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Physiology, Pharmacology and Pathology of the Blood-Brain Barrier



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Zameel Cader • Winfried Neuhaus
Editors

Physiology, Pharmacology and Pathology of the Blood-Brain Barrier

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Preface

Around 1900 and in the decades following, the existence of a barrier between the brain and the rest of the body was first postulated and subsequently proven. Experiments with dyes that were either applied systemically and not recovered in the brain, or injected into the brain and not detected in the rest of the body, led to the concept of a barrier in the brain preventing the movement of substances. Researchers such as Goldmann, Lewandowski, Bouffard and Franke conducted crucial dyeing experiments and participated in the development of the concept of the blood-brain barrier. The term “blood-brain barrier” may have first been used by Lina Stern, the first female professor of the University of Geneva. A further milestone for the understanding of the blood-brain barrier was the electron microscopic analyses at the end of the 1960s and beginning of the 1970s identifying the endothelial cells of the brain capillaries as the main sealing component of the blood-brain barrier. This was followed by anatomical investigations, the measurements of the electrical resistance of the blood vessels in the brain of animals and the successful isolation and cultivation of brain endothelial cells to establish cell culture models in the 1980s–1990s. Together with the technologies available today, these advances have enabled wide ranging research of the blood-brain barrier.

The blood-brain barrier is a substantial topic with respect to pharmacology. On the one hand, drugs have to overcome the blood-brain barrier in order to treat neurological, neurodevelopmental or neurodegenerative diseases; on the other hand, substances that should act in the periphery should not enter the brain in order to avoid adverse side effects. The blood-brain barrier has a number of instruments to fulfil its main task to maintain the homeostasis in the CNS such as restriction of paracellular transport by sealing the gaps at the cell–cell contacts with tight junctions, regulation of transcellular transport by influx as well as efflux transporters or conversion of compounds by intracellular enzymes. The knowledge about properties necessary for small molecules to permeate across the blood-brain barrier is already quite comprehensive, whereas the understanding of pharmacokinetics of biologics such as antibodies is very limited and a current hot topic.

Even after successful entry into the CNS, an active substance still has to reach its intended target in the specific brain region to optimally achieve the desired effect. For this, the routes within the CNS to the target play a critical role. The liquid flows, the interaction of interstitial and cerebrospinal fluid and the disposal pathways for

substances are crucial for their residence and action time. In this respect, it has become clear that the “physics” in the brain is a major factor for drug distribution within the CNS and can also contribute significantly to observed species differences.

It is very clear that the function of the blood-brain barrier is altered in many CNS diseases. Elucidation of underlying mechanisms led to the discussion of whether these changes at the blood-brain barrier are merely symptoms or if the blood-brain barrier contributes actively or is even causally linked to disease progression. In this regard, the concept of neurovascular units and the mutual regulatory influence of the single, neighboured cell types is important. It is highly plausible that changes of brain endothelial cells will lead to altered communication with their microenvironment subsequently influencing both physiological and pathophysiological processes in the CNS. Hence, improved understanding and knowledge about disease specific changes at the blood-brain barrier is indispensable to optimize the pharmacokinetic and pharmacodynamic behaviour of therapeutics.

In light of advances in our knowledge and available technologies, we felt this was the perfect time for a book about the blood-brain barrier with a comprehensive compilation of the manifold aspects relevant to the pharmaceutical perspective. The chapters of the book are organized in four main parts providing basic information and novel insights about the physiology of the blood-brain barrier, the challenges related to finding and developing drugs crossing the blood-brain barrier, experimental methods to study the blood-brain barrier and the role of the blood-brain barrier in disease mechanisms and its consequences for drug development. Addressing all these topics to achieve both the broad coverage and also the key details, we have been very lucky and are incredibly thankful that so many outstanding experts in the specific fields of blood-brain barrier research agreed to contribute to this effort. In addition, we are very grateful for the support and patience of the publisher during the process of book preparation and editing. Finally, we hope that the book provides the intended up-to-date overview about the physiology, pharmacology and pathophysiology of the blood-brain barrier and that it will be helpful, interesting and inspiring for its readers.

