonard. 2011. "IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation." Curr Opin Immunol. 23(5):598-604. doi: 10.1016/j.coi.2011.08.003.

Liebner, S., R.M. Dijkhuizen, Y. Reiss, K.H. Plate, D. Agalliu, and G. Constantin. 2018. "Functional morphology of the blood-brain barrier in health and disease." Acta Neuropathol. 135(3):311-336. doi: 10.1007/s00401-018-1815-1.

Linker, K.E., S.J. Cross, and F.M. Leslie. 2019. "Glial mechanisms underlying substance use disorders." Eur J Neurosci. 50(3):2574-2589. doi: 10.1111/ejn.14163. Review.

Liu, H.C., J.K. Anday, S.D. House, and S.L. Chang. 2004. "Dual effects of morphine on permeability and apoptosis of vascular endothelial cells: morphine potentiates lipopolysaccharide-induced permeability and apoptosis of vascular endothelial cells." J Neuroimmunol 146 (1-2):13-21. doi: 10.1016/j.jneuroim.2003.09.016.

Lynch, J.L., and W.A. Banks. 2008. "Opiate modulation of IL-1alpha, IL-2, and TNF-alpha transport across the blood-brain barrier." Brain Behav Immun 22 (7):1096-102. doi: 10.1016/j.bbi.2008.04.004.

Mahajan, S.D., R. Aalinkeel, D.E. Sykes, J.L. Reynolds, B. Bindukumar, A. Adal, M. Qi, J. Toh, G. Xu, P.N. Prasad, and S.A. Schwartz. 2008a. "Methamphetamine alters blood brain barrier permeability via the modulation of tight junction expression: Implication for HIV-1 neuropathogenesis in the context of drug abuse." Brain Res. 1203:133-48. doi: 10.1016/j.brainres.2008.01.093.

Mahajan, S.D., R. Aalinkeel, D.E. Sykes, J.L. Reynolds, B. Bindukumar, S.F. Fernandez, R. Chawda, T.C. Shanahan, and S.A. Schwartz. 2008b. "Tight junction regulation by morphine and HIV-1 tat modulates blood-brain barrier permeability." J Clin Immunol 28 (5):528-41. doi: 10.1007/s10875-008-9208-1.

Marie, N., C. Canestrelli, and F. Noble. 2019. "Role of pharmacokinetic and pharmacodynamic parameters in neuroadaptations induced by drugs of abuse, with a focus on opioids and psychostimulants." Neurosci Biobehav Rev. 106:217-226. doi: 10.1016/j.neubiorev.2018.06.006.

Martins, T., S. Baptista, J. Gonçalves, E. Leal, N. Milhazes, F. Borges, C.F. Ribeiro, O. Quintela, E. Lendoiro, M. López-Rivadulla, A.F. Ambrósio, and A.P. Silva. 2011. "Methamphetamine transiently increases the blood-brain barrier permeability in the hippocampus: role of tight junction proteins and matrix metalloproteinase-9." Brain Res. 1411:28-40. doi: 10.1016/j.brainres.2011.07.013.

Martins, T., T. Burgoyne, B.A. Kenny, N. Hudson, C.E. Futter, A.F. Ambrósio, A.P. Silva, J. Greenwood, and P. Turowski. 2013. "Methamphetamine-induced nitric oxide promotes vesicular transport

in blood-brain barrier endothelial cells." Neuropharmacology. 65:74-82. doi: 10.1016/j.neuropharm.2012.08.021.

McArthur, J.C., J. Steiner, N. Sacktor, and A. Nath. 2010. "Human immunodeficiency virus-associated neurocognitive disorders: Mind the gap." Ann Neurol. 67(6):699-714. doi: 10.1002/ana.22053.

Mecha, M., A. Feliu, P.M. Inigo, L. Mestre, F.J. Carrillo-Salinas, and C. Guaza. 2013. "Cannabidiol provides long-lasting protection against the deleterious effects of inflammation in a viral model of multiple sclerosis: a role for A2A receptors." Neurobiol Dis 59:141-50. doi: 10.1016/j.nbd.2013.06.016.

Morais, A.P.D., I.R. Pita, C.A. Fontes-Ribeiro, and F.C. Pereira. 2018. "The neurobiological mechanisms of physical exercise in methamphetamine addiction." CNS Neurosci Ther. 24(2):85-97. doi: 10.1111/cns.12788. Epub 2017 Dec 20. Review.

Moretti, M., G. Belli, L. Morini, M.C. Monti, A.M.M. Osculati, and S.D. Visonà. 2019. "Drug abuse-related neuroinflammation in human postmortem brains: an immunohistochemical approach." J Neuropathol Exp Neurol. pii: nlz084. doi: 10.1093/jnen/nlz084.

Nebert, D.W., and F.J. Gonzalez. 1987. "P450 genes: structure, evolution, and regulation." Annu Rev Biochem. 56:945-93.

Niu, F., K. Liao, G. Hu, S. Sil, S. Callen, M.L. Guo, L. Yang, and S. Buch. 2019. "Cocaine-induced release of CXCL10 from pericytes regulates monocyte transmigration into the CNS." J Cell Biol. 218(2):700-721. doi: 10.1083/jcb.201712011.

Orio, L., E. O'Shea, V. Sanchez, J.M. Pradillo, I. Escobedo, J. Camarero, M.A. Moro, A.R. Green, and M.I. Colado. 2004. "3,4-Methylenedioxymethamphetamine increases interleukin-1beta levels and activates microglia in rat brain: studies on the relationship with acute hyperthermia and 5-HT depletion." J Neurochem. 89(6):1445-53.3

Osborne, G.B., and C. Fogel. 2008. "Understanding the motivations for recreational marijuana use among adult Canadians." Subst Use Misuse. 43(3-4):539-87. doi: 10.1080/10826080701884911. Review.

O'Shea, E., A. Urrutia, A.R. Green, and M.I. Colado. 2014. "Current preclinical studies on neuroinflammation and changes in blood-brain barrier integrity by MDMA and methamphetamine." Neuropharmacology. 87:125-34.
doi: 10.1016/j.neuropharm.2014.02.015. Review.

Pacher, P., S. Bátkai, and G. Kunos. 2006. "The endocannabinoid system as an emerging target of pharmacotherapy." Pharmacol Rev. 58(3):389-462. doi: 10.1124/pr.58.3.2. Review.

Pacher, P., and R. Mechoulam. 2011. “Is lipid signaling through cannabinoid 2 receptors part of a protective system?” Prog Lipid Res. 50(2):193-211. doi: 10.1016/j.plipres.2011.01.001.

Park, B.J., K.A. Wannemuehler, B.J. Marston, N. Govender, P.G. Pappas, and T.M. Chiller. 2009. “Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS”. AIDS. 23:525–30. doi: 10.1093/infdis/jit117.

Park, M., B. Hennig, and M. Toborek. 2012. “Methamphetamine alters occludin expression via NADPH oxidase-induced oxidative insult and intact caveolae.” J Cell Mol Med. 16(2):362-75. doi: 10.1111/j.1582-4934.2011.01320.x.

Park, M., H.J. Kim, B. Lim, A. Wylegala, and M. Toborek. 2013. “Methamphetamine-induced occludin endocytosis is mediated by the Arp2/3 complex-regulated actin rearrangement.” J Biol Chem. 288(46):33324-34. doi: 10.1074/jbc.M113.483487.

Pérez-Hernández, M., M.E. Fernández-Valle, A. Rubio-Araiz, R. Vidal, M.D. Gutiérrez-López, E. O'Shea, and M.I. Colado. 2017. “3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) produces edema due to BBB disruption induced by MMP-9 activation in rat hippocampus.” Neuropharmacology. 118:157-166. doi: 10.1016/j.neuropharm.2017.03.019.

Petitto, J.M., R.K. McNamara, P.L. Gendreau, Z. Huang, and A.J. Jackson. 1999. "Impaired learning and memory and altered hippocampal neurodevelopment resulting from interleukin-2 gene deletion." Neuroscience Research 56, 441–446.

Pierce, R.C., B. Fant, S.E. Swinford-Jackson, E.A. Heller, W.H. Berrettini, and M.E. Wimmer. 2018. “Environmental, genetic and epigenetic contributions to cocaine addiction.” Neuropsychopharmacology 43(7):1471-1480. doi: 10.1038/s41386-018-0008-x.

Quinton, M.S., and B.K. Yamamoto. 2006. “Causes and consequences of methamphetamine and MDMA toxicity.” AAPS J. 8(2):E337-47.

Raborn, E.S., M. Jamerson, F. Marciano-Cabral, and G.A. Cabral. 2014. “Cannabinoid inhibits HIV-1 Tat-stimulated adhesion of human monocyte-like cells to extracellular matrix proteins.” Life Sci 104 (1-2):15-23. doi: 10.1016/j.lfs.2014.04.008.

Rahman, S. 2008. “Drug addiction and brain targets: from preclinical research to pharmacotherapy.” CNS Neurol Disord Drug Targets. 7(5):391-2.