Oxford, United Kingdom
Wien, Austria

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Winfried Neuhaus

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Part I

Background on the Blood-Brain Barrier



Structure and Function of the Blood–Brain Barrier (BBB)

Fabienne Benz and Stefan Liebner

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Abstract

The blood-brain barrier (BBB) protects the vertebrate central nervous system from harmful blood-borne, endogenous and exogenous substances to ensure proper neuronal function. The BBB describes a function that is established by endothelial cells of CNS vessels in conjunction with pericytes, astrocytes, neurons and microglia, together forming the neurovascular unit (NVU). Endothelial barrier function is crucially induced and maintained by the Wnt/ β -catenin pathway and requires intact NVU for proper functionality. The BBB and the NVU are characterized by a specialized assortment of molecular specializations, providing the basis for tightening, transport and immune response functionality.

The present chapter introduces state-of-the-art knowledge of BBB structure and function and highlights current research topics, aiming to understanding in more depth the cellular and molecular interactions at the NVU, determining functionality of the BBB in health and disease, and providing novel potential targets for therapeutic BBB modulation. Moreover, we highlight recent advances in understanding BBB and NVU heterogeneity within the CNS as well as their contribution to CNS physiology, such as neurovascular coupling, and pathophysiology, is discussed. Finally, we give an outlook onto new avenues of BBB research.

Keywords

BBB development · Blood–brain barrier · Circumventricular organs · Neurovascular unit · Vascular heterogeneity · Wnt signalling

1 Background on the BBB Structure and Function

1.1 Definition of the BBB

The central nervous system (CNS) is the most complex structure in the body of higher metazoan organisms. Neurons require – for their proper functionality – a specific micro-environment and micro-milieu that considerably differs in cellular and molecular composition from peripheral organs. To establish and maintain a physical segregation of compartments – the blood from the brain – all vertebrate animals have developed a blood–brain barrier (BBB) (Abbott et al. 2010). With the exception of elasmobranchs (sharks, skates) and chondrostei (sturgeons) which possess a glial barrier, all other species within the vertebrate phylum have independently developed an endothelial barrier (Bundgaard and Abbott 2008). This suggests that the passage of substances from blood to the brain parenchyma is best controlled directly at the blood-to-brain interface. Hence, the BBB is localized to brain capillary endothelial cells (BCECs) that strictly control the passage of metabolites into and out of the CNS (Brightman and Reese 1969; Reese and Karnovsky 1967). To accomplish this control, BCECs form complex and continuous, interendothelial tight junctions (TJs), sealing off the paracellular pathway and thereby controlling the

passage of molecules between two BCECs (Wolburg et al. 2003; Cording et al. 2012). Moreover, due to their barrier function, BCECs lack fenestrae and show significantly lower vesicular traffic, hence transcytosis, making the BBB endothelium a tight barrier for most polar, water soluble molecules larger than 450 Da (Abbott et al. 2010). In combination with the expression of specific enzymes and transporters the BBB endothelium is unique and thereby distinguishable from all other endothelial cells (ECs) in the body (Fig. 1).

Although the barrier proper is confined to BCECs, the specific differentiation of brain ECs towards the BBB phenotype is linked to their morphological connection to other cells of the CNS. Because of their close interactions, ECs together with pericytes (PCs), perivascular fibroblasts (FBs), astrocytes (ACs), microglia (MGs) and nerve endings (Ns) are considered as the so-called neurovascular unit (NVU), which is key in developing and maintaining structural and consequently, functional integrity of the BBB (Daneman et al. 2015; Engelhardt and Sorokin 2009).

The vascular basal lamina (BM) is composed of collagen IV, laminin $\alpha 4$, fibronectin and several other components (for review, see Hallmann et al. (2015)) and is distinct from the parenchymal BM. PCs are the closest cell type abluminally apposed to the endothelial vessel wall, embedded in a common basal lamina with ECs. Although PCs are also regularly found in the BM of non-BBB microvessels, the number of PCs along the CNS vasculature is considerably higher (Sweeney et al. 2019). Although this morphological peculiarity was known for a long time, only in the last decade the relevance of PCs for BBB integrity has been elucidated in more detail (Daneman et al. 2010a; Armulik et al. 2010). Specifically, upon interfering with PC differentiation and vascular attachment by manipulating the *Pdgfrb*/*Pdgfr β* signalling pathway, brain vessels show increased paracellular leakage and depolarization of astrocytic endfeet (Armulik et al. 2010). Although the specific function of PCs for maintaining a BBB-like expression profile in BCECs and for supporting TJ integrity remains controversial, PCs are clearly crucial for the barrier function. This effect is currently also exploited to improve in vitro models of the BBB, through co-culture paradigms of PCs and ECs (Daneman et al. 2015; Neal et al. 2019). Due to the fact that PCs develop much earlier during ontogeny than ACs, they are likely relevant for the early differentiation steps in embryonic development, whereas ACs may be required for BBB maintenance (Blanchette and Daneman 2015). However, the latter has not systematically been explored. What became apparent was that communication between PCs and ACs is essential for the proper polarization and investment of ACs along the vascular wall (Armulik et al. 2010). Hence, potentially the parenchymal BM, located between PCs and ACs might fulfil a crucial role herein. The parenchymal BM is deposited by astrocytic endfeet that almost completely cover microvessels in the brain parenchyma. Besides laminin $\alpha 1$ and $\alpha 2$, AC endfeet secrete also the heparansulfate proteoglycan agrin, which is necessary for endfeet polarization (Noell et al. 2007, 2009; Yao et al. 2014). Specifically, agrin is crucial for the correct targeting of the water channel aquaporin-4 (Aqp4) to the endfoot membrane, at which it has a tenfold higher density than at non-endfoot membranes. Aqp4 accumulates together with the inwardly rectifying potassium channel Kir4.1, which is mainly responsible for the maintenance of the negative