Ramirez, S.H., R. Potula, S. Fan, T. Eidem, A. Papugani, N. Reichenbach, H. Dykstra, B.B. Weksler, I.A. Romero, P.O. Couraud, and Y. Persidsky. 2009. “Methamphetamine disrupts blood-brain barrier

function by induction of oxidative stress in brain endothelial cells." J Cereb Blood Flow Metab. 29(12):1933-45. doi: 10.1038/jcbfm.2009.112.

Reich, A., and J.C. Szepietowski. 2007. "Mediators of pruritus in psoriasis." Mediators Inflamm. 2007:64727. doi: 10.1155/2007/64727.

Rosas-Hernandez, H., E. Cuevas, S.M. Lantz, K.C. Rice, B.M. Gannon, W.E. Fantegrossi, C. Gonzalez, M.G. Paule, and S.F. Ali. 2016. "Methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxypyrovalerone (MDPV) induce differential cytotoxic effects in bovine brain microvessel endothelial cells." Neurosci Lett. 629:125-130. doi: 10.1016/j.neulet.2016.06.029.

Rosenberg, G.A. 2009. "Matrix metalloproteinases and their multiple roles in neurodegenerative diseases." Lancet Neurol. 8(2):205-16. doi: 10.1016/S1474-4422(09)70016-X. Review.

Ruiz-Valdepenas, L., J.A. Martinez-Orgado, C. Benito, A. Millan, R.M. Tolon, and J. Romero. 2011. "Cannabidiol reduces lipopolysaccharide-induced vascular changes and inflammation in the mouse brain: an intravital microscopy study." J Neuroinflammation 8 (1):5. doi: 10.1186/1742-2094-8-5.

Sajja, R.K., S. Rahman, and L. Cucullo. 2016. "Drugs of abuse and blood-brain barrier endothelial dysfunction: A focus on the role of oxidative stress." J Cereb Blood Flow Metab. 36(3):539-54. doi: 10.1177/0271678X15616978. Review.

Sanmarti, M., L. Ibáñez, S. Huertas, D. Badenes, D. Dalmau, M. Slevin, J. Krupinski, A. Popa-Wagner, and A. Jaen. 2014. "HIV-associated neurocognitive disorders." J Mol Psychiatry. 2(1):2. doi: 10.1186/2049-9256-2-2.

Seleman, M., H. Chapy, S. Cisternino, C. Courtin, M. Smirnova, J. Schlatter, F. Chiadmi, J.M. Scherrmann, F. Noble, and C. Marie-Claire. 2014. "Impact of P-glycoprotein at the blood-brain barrier on the uptake of heroin and its main metabolites: behavioral effects and consequences on the transcriptional responses and reinforcing properties." Psychopharmacology (Berl) 231 (16):3139-49. doi: 10.1007/s00213-014-3490-9.

Sharma, H.S., and S.F. Ali. 2006. "Alterations in blood-brain barrier function by morphine and methamphetamine." Ann N Y Acad Sci. 1074:198-224. doi: 10.1196/annals.1369.020

Sharma, H.S., and S.F. Ali. 2008. "Acute administration of 3,4-methylenedioxymethamphetamine induces profound hyperthermia, blood-brain barrier disruption, brain edema formation, and cell injury." Ann N Y Acad Sci. 1139:242-58. doi: 10.1196/annals.1432.052.

Sharma, H.S., D. Muresanu, A. Sharma, and R. Patnaik. 2009. “Cocaine-induced breakdown of the blood-brain barrier and neurotoxicity.” Int Rev Neurobiol. 88:297-334. doi: 10.1016/S0074-7742(09)88011-2.

Sharma, H.S., P.O. Sjoquist, and S.F. Ali. 2010. “Alterations in blood-brain barrier function and brain pathology by morphine in the rat. Neuroprotective effects of antioxidant H-290/51.” Acta Neurochir Suppl 106:61-6. doi: 10.1007/978-3-211-98811-4_10.

Silva, A.P., T. Martins, S. Baptista, J. Gonçalves, F. Agasse, and J.O. Malva. 2010. “Brain injury associated with widely abused amphetamines: neuroinflammation, neurogenesis and blood-brain barrier.” Curr Drug Abuse Rev. 3(4):239-54. Review.

Strazza, M., V. Pirrone, B. Wigdahl, W. Dampier, W. Lin, R. Feng, M.E. Maubert, B. Weksler, I.A. Romero, P.O. Couraud, and M.R. Nonnemacher. 2016. “Prolonged morphine exposure induces increased firm adhesion in an in vitro model of the blood-brain barrier.” Int J Mol Sci 17 (6). doi: 10.3390/ijms17060916.

Toborek, M., M.J. Seelbach, C.S. Rashid, I.E. András, L. Chen, M. Park, and K.A. Esser. 2013. “Voluntary exercise protects against methamphetamine-induced oxidative stress in brain microvasculature and disruption of the blood-brain barrier.” Mol Neurodegener. 8:22. doi: 10.1186/1750-1326-8-22.

Torres, E., M.D. Gutierrez-Lopez, A. Mayado, A. Rubio, E. O'Shea, and M.I. Colado. 2011. “Changes in interleukin-1 signal modulators induced by 3,4-methylenedioxymethamphetamine (MDMA): regulation by CB2 receptors and implications for neurotoxicity.” J Neuroinflammation. 8:53. doi: 10.1186/1742-2094-8-53.

Trancredi, V., C. Zona, F. Velotti, F. Eusebi, and A. Santoni. 1990. "Interleukin-2 suppresses established long-term potentiation and inhibits its induction in the rat hippocampus." Brain Research 525, 149–161.

Urrutia, A., A. Rubio-Araiz, M.D. Gutierrez-Lopez, A. ElAli, D.M. Hermann, E. O'Shea, and M.I. Colado. 2013. “A study on the effect of JNK inhibitor, SP600125, on the disruption of blood-brain barrier induced by methamphetamine.” Neurobiol Dis. 50:49-58. doi: 10.1016/j.nbd.2012.10.006.

Wen, H., Y. Lu, H. Yao, and S. Buch. 2011. “Morphine induces expression of platelet-derived growth factor in human brain microvascular endothelial cells: implication for vascular permeability.” PLoS One 6 (6):e21707. doi: 10.1371/journal.pone.0021707.

World Drug report 2019 - United Nations Office on Drugs and Crime. https://wdr.unodc.org/wdr2019/index.html.

Wylezinski, L.S., and J. Hawiger J. 2016. "Interleukin 2 activates brain microvascular endothelial cells resulting in destabilization of adherens junctions." J Biol Chem. 28;291(44):22913-22923.

Yamamoto, B.K., and M.G. Bankson. 2005. “Amphetamine neurotoxicity: cause and consequence of oxidative stress.” Crit Rev Neurobiol. 17(2):87-117. Review.

Yamamoto, B.K., and J. Raudensky. 2008. “The role of oxidative stress, metabolic compromise, and inflammation in neuronal injury produced by amphetamine-related drugs of abuse.” J Neuroimmune Pharmacol. 3(4):203-17. doi: 10.1007/s11481-008-9121-7. Review.

Yao, H., Y. Yang, K.J. Kim, C. Bethel-Brown, N. Gong, K. Funa, H.E. Gendelman, T.P. Su, J.Q. Wang, and S. Buch. 2010. “Molecular mechanisms involving sigma receptor-mediated induction of MCP-1: implication for increased monocyte transmigration.” Blood. 115(23):4951-62. doi: 10.1182/blood-2010-01-266221.

Yao, H., M. Duan, and S. Buch. 2011a “Cocaine-mediated induction of platelet-derived growth factor: implication for increased vascular permeability.” Blood. 117(8):2538-47. doi: 10.1182/blood-2010-10-313593.

Yao, H., M. Duan, G. Hu, and S. Buch. 2011b. “Platelet-derived growth factor B chain is a novel target gene of cocaine-mediated Notch1 signaling: implications for HIV-associated neurological disorders.” J Neurosci. 31(35):12449-54. doi: 10.1523/JNEUROSCI.2330-11.2011.

Yao, H., K. Kim, M. Duan, T. Hayashi, M. Guo, S. Morgello, A. Prat, J. Wang, T.P. Su, and S. Buch. 2011c. “Cocaine hijacks σ1 receptor to initiate induction of activated leukocyte cell adhesion molecule: implication for increased monocyte adhesion and migration in the CNS.” J Neurosci. 31(16):5942-55. doi: 10.1523/JNEUROSCI.5618-10.2011.

Yousif, S., B. Saubamea, S. Cisternino, C. Marie-Claire, S. Dauchy, J.M. Scherrmann, and X. Decleves. 2008. “Effect of chronic exposure to morphine on the rat blood-brain barrier: focus on the P-glycoprotein.” J Neurochem 107 (3):647-57. doi: 10.1111/j.1471-4159.2008.05647.x.

Yousif, S., C. Chaves, S. Potin, I. Margaill, J.M. Scherrmann, and X. Decleves. 2012. “Induction of P-glycoprotein and Bcrp at the rat blood-brain barrier following a subchronic morphine treatment is mediated through NMDA/COX-2 activation.” J Neurochem 123 (4):491-503. doi: 10.1111/j.1471-4159.2012.07890.x.

Zhang, L., D. Looney, D. Taub D, S.L. Chang, D. Way, M.H. Witte, M.C. Graves, and M. Fiala. 1998. “Cocaine opens the blood-brain barrier to HIV-1 invasion.” J Neurovirol. 4(6):619-26.

doi: 10.3109/13550289809114228.
Zhang, R., and N.D. Volkow. 2019. "Brain default-mode network dysfunction in addiction." Neuroimage. 200:313-331. doi: 10.1016/j.neuroimage.2019.06.036. Review.
Zlokovic, B.V. 2008. "The blood-brain barrier in health and chronic neurodegenerative disorders." Neuron. 57(2):178-201. doi: 10.1016/j.neuron.2008.01.003. Review.

CHAPTER EIGHT

CURRENT USE OF IN VITRO MODELS OF THE BLOOD-BRAIN BARRIER FOR THE STUDY OF ALZHEIMER'S DISEASE: FOCUS ON THE EFFECT OF Aβ PEPTIDES ON BBB CELLS

PIETRA CANDELA AND LAURENCE FENART

Introduction

The blood-brain barrier (BBB) is a physiological barrier that controls the passage of electrolytes, xenobiotics and circulating immune cells between the systemic circulation and the central nervous system (CNS) to maintain an optimal environment for neuronal function (Alvarez, Cayrol, & Prat, 2011) (Alvarez et al., 2011a). It is universally accepted that this barrier is histologically formed by endothelial cells (EC) lining the brain microvessels (Ballabh, Braun, & Nedergaard, 2004; Cardoso, Brites, & Brito, 2010). EC are in dynamic contact with astrocytes, pericytes, and neurons and together form the neurovascular unit (NVU). This specific brain environment provides the BBB's properties and is essential for maintaining brain homeostasis (Armulik et al., 2010; Bell et al., 2010). Compared with other endothelia, the morphological and functional properties of EC are different as they lack fenestrations to limit the movement of molecules (Fenstermacher et al., 1988), have a low rate of vesicular transport to prevent transport of large hydrophilic molecules to the CNS (Sedlakova, Shivers, & Del Maestro, 1999), and have an elevated mitochondrial content (Oldendorf, Cornford, & Brown, 1977). Moreover, EC are tightly sealed through junctional complexes including tight junctions (TJ) (e.g. scaffolding proteins such as zonula occludens protein (ZO)-1, ZO-2 and ZO-3) and transmembrane proteins (e.g. claudins and occludin) to limit the flux between adjacent endothelial cells (Hawkins &

Davis, 2005) and protect the CNS from exposure to potential toxicants. The junctional complex contributes to high transendothelial electrical resistance (TEER) and low paracellular permeability of the BBB by limiting the paracellular movement of endogenous and exogenous compounds (Bazzoni & Dejana, 2004). Due to their limited permeability, EC express specific transporters and receptors at the apical (blood side) and basolateral (brain side) membranes to ensure efficient nutrient supply and brain waste elimination. Of major importance are the glucose transporter (GLUT1), monocarboxylate transporters (MCT) and the low-density lipoprotein receptor (LDL-R), which participate in the transport of glucose, ketone bodies, and lipoproteins respectively (Abbott, Patabendige, Dolman, Yusof, & Begley, 2010; Sweeney, Ayyadurai, & Zlokovic, 2016). Furthermore, EC express several members of the ATP-binding cassette (ABC) family, such as ABCB1 (or P-glycoprotein), ABCA1, ABCC1 and the ABCG2 (or BCRP). These proteins are known to pump substrates, including several toxic substances, back into the blood thereby using ATP as an energy source. All these receptors and transporters have been identified as key elements of the BBB and contribute to the polarity of EC. An extensive list of BBB transport systems has been recently reviewed elsewhere (Gosselet et al., 2009; Sweeney et al., 2016; Sweeney, Kisler, Montagne, Toga, & Zlokovic, 2018).

Because of the complexity of the in vivo BBB, a number of in vitro BBB models have been developed over the past 40 years, including monolayer models, co-culture models, dynamic models and microfluid models. These models are based on primary or immortalized endothelial cells isolated from different species, in particular mice, rats, bovines, pigs, and humans. All these BBB models have different properties. No perfect in vitro BBB model exists, but each model has specific advantages as well as disadvantages. The choice of the most suitable BBB model is essential to develop effective treatments and to explore preventive and therapeutic strategies. The selection of the right model will be highly dependent on the goal of the research (Helms et al., 2016). This is particularly important when investigating BBB alterations in pathological and neurological diseases such as Alzheimer's disease (AD), where many specific BBB properties are altered (Marques, Sousa, Sousa, & Palha, 2013; Montagne, Zhao, & Zlokovic, 2017).

This chapter summarizes some of the experimental in vitro BBB models currently used to decipher the cellular and molecular mechanisms involved in AD pathogenesis, with particular attention on the effects of β-amyloid peptides (Aβ) on the microvascular cells and their transport

across the BBB. The benefits and limitations of each model will be also discussed.

Alzheimer Disease: pathological features and molecular etiology

Affecting more than 47 million people worldwide, AD is now considered to be the most common cause of dementia in the elderly population (World Health Organization (WHO) with an increase of 7.7 million new cases every year. The disease mainly concerns individuals over 65 years old and constitutes an important threat to national and global public health in terms of prevalence and economic burden (Gillis, Mirzaei, Potashman, Ikram, & Maserejian, 2019). Despite decades of research, no curative therapies are currently available for this disease. The latter may partly be due to the lack of early and accurate diagnostic tools that enable AD diagnosis before pathological features emerge. These features include neurofibrillary tangles (reviewed in (Castellani & Perry, 2019) and an accumulation of Aβ peptides within senile plaques and around cerebral microvessels resulting in cerebral amyloid angiopathy (CAA) (Selkoe, 2000; Thal et al., 2002).

Aβ peptide is a ~4 kDa protein composed of 36 to 48 amino acids. It is normally produced in different subcellular compartments and exists in different biophysical states, of which some are potentially toxic for neurons under certain circumstances (Brouillette et al., 2012; Haass & Selkoe, 2007). Neurons are the main source of Aβ peptide, but other cell types such as liver cells and endothelial cells are also involved in the biosynthesis of Aβ (Devraj et al., 2016; Schweinzer et al., 2011). In the brain, different Aβ species can be detected, and the most abundant are those ending at position 40 (Aβ1-40 ~80-90%) and 42 (Aβ1-42, ~5-10%) (Glenner & Wong, 2012; Masters et al., 1985).

As is known, Aβ peptides are normally cleared in healthy brains through proteinase-mediated degradation processes, including by neprilysin and insulin-degrading enzymes (Miners et al., 2008), which are expressed by neurons or glial cells. Instead, in elderly patients as well as in AD pathology, it has been clearly demonstrated that the production of Aβ peptides is not altered compared to non-AD patients as stated in the nineties by the “amyloid cascade hypothesis” (Selkoe, 2000). Indeed, this theory explains familial AD disease, which represents between 1% to 5% of all cases but does not explain the sporadic forms of AD.

In the latter case, if no overproduction of Aβ peptides exists, the question is what process then leads to the formation of senile plaques,

neurofibrillary tangles, inflammation, oxidative stress and ultimately dementia in AD patients?

To solve this enigma, another theory called "the amyloid hypothesis" has been put forward suggesting that altered clearance is probably the main cause of the disease in sporadic AD (Mawuenyega et al., 2010; Ries & Sastre, 2016; Tarasoff-Conway et al., 2015; Zuroff, Daley, Black, & Koronyo-Hamaoui, 2017). The plausibility of the hypothesis is evidenced by numerous experimental and clinical trials aiming to reduce the production or promote the clearance of Aβ (e.g. secretase inhibitors, secretase modulators, anti-Aβ immunotherapy). However, this theory also bears limitations considering the neurotoxicity of only parenchymal Aβ and ignores vascular Aβ which is frequently detected in AD brains and CAA (Grinberg & Thal, 2010). Considering this new element, a new theory called the "vascular theory" has emerged in the early 2000s. This new theory suggests the potential contribution of Aβ to dementia through vascular mechanisms and implies the thus-far underestimated role of the BBB in AD pathogenesis, which is now becoming one of the most studied topics in AD research.

Indeed, it is now accepted that some specific sets of receptors and transporters that are expressed at BBB level are involved in bidirectional Aβ exchanges between the blood and brain compartments (Zlokovic 2008) and their dysfunction induces failure of Aβ transport across the BBB (Sweeney, Zhao, Montagne, Nelson, & Zlokovic, 2019). In particular, the altered expression of several members of the ABC transporter family, including P-glycoprotein and BCRP (Cirrito et al., 2005), decreased levels of LRP-1 (low-density lipoprotein receptor-related protein 1) and increased levels of RAGE (receptor for advanced glycation end-products) at the BBB (Donahue et al., 2006) can cause Aβ transport to fail, leading to neuroinflammation, oxidative stress and finally BBB dysfunction. This feedback loop gives rise to cognitive impairment and the onset of dementia.