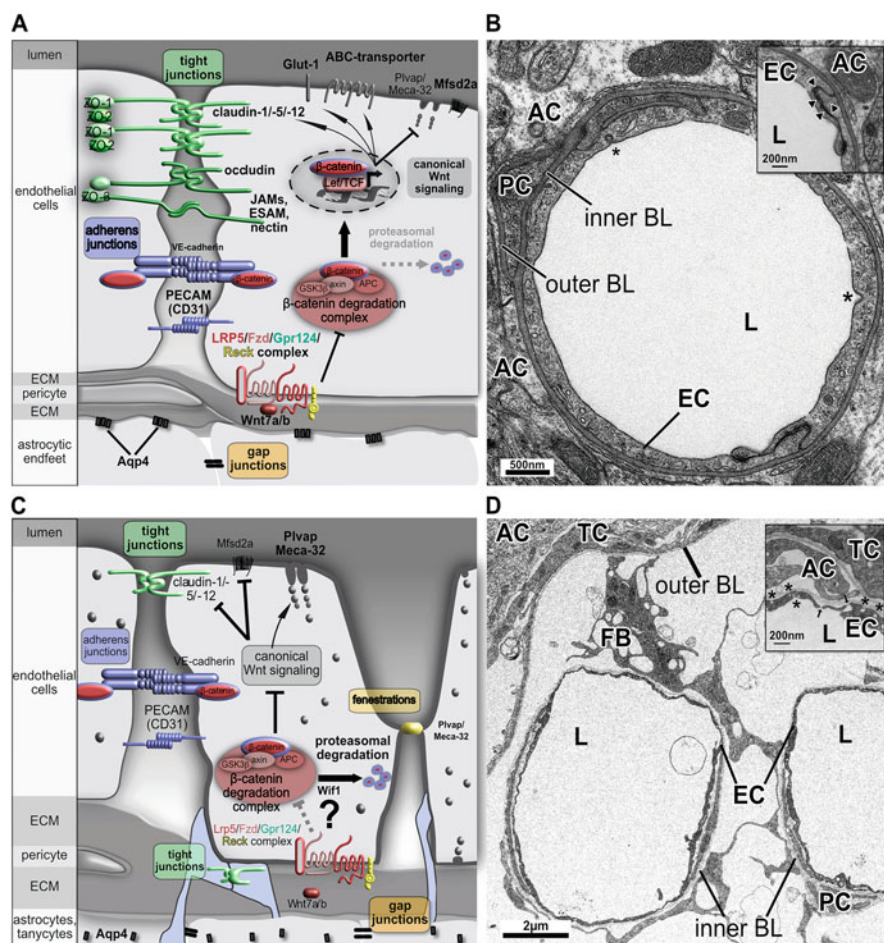


Fig. 1 Scheme of the cellular and molecular organization of the NVU proper and leaky vessels in the CVOs. **(a)** NVU of the prototypic BBB with continuous, tight endothelia connected by elaborate cell-cell junctions and showing low levels of transcytosis. Continuous levels of active Wnt/β-catenin signalling are required to maintain the BBB phenotype in ECs of the NVU. Close attachment of PCs and ACs with endfeet structures that show polarized expression of Aqp4. **(b)** Transmission electron micrograph of a prototypical, cortical BBB capillary. Asterisks mark vesicular transport. Inset shows higher magnification of the interendothelial junction. Arrowheads point to membrane “kisses” indicative for TJs. **(c)** NVU scheme of leaky, fenestrated vessels in areas of the CVOs, characterized by low level expression of tight junction proteins, high level of transcytosis and discontinuous ECs with fenestrations. Tanycytes (TCs) that are connected by tight junctions limit the leakage of blood-borne substances into the brain parenchyma. Absent or very low levels of Wnt/β-catenin signalling allow the formation of fenestrations. The question mark indicates that the mechanisms, responsible for the undetectable levels of Wnt/β-catenin signalling in CVO vessels are currently poorly understood. **(d)** Transmission electron micrograph of leaky, fenestrated vessels in the SFO, as a typical sensory CVO. Note the large perivascular space hosting fibroblast-like cells that wrap the vessels. Inset shows fenestrations and vesicular transport indicated by arrows and asterisks, respectively. AC astrocyte, BL basal lamina, EC endothelial cell, ECM extracellular matrix, ESAM endothelial cell-specific adhesion molecule, FB fibroblast, JAM junctional adhesion molecule, L lumen, PC pericyte, TC tanycytes, Wif1 Wnt inhibitory factor 1

resting membrane potential of ACs (Kofuji et al. 2000). This clustering is mediated by the anchoring of dystroglycan to the basement membrane in general and to agrin and laminin in particular (Guadagno and Moukhles 2004). Aqp4 is responsible for maintaining water homeostasis in the CNS and is part of the so-called spatial buffering property of ACs. Herein endfoot swelling is commonly seen in pathological conditions, allowing for the reduction of cytotoxic oedema in the CNS (Abbott et al. 2006; Warth et al. 2005; Kovács et al. 2012).

Besides PCs and ACs, MGs are frequently detected along the microvascular tree in the CNS, probing the vasculature for the entry of inflammatory compounds and cells (Thurgur and Pinteaux 2019) (Fig. 1). More recently, MGs have attracted scientific attention given that in all pathological conditions MGs become activated and may have, depending on their activation status into pro- and anti-inflammatory MGs, beneficial or detrimental effects on disease progression (see Part IV: BBB and Diseases: Novel treatment approaches from a BBB perspective).

Although neurons are regularly considered as a part of the NVU, their actual contribution to BBB functionality is poorly defined. Neuronal nerve endings at ACs, PCs as well as at smooth muscle cells (SMCs) were shown to influence the vascular tone and therefore cerebral blood flow (CBF) and oxygen supply of the surrounding CNS tissue (Iadecola 2017; Andreone et al. 2015) (discussed in more detail below).

1.2 Molecular Composition of BVEC Junctions

Similarly, the molecular composition of the BBB was for long considered to be uniformly established throughout the CNS. However, increasing evidence points to considerable region-specific variations of BBB signature genes (Wilhelm et al. 2016; Noubissi et al. 2018).