This information has partly been obtained using in vitro models of the BBB. Indeed, these models have made it possible not only to complement in vivo studies but have proved extremely useful for understanding endothelial cell functionality and gaining insight into AD mechanisms.

Isolated brain capillaries: an interesting first global approach to study Aβ effect on the BBB

Brain capillaries or microvessels were often used as an in vitro BBB model. Brain capillaries can be isolated from animal brains as well as from

human brain tissues using different protocols (Pardridge, 1998). Typically, brain capillaries are composed of EC, pericytes embedded within the basement membrane and astrocytic end-feet processes (Allt & Lawrenson, 2001; Armulik et al., 2010; Cecchelli et al., 2007). Despite the fact that isolated capillaries lose their metabolic activity during the isolation procedure (Lasbennes et al., 1984), the major advantage is that it is possible to isolate BBB specific messenger ribonucleic acid (mRNA) and proteins (Li, Boado, & Pardridge, 2001). The latter is particularly interesting when using capillaries isolated from animal and human brains, both suffering from AD, to elucidate the role of the BBB in pathophysiology. Indeed, several researchers have already reported that brain capillaries isolated from animal models for AD or from AD patients, express receptors and transporters implicated in Aβ peptide transport (Ito, Ohtsuki, & Terasaki, 2006; Moestrup & Hokland, 1992; Ohtsuki et al., 2013; Shibata et al., 2000), rapidly generating information about the pathophysiology of the BBB.

However, due to the anatomical complexity of microvessels, receptor expression patterns in BBB cells (EC, pericytes, astrocytes) remain difficult to explore (Candela et al., 2015). For this reason, the use of BBB cell models is required.

In vitro BBB approaches to study Aβ effect on the BBB

Several in vitro (or ex vivo) BBB models have been used to decipher the role of the BBB in Aβ metabolism and to evaluate potential therapies in AD. Commonly, these BBB models are based on endothelial cells isolated and cultured from brain capillaries from different species (e.g. humans, rats, pigs, mice and bovines), and also non-endothelial cells or immortalized brain endothelial cells. Endothelial cells are seeded on a permeable insert, coated with different components (e.g. collagen, Matrigel and fibronectin), to create a two-compartment system with a luminal (i.e. blood compartment) and abluminal (i.e. brain compartment) side. In such a set-up, other cell types such as astrocytes or pericytes are seeded at the bottom of the culture well providing a range of co- and triple cultures using various configurations.

In these BBB models, different isoforms of Aβ peptides (i.e. synthetic, radiolabeled or fluorescent), alone or coupled with several chaperone proteins (i.e. ApoE, Apo A-1, α-2 macroglobulin), are added in the luminal or abluminal compartment using a variety of different protocols. In general, the most commonly used forms are $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$ peptides in monomeric, oligomeric or fibrillary states. Other isoforms include $A\beta_{25\text{-}}$

$_{35}$ due to its particular aggregation properties (Millucci, Ghezzi, Bernardini, & Santucci, 2010). It is important to note that interaction with chaperone proteins may modulate the conformation of Aβ (Wisniewski, Castaño, Golabek, Vogel, & Frangione, 1994) and that in these conditions the clearance of Aβ across the BBB can be changed (Cirrito et al., 2005; Deane, Sagare, & Zlokovic, 2008; Sagare et al., 2007). In general, physiological concentrations of Aβ in the picomolar to nanomolar range are most widely used to study the mechanisms involved in Aβ transport across the BBB (Candela et al., 2010; Saint-Pol et al., 2012). Instead, a micromolar range of Aβ is usually used to mimic a pathological condition and study endothelial viability and BBB integrity (Marco & Skaper, 2006). However, it should be noted that differences in sample preparation protocols or experimental conditions (especially temperature and salt concentration) can lead to an increase in the number of aggregates, in particular for the $A\beta_{1\text{-}42}$ peptide (Novo, Freire, & Al-Soufi, 2018). In these experimental models, inulin is often used as a paracellular marker, since its molecular weight (4.5 kDa) is very similar to that of Aβ peptides, and because of the absence of a specific membrane receptor on ECs (Candela et al., 2010; Saint-Pol et al., 2012).

In these experiments and these conditions, several factors can be investigated, including (i) integrity of the BBB, measured by the TEER or by diffusion of paracellular markers (e.g. Lucifer yellow (LY) or radiolabeled sucrose) across the insert; (ii) transport of Aβ peptides across the BBB (apical-to-basolateral and vice versa) to explore molecular and cellular mechanisms leading to AD; (iii) inflammatory responses to study the role of BBB cells in an environment that simulates AD pathology; (iv) isolation and identification of BBB-specific mRNA and proteins; and finally (v) rapid screening of potential therapeutic agents.

For these experiments, the most commonly used detection method for Aβ peptides is the enzyme-linked immunosorbent assay (ELISA) technique. This analytical method provides accurate results to specifically detect physiological concentrations of Aβ. However, the method is relatively expensive compared to other analytical techniques such as electrophoresis (Picou, Moses, Wellman, Kheterpal, & Gilman, 2010; Pryor, Moss, & Hestekin, 2014), radioactivity or even spectrophotometry when fluorescent Aβ peptides are used in the experiments (Candela et al., 2015).

In any case, many discrepancies can be detected in the calculated values. A possible reason for these discrepancies may be the strong adsorption of Aβ peptides to interfaces that can lead to great differences between the nominal and real solution concentrations of Aβ (Novo et al.,

2018). Therefore, evaluation of potential adsorption or accumulation is very important and the mass balance (the percentage of compound recovered at the end of the experiment) should be in the analysis.

Nevertheless, these BBB models represent a useful tool to study Aβ metabolism and Aβ transport thereby providing important indications about disease mechanisms and potential pharmacological targets. However, no "gold standard" in vitro BBB model exists (Aday, Cecchelli, Hallier-Vanuxeem, Dehouck, & Ferreira, 2016; Reichel, Begley, & Abbott, 2003), especially to study neurological disorders such as AD. Each BBB model has specific limitations, however, regardless of the complexity of the model, all models are valuable when it is clear what exact purpose they can be used for.

Cell lines and immortalized brain endothelial cells

A significant number of authors have used different cell lines of epithelial cells (e.g. Madin–Darby canine kidney (MDCK) and the epithelial-like kidney cell strain LLC clone PK1 (LLC-PK1)) to study Aβ transport. The latter is due to the relative ease with which these cells grow as polarized monolayer cultures and because of their low permeability for paracellular markers (e.g. LY or sucrose) (Gumbleton & Audus, 2001). Nevertheless, there are important differences between epithelial and endothelial cells in terms of junction structures and transporter expression (Mária A. Deli, 2009). Moreover, it has been shown that some cellular receptors, including LRP1, may have different functions. For this, it has been shown that in endothelial cells LRP1 mediates Aβ efflux from blood to the brain (Shibata et al., 2000), instead, in MDCK it is LRP1 that is implicated in Aβ endocytosis and degradation rather than transcytosis (Nazer, Hong, & Selkoe, 2008). As a result of these differences, these models cannot completely be considered as fully BBB models and are therefore not particularly suitable for the study of Aβ peptide transport. Indeed, any in vitro BBB model should be based on the culture of brain endothelial cells, especially for the study of neurodegenerative diseases.

The ideal in vitro BBB model to investigate the role of endothelial cells in AD would be one based on the culture of human brain endothelial cells. The limited availability of human material for the isolation of primary human brain endothelial cells led researchers to use immortalized cell lines. The most widely used human cell line to investigate the role of endothelial cells in AD is the human cell line hCMEC/D3, which has been shown to expose important in vivo BBB characteristics, such as the expression of junctional proteins and efflux transporters (Förster et al.,

2008). Indeed, the hCMEC/D3 cells are shown to bear receptors for Aβ peptide transport including LRP1, RAGE (András et al., 2010; Andras et al., 2008; Tai et al., 2009). However, they do not form tight monolayers as shown by low TEER values (Biemans, Jäkel, de Waal, Kuiperij, & Verbeek, 2017), which is mainly due to a reduced expression of tight junction proteins claudin-5 and ZO-1 (Biemans et al., 2017) and are not polarized. Therefore, the use of this cell line is rather limited for the evaluation of Aβ permeability and Aβ transport. Instead, this cell line remains a useful tool for mechanistic studies and Aβ uptake.