The prototype of BBB capillaries is characterized by elaborate TJs that are mainly formed by claudin-5 (Cldn5) that has been shown to regulate barrier function for small molecular components below 800 Da (Nitta et al. 2003). Several other claudin (Cldn) family members have been shown and discussed to be expressed in ECs of the BBB, although more recently the contribution of Cldn1, Cldn12, and particularly of Cldn3 has been challenged, given that commercially available antibodies recognized junctional proteins in Cldn3-deficient mice (Castro Dias et al. 2019) (Fig. 1). Certainly, these findings demonstrate that quantitatively, other claudins than Cldn5 do not play a major role in BBB ECs under steady state conditions. However, there is recent evidence that under pathological conditions such as stroke, Cldn3 together with occludin (Ocln) may support TJ and BBB integrity (Winkler et al. 2020). Whether this holds true for other pathologies and how Cldn family members functionally work together for barrier function still has to be determined in more detail. Cldns are linked to the cytoskeleton via members of the zonula occludens proteins (ZO-1/-2/-3) that bind to the PDZ-binding motive at the C-terminus of Cldns (van Itallie and Anderson 2014). The binding of Cldns to the cytoskeleton has been considered to influence the appearance of TJs in classical freeze fracture preparations, a technique that uniquely offers the possibility to characterize TJs

with regard to their complexity and to their particle association to either the exocyttoplasmic (E-face) or protoplasmic (P-face) fracture face (Kniesel et al. 1994; Liebner et al. 2000). Although it is unclear which molecular cues determine the E-/P-face association, it is a robust parameter to determine Cldn characteristics. At the BBB, it has been shown that Cldn5 forms E-face-associated TJ strands, whereas Cldn1 and Cldn3 form P-face associated TJ strands in single fibroblast transfectants in vitro (Furuse et al. 1999; Morita et al. 1999). In BBB ECs, the ratio of E- and P-face particle distribution has been described to be 40/60 in vivo (Wolburg et al. 2003). Despite the predominant role of Cldn5, this observation suggests that other Cldns participate in TJ formation in vivo (Berndt et al. 2019). The role of other Cldns than Cldn5 might be particularly important at contact points of three ECs, so-called tricellular junctions. At these sites additional proteins such as tricellulin and LSR/angulin are required to organize TJ stability (Sohet et al. 2015; Ikenouchi et al. 2005; Masuda et al. 2011). However, the precise molecular composition at tricellular junctions, as well as the correlation of freeze-fracture morphology and molecular composition of TJ strands requires further investigation.

Within the TJ region, BCECs express members of the IG superfamily like junctional adhesion molecules (JAMs) and endothelial cell-selective adhesion molecule (ESAM), which have regulatory function at the BBB (Garrido-Urbani et al. 2014; Tietz and Engelhardt 2015). As opposed to epithelial cells, brain ECs have intermingled adherens junctions (AJs) within the TJ zone that are formed by vascular endothelial cadherin (Cdh5, cadherin-5, VE-cadherin) that, like other classical cadherins, is connected via β - or γ -catenin as well as α -catenin to the actin cytoskeleton (Dejana and Orsenigo 2013) (Fig. 1). In the cytoplasm, additional proteins bind to the complex of VE-cadherin and β -catenin that were shown in the last years to be crucial for vascular function particularly in the CNS, given that their ablation leads to the formation of cerebral cavernous malformations (CCM). The homologous proteins are named after the pathology as CCM protein 1 (also known as Krev interaction trapped protein 1, Krit1), CCM2 and CCM3. CCM1 links β -catenin to CCM2 and CCM3, as well as to the mucin-like membrane protein heart of glass (HEG1), which was first described to be important for zebrafish heart development (Lampugnani et al. 2017). Loss-of-function mutations in either CCM1, 2 or 3 lead to vascular instability specifically in the CNS, due to defects in endothelial polarization and promotion of endothelial-mesenchymal transition (EndMT), resulting in neurological symptoms and frequently to haemorrhagic stroke (Flemming et al. 2017). The functional relevance of AJs in brain ECs is not only based on their structural function, bringing and holding ECs together, but in the last decade, endothelial AJs evolved as highly specific signalling platforms that are involved in developmental as well as pathological vascular responses (see below).