Co-culture BBB model

Several of the EC characteristics that were mentioned above are demonstrated not to be intrinsic to brain EC, however, they result from the regulation of cellular and non-cellular factors produced by different cell types of the NVU (Daneman et al., 2010; Obermeier, Daneman, & Ransohoff, 2013). For example, by releasing soluble mediators, such as sonic hedgehog, astrocytes induce BBB properties in brain EC (Alvarez et al., 2011). However, pericytes have also been shown to play a key role in the regulation of endothelial proliferation and inflammatory processes (Dore-Duffy, 2008). Similar to pericytes, microglia influence BBB permeability and contribute to angiogenetic processes (da Fonseca et al., 2014). For this reason, including these cell types in in vitro BBB models is very important to increase our understanding of cell-cell interaction at the NVU and to delineate BBB function in physiological and (or) pathological conditions such as AD. Although numerous "complex" BBB models exist, the in vitro BBB models that are known to best reproduce in vivo BBB features are based on the co-culture of EC and BBB-inducing cells such as glial cells, astrocytes, and brain pericytes (Máiria A Deli, Abrahám, Kataoka, & Niwa, 2005).

According to their high level of tightness, these co-culture models have been successfully used to study BBB permeability changes that are responsible for the loss of BBB integrity observed in AD brains (van de Haar et al., 2016). By treating different in vitro BBB models with different forms of Aβ peptides, researchers have demonstrated that Aβ peptides are toxic to brain EC resulting in an alteration of their structure and function (Blanc, Toborek, Mark, Hennig, & Mattson, 1997; Folin et al., 2005). This leakage results from a modification of the distribution of TJ proteins (Marco & Skaper, 2006; Nagababu, Usatyuk, Enika, Natarajan, & Rifkind, 2009). These modifications are probably mediated by the production and activation of matrix metalloproteases, especially MMP2 and MMP9,

which are capable of degrading TJ proteins and which contribute to BBB breakdown (Spampinato, Merlo, Sano, Kanda, & Sortino, 2017). Moreover, it has been suggested that Aβ exposure induces the expression of Intercellular Adhesion Molecule 1 (ICAM-1) and vascular cell adhesion molecule 1(VCAM-1) (Liu, Yang, & Qi, 2012) resulting in decreased barrier tightness (Gonzalez-Velasquez, Kotarek, & Moss, 2008).

As a result of their good polarized distribution of specific receptors and transporters, these co-culture models have been successfully used to study faulty Aβ transport across the BBB, which leads to the pathogenesis of AD and CAA (Mawuenyega et al., 2010). Indeed, thanks to their configuration, these co-culture models enable Aβ peptide transport from the luminal to the abluminal direction (influx) and from the abluminal to the luminal direction (efflux) to be measured and help to decipher molecular mechanisms involved in these processes. Furthermore, since EC are well polarized it is possible to more accurately decipher the role of each receptor and/or transporter in the influx or efflux process. For instance, by using an animal in vitro BBB model in combination with specific competition experiments, our laboratory and others have confirmed that the influx of Aβ across BBB cells is mediated by RAGE (Candela et al., 2010; Tai et al., 2009). This transcellular transport is a caveolae-dependent process and coincides with the apical location of RAGE (Candela et al., 2010).

Moreover, according to their configuration (i.e. monolayer, coculture or triculture), it is possible to better decipher the role of each cell type in the different molecular mechanisms leading to AD. Recently, using an in vitro BBB model (microvascular EC co-cultured with astrocytes) Spampinato and colleagues evidenced that downregulation of claudin-5 was not related to the direct effects of Aβ peptides, but was rather the consequence of Aβ-induced vascular endothelial growth factor (VEGF) expression in astrocytes (Spampinato et al., 2017). The latter points out that the presence of astrocytes is essential to induce endothelial responses to $A\beta_{1\text{-}42}$, which results in increased barrier permeability and TJ disruption. In a different way, pericytes can also modulate the response of EC, thereby attenuating Aβ-induced changes of BBB properties through the inhibition of the RhoA-ROCK signaling pathway (Park et al., 2017). Furthermore, a few years ago, our laboratory suggested that LRP1 is not directly involved in Aβ efflux by EC as was widely described (Sagare et al., 2007). Instead, we evidenced the presence of LRP1 in pericytes and its important role in Aβ internalization (Candela et al., 2015).

Finally, these in vitro BBB models are powerful tools for identifying or confirming cell targets for drug screening. For instance, these models have

been used to investigate the effects of pharmacological P-glycoprotein modulation by using potent inhibitors. With this approach, some groups have demonstrated the involvement of P-glycoprotein but also of BCRP in Aβ transport (Candela et al., 2010; Tai et al., 2009). In these cases, the BBB model and specific P-glycoprotein inhibitor should be chosen carefully to highlight the P-glycoprotein and BCRP contribution. Furthermore, the anticancer drug bexarotene agonist of RXR has been shown to result in an upregulation of the P-glycoprotein transporter and hence a decrease in Aβ entry into the brain compartment (Kuntz et al., 2015). Recently, using immunoprecipitation experiments, Pietrzik and colleagues confirmed that PICALM, another genetic risk factor for AD, is expressed at the BBB and functionally linked with LRP1 and P-glycoprotein in Aβ clearance (Storck et al., 2018). These studies, confirmed by in vivo experiments, showed that the induction of ABC transporters such as P-glycoprotein or LRP1 may be a novel therapeutic strategy to protect the brain from Aβ accumulation (Bomben et al., 2014), thereby restoring the balance between Aβ entry and clearance.

New BBB model and challenges

Even though the co-culture static models have numerous advantages for studying molecular mechanisms that may be involved in AD pathogenesis, they do not reflect all the physiologic conditions in which the in vivo BBB operates and have limitations. For example, they neglect cerebral blood flow (CBF) which could be a considerable advantage to study the pathological process leading to AD. To bypass this problem, flow and microfluidic models (with a controlled flow of fluid mimicking natural blood flow in cerebral microvessels) have been built (Booth & Kim, 2014; Cochrane et al., 2018; Wegener & Seebach, 2014). These systems represent a useful tool to evaluate the destructive effects of various neuroinflammatory mediators on the integrity of the BBB (Cho et al., 2015). Therefore, they are useful tools for studying inflammatory processes leading to changes affecting barrier integrity in AD.

In any case, the greater limitation of all BBB in vitro models includes species differences in receptor and transporter expression levels and homology (Helms et al., 2016; Syvänen et al., 2009).

Recently, human brain endothelial cells have been derived from human cord blood endothelial progenitor stem cells (Cecchelli et al., 2014) to create an in vitro BBB model that displays improved BBB properties closed to those observed in vivo. This model has already proved valuable in the study of some pathologies related to the BBB (Drolez et al., 2016)

and validated to study the transport of Aβ across the BBB (Kuntz et al., 2015). However, building an in vitro BBB model with various cell types generated from human stem cells (hPSC) (Lippmann et al., 2012) represents the best hope to study different diseases including AD. Indeed, the generation of endothelial cells, pericytes, astrocytes (in isolation or combination) based on patient-derived stem cells offers an important opportunity to create in vitro BBB models for mechanistic studies and drug delivery (see review of (Page, Patel, Raut, & Al-Ahmad, 2018)). In addition, patient-derived models of BBB open the avenue to explore the contribution of genetic factors associated with the BBB pathophysiology. In this sense, using an in vitro model based on iPSC-derived monocultures, Zhao and colleagues demonstrated the central role of PICALM in Aβ BBB transcytosis and identified the polymorphism rs3851179 in PICALM gene as a potential contributing risk factor for AD (Zhao et al., 2015).

Despite these advantages, one of the main challenges in BBB modeling derived from AD patients is the limited number of iPSC lines, as well as the limited availability of matched controls (e. g. siblings, parents) and the high tendency of mutations.

In any case, these iPSC-based models of the BBB for studying AD remain an interesting tool compared to existing BBB animal-based models, but there is still a long way to go in this area to demonstrate its ability to reflect the phenomena observed in vivo.

Conclusion

AD is a neurological disorder characterized by an abnormal accumulation of Aβ peptides in the brain. The pathological process is not clarified yet, but a dysfunction of the BBB is definitely ascertained. For this reason, to accurately investigate the cellular and molecular mechanisms occurring at the level of this complex physiological barrier, an in vitro approach is required. Several in vitro BBB models are currently used. These in vitro BBB models offer advantages and disadvantages and the choice of each model is dependent on the research goals and the objective of the study. Altogether, these models, discussed in this chapter, are a complement to in vivo studies and provide additional information to investigate the cellular and molecular mechanisms occurring at BBB level leading to AD pathogenesis. In the meantime, there are presently no models able to reproduce all the characteristics of the BBB and therefore respond to the needs required to study AD experimentally. Thus, current BBB models require additional modification and further optimization. In

concert with other models, the use of patient iPSC to create the BBB in the future will help to better understand the complicated AD mechanisms related to the BBB that might lead to developing new strategies to identify new therapeutic targets/approaches.

References

Abbott, N. J., Patabendige, A. A. K., Dolman, D. E. M., Yusof, S. R., & Begley, D. J. (2010). "Structure and function of the blood–brain barrier". *Neurobiology of Disease*, 37(1), 13–25.

Aday, S., Cecchelli, R., Hallier-Vanuxeem, D., Dehouck, M. P., & Ferreira, L. (2016). "Stem Cell-Based Human Blood-Brain Barrier Models for Drug Discovery and Delivery". *Trends in Biotechnology*, 34(5), 382–393.