1.3 Transport and Metabolic Barrier

The obliteration of the paracellular pathway, accomplished by the formation of complex TJs between brain capillary ECs is not the only measure of the BBB to

protect the CNS from blood-borne noxious compounds. BBB ECs additionally express high levels of ATP-binding cassette (ABC) transporters, specifically Abcb1a (P-gp, MDR1) and Abcg2 (breast cancer resistance protein, BCRP) (Campos et al. 2012; Miller 2015; Mahringer and Fricker 2016) (see chapters 4 & 6). As already indicated by their names, ABC transporters are frequently upregulated in tumour cells, conferring resistance to chemotherapeutic agents. In brain ECs, the ABC transporters are mainly located at the luminal membrane and shuttle a large variety of substrates back into the blood. This transport barrier is responsible for the low bioavailability of a large number of compounds and drugs in the CNS. In addition to their role as a metabolic barrier, specifically P-gp has been shown to play a major role in the clearance of brain-derived waste products such as amyloid β (A β) in Alzheimer's disease (AD; see Chap. 13).

Besides the transport barrier, metabolic processes within ECs clearly influence and modulate barrier properties. In particular the cytochrome P450 enzyme family member Cyp1b1 has been demonstrated to be enriched in brain ECs and to contribute to the metabolism of retinoic acid and arachidonic acid, thereby modulating ABC transporter and barrier gene expression, respectively (Dauchy et al. 2008; Ziegler et al. 2016). How EC metabolism contributes in detail to BBB function and regulation still requires additional investigation. This field of research might be particularly interesting in the context of regional vascular and barrier heterogeneity in the CNS, which is discussed in more detail below.

1.4 BBB Formation and Maintenance

As mentioned above, the barrier function of ECs in the CNS of adult vertebrates is closely linked to the establishment and integrity of the NVU. During development however, the NVU might not consistently be formed as long as angiogenic vessel growth is ongoing in the CNS, which is the case in rodents up to 2 weeks after birth (Daneman et al. 2010b; Ek et al. 2010). Nevertheless, during embryonic development, CNS vessels start to develop the BBB phenotype along with brain angiogenesis, initiating around embryonic day (E) 9.5, and show detectable BBB characteristics with regard to permeability and TJ markers around E15. This suggests that even in the absence of a defined NVU, ECs can develop barrier properties (Blanchette and Daneman 2015; Saunders et al. 2018; Hagan and Ben-Zvi 2015). Although in the past the definite timepoint of BBB functionality was passionately discussed, the present data suggest that vessels are indeed largely tight already at ~E15 in the mouse, whereas the development of the full BBB expression profile, as well as TJ morphology reaches maturity postnatally (Sweeney et al. 2019; Blanchette and Daneman 2015).

With starting angiogenesis of the neuroectoderm, vessels receive signals from neuroectodermal cells, mainly from neuroblasts, that induce BBB characteristics (Stewart and Wiley 1981). Herein the Wnt/ β -catenin signalling pathway, specifically induced by the ligands Wnt7a and Wnt7b, has been identified as the major driver of CNS angiogenesis and BBB induction in BCECs (Liebner et al. 2008; Daneman