Allt, G., & Lawrenson, J. G. (2001). "Pericytes: cell biology and pathology". *Cells, Tissues, Organs*, 169(1), 1–11. https://doi.org/10.1159/000047855

Alvarez, J. I., Cayrol, R., & Prat, A. (2011). "Disruption of central nervous system barriers in multiple sclerosis". *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 252–264. doi: 10.1016/j.bbadis.2010.06.017.

András, I. E., Eum, S. Y., Huang, W., Zhong, Y., Hennig, B., & Toborek, M. (2010). "HIV-1-induced amyloid beta accumulation in brain endothelial cells is attenuated by simvastatin". *Molecular and Cellular Neuroscience* 232–243. doi: org/10.1016/j.mcn.2009.11.004.

Andras, I. E., Rha, G., Huang, W., Eum, S., Couraud, P.-O., Romero, I. A., Toborek, M. (2008). "Simvastatin Protects against Amyloid and HIV-1 Tat-Induced Promoter Activities of Inflammatory Genes in Brain Endothelial Cells". *Molecular Pharmacology*, 73(5), 1424–1433. doi: 10.1124/mol.107.042028.

Armulik, A., Genové, G., Mäe, M., Nisancioglu, M. H., Wallgard, E., Niaudet, C., Betsholtz, C. (2010). "Pericytes regulate the blood–brain barrier". *Nature,* 468(7323), 557–561. Doi: 10.1038/nature09522.

Ballabh, P., Braun, A., & Nedergaard, M. (2004). The blood–brain barrier: an overview. Neurobiology of Disease, 16(1), 1–13. https://doi.org/10.1016/j.nbd.2003.12.016.

Bazzoni, G., & Dejana, E. (2004). "Endothelial Cell-to-Cell Junctions: Molecular Organization and Role in Vascular Homeostasis". *Physiological Reviews*, 84(3), 869–901. doi:10.1152/physrev.00035.2003.

Bell, R. D., Winkler, E. A., Sagare, A. P., Singh, I., LaRue, B., Deane, R., & Zlokovic, B. V. (2010). "Pericytes Control Key Neurovascular Functions and Neuronal Phenotype in the Adult Brain and during Brain Aging". *Neuron,* 68(3), 409–427. doi:10.1016/j.neuron.2010.09.043.

Biemans, E. A. L. M., Jäkel, L., de Waal, R. M. W., Kuiperij, H. B., & Verbeek, M. M. (2017). "Limitations of the hCMEC/D3 cell line as a model for Aβ clearance by the human blood-brain barrier". *Journal of Neuroscience Research,* 95(7), 1513–1522. doi: 10.1002/jnr.23964.

Blanc, E. M., Toborek, M., Mark, R. J., Hennig, B., & Mattson, M. P. (1997). "Amyloid beta-peptide induces cell monolayer albumin permeability, impairs glucose transport, and induces apoptosis in vascular endothelial cells". *Journal of Neurochemistry*, 68(5), 1870–1881.

Bomben, V., Holth, J., Reed, J., Cramer, P., Landreth, G., & Noebels, J. (2014). "Bexarotene reduces network excitability in models of Alzheimer's disease and epilepsy". *Neurobiology of Aging*, 35(9), 2091–2095. doi: 10.1016/j.neurobiolaging.2014.03.029.

Booth, R., & Kim, H. (2014). "Permeability Analysis of Neuroactive Drugs Through a Dynamic Microfluidic In Vitro Blood–Brain Barrier Model". *Annals of Biomedical Engineering,* 42(12), 2379–2391. doi:10.1007/s10439-014-1086-5.

Brouillette, J., Caillierez, R., Zommer, N., Alves-Pires, C., Benilova, I., Blum, D., Buée, L. (2012). "Neurotoxicity and memory deficits induced by soluble low-molecular-weight amyloid-β1-42 oligomers are revealed in vivo by using a novel animal model." *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience,* 32(23), 7852–7861. doi: 10.1523/JNEUROSCI.5901-11.2012.

Candela, P., Gosselet, F., Saint-Pol, J., Sevin, E., Boucau, M.-C., Boulanger, E., Fenart, L. (2010). "Apical-to-basolateral transport of amyloid-β peptides through blood-brain barrier cells is mediated by the receptor for advanced glycation end-products and is restricted by p-glycoprotein". *Journal of Alzheimer's Disease,* 22(3). doi.10.3233/JAD-2010-100462.

Candela, P., Saint-Pol, J., Kuntz, M., Boucau, M.-C., Lamartiniere, Y., Gosselet, F., & Fenart, L. (2015)." In vitro discrimination of the role of LRP1 at the BBB cellular level: Focus on brain capillary endothelial cells and brain pericytes". *Brain Research*, 1594. doi: 10.1016/j.brainres.2014.10.047.

Cardoso, F. L., Brites, D., & Brito, M. A. (2010). "Looking at the blood-brain barrier: molecular anatomy and possible investigation approaches". *Brain Research Reviews*, 328–363.

doi: 10.1016/j.brainresrev.2010.05.003.

Castellani, R. J., & Perry, G. (2019). "Tau Biology, Tauopathy, Traumatic Brain Injury, and Diagnostic Challenges". *Journal of Alzheimer's Disease: JAD,* 67 447–467. doi:10.3233/JAD-180721.

Cecchelli, R., Aday, S., Sevin, E., Almeida, C., Culot, M., Dehouck, L., Ferreira, L. (2014). "A Stable and Reproducible Human Blood-Brain Barrier Model Derived from Hematopoietic Stem Cells". *PLoS ONE*, 9(6), e99733. doi:10.1371/journal.pone.0099733.

Cecchelli, R., Berezowski, V., Lundquist, S., Culot, M., Renftel, M., Dehouck, M.-P., & Fenart, L. (2007). "Modelling of the blood-brain barrier in drug discovery and development". *Nature Reviews. Drug Discovery,* 6(8), 650–661. doi:10.1038/nrd2368.

Cho, H., Seo, J. H., Wong, K. H. K., Terasaki, Y., Park, J., Bong, K., … Irimia, D. (2015). "Three-Dimensional Blood-Brain Barrier Model for in vitro Studies of Neurovascular Pathology". *Scientific Reports*, 5(1), 15222. doi:10.1038/srep15222.

Cirrito, J. R., Deane, R., Fagan, A. M., Spinner, M. L., Parsadanian, M., Finn, M. B., Holtzman, D. M. (2005). "P-glycoprotein deficiency at the blood-brain barrier increases amyloid- deposition in an Alzheimer disease mouse model". *Journal of Clinical Investigation*, 115(11), 3285–3290. doi:10.1172/JCI25247.

Cochrane, A., Albers, H. J., Passier, R., Mummery, C. L., van den Berg, A., Orlova, V. V, & van der Meer, A. D. (2018). "Advanced in vitro models of vascular biology: Human induced pluripotent stem cells and organ-on-chip technology". *Advanced Drug Delivery Reviews*. doi: 10.1016/j.addr.2018.06.007.

Da Fonseca, A. C. C., Matias, D., Garcia, C., Amaral, R., Geraldo, L. H., Freitas, C., & Lima, F. R. S. (2014). "The impact of microglial activation on blood-brain barrier in brain diseases". *Frontiers in Cellular Neuroscience,* 8, 362. doi: 10.3389/fncel.2014.00362.

Daneman, R., Zhou, L., Agalliu, D., Cahoy, J. D., Kaushal, A., & Barres, B. A. (2010). "The mouse blood-brain barrier transcriptome: a new resource for understanding the development and function of brain endothelial cells". *PloS One*, 5(10), e13741. doi: 10.1371/journal.pone.0013741.

Deane, R., Sagare, A., & Zlokovic, B. V. (2008). "The role of the cell surface LRP and soluble LRP in blood-brain barrier Abeta clearance in Alzheimer's disease". *Current Pharmaceutical Design*, 14(16), 1601–1605.

Deli, Máiria A, Abrahám, C. S., Kataoka, Y., & Niwa, M. (2005). "Permeability studies on in vitro blood-brain barrier models:

physiology, pathology, and pharmacology". *Cellular and Molecular Neurobiology,* 25(1), 59–127.

Deli, Mária A. (2009). "Potential use of tight junction modulators to reversibly open membranous barriers and improve drug delivery". *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1788(4), 892–910. doi: 10.1016/j.bbamem.2008.09.016.

Devraj, K., Poznanovic, S., Spahn, C., Schwall, G., Harter, P. N., Mittelbronn, M., Liebner, S. (2016). BACE-1 is expressed in the blood-brain barrier endothelium and is upregulated in a murine model of Alzheimer's disease. Journal of Cerebral Blood Flow and Metabolism: Official Journal of the International Society of Cerebral Blood Flow and Metabolism, 36(7), 1281–1294. doi: 10.1177/0271678X15606463.

Donahue, J. E., Flaherty, S. L., Johanson, C. E., Duncan, J. A., Silverberg, G. D., Miller, M. C., … Stopa, E. G. (2006). RAGE, LRP-1, and amyloid-beta protein in Alzheimer's disease. Acta Neuropathologica, 112(4), 405–415. https://doi.org/10.1007/s00401-006-0115-3.

Dore-Duffy, P. (2008). "Pericytes: pluripotent cells of the blood brain barrier". *Current Pharmaceutical Design*, 14(16), 1581–1593.

Drolez, A., Vandenhaute, E., Julien, S., Gosselet, F., Burchell, J., Cecchelli, R., Mysiorek, C. (2016). "Selection of a Relevant In Vitro Blood-Brain Barrier Model to Investigate Pro-Metastatic Features of Human Breast Cancer Cell Lines". *PLOS ONE*, 11(3), e0151155. doi:10.1371/journal.pone.0151155.

Fenstermacher, J., Gross, P., Sposito, N., Acuff, V., Pettersen, S., & Gruber, K. (1988). "Structural and functional variations in capillary systems within the brain". *Annals of the New York Academy of Sciences,* 529, 21–30.

Folin, M., Baiguera, S., Tommasini, M., Guidolin, D., Conconi, M. T., De Carlo, E., Parnigotto, P. P. (2005). "Effects of beta-amyloid on rat neuromicrovascular endothelial cells cultured in vitro". *International Journal of Molecular Medicine*, 15(6), 929–935.

Förster, C., Burek, M., Romero, I. A., Weksler, B., Couraud, P.-O., & Drenckhahn, D. (2008). "Differential effects of hydrocortisone and TNFalpha on tight junction proteins in an in vitro model of the human blood-brain barrier". *The Journal of Physiology*, 586(7), 1937–1949. doi: 10.1113/jphysiol.2007.146852.

Gillis, C., Mirzaei, F., Potashman, M., Ikram, M. A., & Maserejian, N. (2019). "The incidence of mild cognitive impairment: A systematic review and data synthesis". *Alzheimer's & Dementia (Amsterdam, Netherlands),* 11, 248–256. doi: 10.1016/j.dadm.2019.01.004

Glenner, G. G., & Wong, C. W. (2012). "Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein". *Biochemical and Biophysical Research Communications,* 425(3), 534–539. doi: 10.1016/j.bbrc.2012.08.020.

Gonzalez-Velasquez, F. J., Kotarek, J. A., & Moss, M. A. (2008). "Soluble aggregates of the amyloid-beta protein selectively stimulate permeability in human brain microvascular endothelial monolayers". *Journal of Neurochemistry*, 466–477. doi: 10.1111/j.1471-4159.2008.05618.x.

Gosselet, F., Candela, P., Sevin, E., Berezowski, V., Cecchelli, R., & Fenart, L. (2009). "Transcriptional profiles of receptors and transporters involved in brain cholesterol homeostasis at the blood-brain barrier: Use of an in vitro model". *Brain Research*, 1249. doi: 10.1016/j.brainres.2008.10.036.

Grinberg, L. T., & Thal, D. R. (2010). "Vascular pathology in the aged human brain". *Acta Neuropathologica*, 119(3), 277–290. doi: 10.1007/s00401-010-0652-7.

Gumbleton, M., & Audus, K. L. (2001). "Progress and limitations in the use of in vitro cell cultures to serve as a permeability screen for the blood-brain barrier". *Journal of Pharmaceutical Sciences*, 90(11), 1681–1698.

Haass, C., & Selkoe, D. J. (2007). "Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide". *Nature Reviews*. Molecular Cell Biology, 101–112. doi:10.1038/nrm2101.

Hawkins, B. T., & Davis, T. P. (2005). "The blood-brain barrier/neurovascular unit in health and disease". *Pharmacological Reviews*, (57) 173–185. doi:10.1124/pr.57.2.4.

Helms, H. C., Abbott, N. J., Burek, M., Cecchelli, R., Couraud, P.-O., Deli, M. A., Brodin, B. (2016). "In vitro models of the blood-brain barrier: An overview of commonly used brain endothelial cell culture models and guidelines for their use. Journal of Cerebral Blood Flow and Metabolism" *Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, 36(5), 862–890. doi: 10.1177/0271678X16630991.

Ito, S., Ohtsuki, S., & Terasaki, T. (2006). "Functional characterization of the brain-to-blood efflux clearance of human amyloid-beta peptide (1-40) across the rat blood-brain barrier". *Neuroscience Research*, 56(3), 246–252. doi: 10.1016/j.neures.2006.07.006.

Kuntz, M., Candela, P., Saint-Pol, J., Lamartinière, Y., Boucau, M.-C., Sevin, E., Gosselet, F. (2015). "Bexarotene Promotes Cholesterol Efflux and Restricts Apical-to-Basolateral Transport of Amyloid-β Peptides in an in Vitro Model of the Human Blood-Brain Barrier". *Journal of Alzheimer's Disease*, 48(3). doi: 10.3233/JAD-150469.

Li, J. Y., Boado, R. J., & Pardridge, W. M. (2001). "Blood-brain barrier genomics. Journal of Cerebral Blood Flow and Metabolism". *Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, 21(1), 61–68. doi:10.1097/00004647-200101000-00008.

Lippmann, E. S., Azarin, S. M., Kay, J. E., Nessler, R. A., Wilson, H. K., Al-Ahmad, A., Shusta, E. V. (2012). "Derivation of blood-brain barrier endothelial cells from human pluripotent stem cells". *Nature Biotechnology*, 30(8), 783–791. doi: 10.1038/nbt.2247.

Liu, X.-J., Yang, W., & Qi, J.-S. (2012). "[Oxidative stress and Alzheimer's disease]". Acta Physiologica Sinica], 64(1), 87–95.

Marco, S., & Skaper, S. D. (2006). "Amyloid β-peptide1–42 alters tight junction protein distribution and expression in brain microvessel endothelial cells". *Neuroscience Letters*, 401(3), 219–224. doi:10.1016/j.neulet.2006.03.047.

Marques, F., Sousa, J. C., Sousa, N., & Palha, J. A. (2013). "Blood-brain-barriers in aging and in Alzheimer's disease". *Molecular Neurodegeneration*, 8(1), 38. doi: 10.1186/1750-1326-8-38.

Masters, C. L., Multhaup, G., Simms, G., Pottgiesser, J., Martins, R. N., & Beyreuther, K. (1985). "Neuronal origin of a cerebral amyloid: neurofibrillary tangles of Alzheimer's disease contain the same protein as the amyloid of plaque cores and blood vessels*". The EMBO Journal*, 4(11), 2757–2763.

Mawuenyega, K. G., Sigurdson, W., Ovod, V., Munsell, L., Kasten, T., Morris, J. C., Bateman, R. J. (2010). Decreased clearance of CNS beta-amyloid in Alzheimer's disease. *Science (New York, N.Y.),* 330(6012), 1774. doi: 10.1126/science.1197623.

Millucci, L., Ghezzi, L., Bernardini, G., & Santucci, A. (2010). "Conformations and biological activities of amyloid beta peptide 25-35". *Current Protein & Peptide Science*, 11(1), 54–67.

Moestrup, S. K., & Hokland, P. (1992). "Surface expression of the alpha 2-macroglobulin receptor on human malignant blood cells". *Leukemia Research,* 16(3), 227–234.

Montagne, A., Zhao, Z., & Zlokovic, B. V. (2017). "Alzheimer's disease: A matter of blood-brain barrier dysfunction?" *The Journal of Experimental Medicine,* 214(11), 3151–3169. doi: 10.1084/jem.20171406.

Nagababu, E., Usatyuk, P. V, Enika, D., Natarajan, V., & Rifkind, J. M. (2009). "Vascular endothelial barrier dysfunction mediated by amyloid-beta proteins". *Journal of Alzheimer's Disease: JAD*, 17(4), 845–854. doi: 10.3233/JAD-2009-1104.

Nazer, B., Hong, S., & Selkoe, D. J. (2008). "LRP promotes endocytosis and degradation, but not transcytosis, of the amyloid-β peptide in a blood–brain barrier in vitro model". *Neurobiology of Disease*, 30(1), 94–102. doi: 10.1016/j.nbd.2007.12.005.

Novo, M., Freire, S., & Al-Soufi, W. (2018). "Critical aggregation concentration for the formation of early Amyloid-β (1–42) oligomers". *Scientific Reports*, 8(1), 1783. doi:10.1038/s41598-018-19961-3.

Obermeier, B., Daneman, R., & Ransohoff, R. M. (2013). "Development, maintenance and disruption of the blood-brain barrier". *Nature Medicine,* 19(12), 1584–1596. doi: 10.1038/nm.3407.

Ohtsuki, S., Ikeda, C., Uchida, Y., Sakamoto, Y., Miller, F., Glacial, F., Terasaki, T. (2013). "Quantitative targeted absolute proteomic analysis of transporters, receptors and junction proteins for validation of human cerebral microvascular endothelial cell line hCMEC/D3 as a human blood-brain barrier model". *Molecular Pharmaceutics,* 10(1), 289–296.doi:10.1021/mp3004308

Oldendorf, W. H., Cornford, M. E., & Brown, W. J. (1977). "The large apparent work capability of the blood-brain barrier: a study of the mitochondrial content of capillary endothelial cells in brain and other tissues of the rat". *Annals of Neurology*, 1(5), 409–417. doi: 10.1002/ana.410010502.

Page, S., Patel, R., Raut, S., & Al-Ahmad, A. (2018). "Neurological diseases at the blood-brain barrier: Stemming new scientific paradigms using patient-derived induced pluripotent cells". *Biochimica et Biophysica Acta. Molecular Basis of Disease*. doi:10.1016/j.bbadis.2018.12.009.

Pardridge, W. M. (1998). "CNS drug design based on principles of blood-brain barrier transport". *Journal of Neurochemistry*, 70(5), 1781–1792.

Park, J.-C., Baik, S. H., Han, S.-H., Cho, H. J., Choi, H., Kim, H. J., Mook-Jung, I. (2017). "Annexin A1 restores Aβ1-42 -induced blood-brain barrier disruption through the inhibition of RhoA-ROCK signaling pathway". *Aging Cell*, 16(1), 149–161. doi:10.1111/acel.12530.

Picou, R., Moses, J. P., Wellman, A. D., Kheterpal, I., & Gilman, S. D. (2010). "Analysis of monomeric Aβ (1–40) peptide by capillary electrophoresis". *The Analyst*, 135(7), 1631. doi:10.1039/c0an00080a.

Pryor, N. E., Moss, M. A., & Hestekin, C. N. (2014). "Capillary electrophoresis for the analysis of the effect of sample preparation on early stages of Aβ1-40 aggregation". *Electrophoresis*, 35(12–13), 1814–1820. doi: 10.1002/elps.201400012.

Reichel, A., Begley, D. J., & Abbott, N. J. (2003). "An overview of in vitro techniques for blood-brain barrier studies". *Methods in Molecular Medicine,* 89, 307–324. doi:10.1385/1-59259-419-0:307.

Ries, M., & Sastre, M. (2016). "Mechanisms of Aβ Clearance and Degradation by Glial Cells". *Frontiers in Aging Neuroscience*, 8, 160. doi: 10.3389/fnagi.2016.00160.

Sagare, A., Deane, R., Bell, R. D., Johnson, B., Hamm, K., Pendu, R., Zlokovic, B. V. (2007). "Clearance of amyloid-beta by circulating lipoprotein receptors". *Nature Medicine,* 13(9), 1029–1031. doi: 10.1038/nm1635.

Saint-Pol, J., Vandenhaute, E., Boucau, M.-C., Candela, P., Dehouck, L., Cecchelli, R., Gosselet, F. (2012). "Brain pericytes abca1 expression mediates cholesterol efflux but not cellular amyloid-β peptide accumulation". *Journal of Alzheimer's Disease,* 30(3). doi: 10.3233/JAD-2012-112090.

Schweinzer, C., Kober, A., Lang, I., Etschmaier, K., Scholler, M., Kresse, A., Panzenboeck, U. (2011). "Processing of endogenous AβPP in blood-brain barrier endothelial cells is modulated by liver-X receptor agonists and altered cellular cholesterol homeostasis". *Journal of Alzheimer's Disease:* 341–360. doi:10.3233/JAD-2011-110854.

Sedlakova, R., Shivers, R. R., & Del Maestro, R. F. (1999). "Ultrastructure of the blood-brain barrier in the rabbit". *Journal of Submicroscopic Cytology and Pathology*, 31(1), 149–161.

Selkoe, D. J. (2000). "Toward a comprehensive theory for Alzheimer's disease. Hypothesis: Alzheimer's disease is caused by the cerebral accumulation and cytotoxicity of amyloid beta-protein". *Annals of the New York Academy of Sciences*, 924, 17–25.

Shibata, M., Yamada, S., Kumar, S. R., Calero, M., Bading, J., Frangione, B., Zlokovic, B. V. (2000). "Clearance of Alzheimer's amyloid-ss (1-40) peptide from brain by LDL receptor-related protein-1 at the blood-brain barrier". *The Journal of Clinical Investigation*, 106(12), 1489–1499. doi: 10.1172/JCI10498.

Spampinato, S. F., Merlo, S., Sano, Y., Kanda, T., & Sortino, M. A. (2017). "Astrocytes contribute to Aβ-induced blood-brain barrier damage through activation of endothelial MMP9". *Journal of Neurochemistry,* 142(3), 464–477. doi:10.1111/jnc.14068.

Storck, S. E., Hartz, A. M. S., Bernard, J., Wolf, A., Kachlmeier, A., Mahringer, A., Pietrzik, C. U. (2018). "The concerted amyloid-beta clearance of LRP1 and ABCB1/P-gp across the blood-brain barrier is linked by PICALM". *Brain, Behavior, and Immunity*, 73, 21–33. doi: 10.1016/j.bbi.2018.07.017.

Sweeney, M. D., Ayyadurai, S., & Zlokovic, B. V. (2016). "Pericytes of the neurovascular unit: key functions and signaling pathways". *Nature Neuroscience*, 19(6), 771–783. doi: org/10.1038/nn.4288.

Sweeney, M. D., Kisler, K., Montagne, A., Toga, A. W., & Zlokovic, B. V. (2018). "The role of brain vasculature in neurodegenerative disorders". *Nature Neuroscience*, 21(10), 1318–1331. https://doi.org/10.1038/s41593-018-0234-x.

Sweeney, M. D., Zhao, Z., Montagne, A., Nelson, A. R., & Zlokovic, B. V. (2019). "Blood-Brain Barrier: From Physiology to Disease and Back". *Physiological Reviews*, 99(1), 21–78. doi: 10.1152/physrev.00050.2017.

Syvänen, S., Lindhe, O., Palner, M., Kornum, B. R., Rahman, O., Långström, B., Hammarlund-Udenaes, M. (2009). "Species differences in blood-brain barrier transport of three positron emission tomography radioligands with emphasis on P-glycoprotein transport". *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, 37(3), 635–643. doi: 10.1124/dmd.108.024745.

Tai, L. M., Reddy, P. S., Lopez-Ramirez, M. A., Davies, H. A., Male, A. D. K., Loughlin, A. J., & Romero, I. A. (2009). "Polarized P-glycoprotein expression by the immortalised human brain endothelial cell line, hCMEC/D3, restricts apical-to-basolateral permeability to rhodamine" *Brain Research*, 1292, 14–24. doi: 10.1016/j.brainres.2009.07.039.

Tarasoff-Conway, J. M., Carare, R. O., Osorio, R. S., Glodzik, L., Butler, T., Fieremans, E., de Leon, M. J. (2015). "Clearance systems in the brain-implications for Alzheimer disease". *Nature Reviews Neurology*, 11(8), 457–470. doi: 10.1038/nrneurol.2015.119.

Thal, D. R., Ghebremedhin, E., Rüb, U., Yamaguchi, H., Del Tredici, K., & Braak, H. (2002). "Two Types of Sporadic Cerebral Amyloid Angiopathy". *Journal of Neuropathology & Experimental Neurology,* 61(3), 282–293. doi: 10.1093/jnen/61.3.282.

Van de Haar, H. J., Burgmans, S., Jansen, J. F. A., van Osch, M. J. P., van Buchem, M. A., Muller, M., Backes, W. H. (2016). "Blood-Brain Barrier Leakage in Patients with Early Alzheimer Disease". *Radiology*, 281 doi:10.1148/radiol.2016152244.

Wegener, J., & Seebach, J. (2014). "Experimental tools to monitor the dynamics of endothelial barrier function: a survey of in vitro approaches". *Cell and Tissue Research*, 355(3), 485–514. doi: 10.1007/s00441-014-1810-3.

Wisniewski, T., Castaño, E. M., Golabek, A., Vogel, T., & Frangione, B. (1994). "Acceleration of Alzheimer's fibril formation by apolipoprotein E in vitro". *The American Journal of Pathology*, 145(5), 1030–1035.

Zhao, Z., Sagare, A. P., Ma, Q., Halliday, M. R., Kong, P., Kisler, K., Zlokovic, B. V. (2015). "Central role for PICALM in amyloid-β blood-brain barrier transcytosis and clearance". *Nature Neuroscience*, 18(7), 978–987. doi: org/10.1038/nn.4025.

Zlokovic, B. V. (2008). "The blood-brain barrier in health and chronic neurodegenerative disorders". *Neuron*, 57
doi: 10.1016/j.neuron.2008.01.003.

Zuroff, L., Daley, D., Black, K. L., & Koronyo-Hamaoui, M. (2017). Clearance of cerebral Aβ in Alzheimer's disease: reassessing the role of microglia and monocytes. "Cellular and Molecular Life Science"s : *CMLS*, 74(12), 2167–2201. doi: 10.1007/s00018-017-2463-7.