et al. 2009; Stenman et al. 2008) (Fig. 1a). In parallel to the induction of barrier properties in BCECs, the endothelium loses markers of peripheral vessels in particular plasmalemma vesicle-associated protein (Plvap), also known as Meca32, that is involved in the formation of endothelial fenestrae diaphragms, as well as in stomata of caveolae (Hallmann et al. 1995). Moreover, the Wnt/ β -catenin pathway is equally crucial for maintaining barrier function in the adult vertebrate organism, given that deletion of one or multiple pathway genes in ECs leads to breakdown of the endothelial barrier, resulting in CNS haemorrhages (Zhou et al. 2014). It turned out in the last couple of years that the specificity of the Wnt/ β -catenin pathway in brain ECs is dependent on a highly complex and specific set of receptors. Wnt7a/b-binding and downstream signalling requires except for frizzled receptors – frizzled-4 (Fzd4) in ECs – and Lrp co-receptors (Lrp5 or 6) the presence of the Wnt7-binding, GPI-anchored protein Reck as well as the G-coupled receptor Gpr124 (Vanhollebeke et al. 2015; Eubelen et al. 2018; Zhou and Nathans 2014; Chang et al. 2017; Vallon et al. 2018; Ulrich et al. 2016). Identification of this receptor complex and the decoding of the Wnt7 signalling specificity in brain ECs was the first description of a unique Wnt-code that confers functional specificity in a given cell type (Eubelen et al. 2018; Vallon et al. 2018). The process of BBB induction is, as known by now, even more complex, based on the observation that the Wnt/ β -catenin pathway in ECs presents in different flavours. Besides Wnt7a/b, the non-Wnt-related Fzd ligand Norrie-disease protein (norrin) has been shown to be able to induce Wnt signalling in CNS ECs (Zhou et al. 2014; Ye et al. 2009; Wang et al. 2018). Apparently, the norrin-mediated signal that requires the co-receptor tetraspanin 12 (Tspan12) is confined, at least during embryonic development, to the inner ear, the optic stalk and the retina as part of the CNS, as well as the olfactory bulb and the cerebellum (Zhou et al. 2014; Junge et al. 2009). Interestingly, in postnatal stages, no clear expression boundaries of norrin can be identified as most of ACs show high expression of norrin in a knock-in reporter mouse line (Ye et al. 2011), suggesting that norrin may have homeostatic function in the adult rodent brain. Given the importance of the Wnt/ β -catenin signalling for barrier induction, maturation and maintenance, the pathway requires precise control. At least, in the retina, the Wnt modulator adenomatous polyposis coli downregulated 1 (Apcdd1) was shown to be involved in retina angiogenesis as well as in barrier formation, as Apcdd1-deficient mice postnatally showed a transient increase in vessel density and precocious paracellular barrier tightening (Mazzoni et al. 2017). Apcdd1 is a Wnt/ β -catenin target and highly expressed throughout the CNS vasculature compared to non-CNS vessels (Daneman et al. 2010b). Besides the membrane-bound glycoprotein Apcdd1, other soluble regulators of the Wnt pathway such as Wnt-inhibitory factor 1 (Wif1) have been implicated in Wnt pathway modulation in the area postrema as well as in cerebellar tumours (Phoenix et al. 2016; Wang et al. 2019). Which factors however are required to control Wnt signalling and thereby BBB homeostasis and maintenance in different regions of the brain, spinal cord and retina has not been determined in more detail and leaves space for further studies (see section below; barrier heterogeneity) (Fig. 1a, b).

Nevertheless, the high specificity of the Wnt/ β -catenin pathway for endothelial barrier formation and the more recent identification of molecular and cellular specificity open possibilities for therapeutic sealing or eventually also opening of the BBB. As proof of concept, tightening of tumour vessels as well as CNS vasculature after stroke has already been shown (Chang et al. 2017; Reis et al. 2012).

Certainly, molecular BBB induction and maintenance in particular reaches far beyond Wnt/ β -catenin signalling. Additional pathways such as retinoic acid (RA) and Sonic hedgehog signalling have been shown to induce barrier properties and to maintain barrier integrity in ECs (Mizee et al. 2013; Alvarez et al. 2011). Although the barrier-inducing and barrier-inhibiting effects of RA are controversially discussed (Mizee et al. 2013; Bonney and Siegenthaler 2017), the barrier-inducing properties of RA are currently exploited for the differentiation of induced pluripotent stem cells into BBB ECs (Neal et al. 2019; Lippmann et al. 2014). Stabilization of the BBB and more specifically of the NVU in the adult vertebrate brain is also influenced by pathways like the angiotensin/Tie pathway that is mostly investigated in the context of developmental and neo-angiogenesis in tumours, stroke and other pathological conditions (Reiss et al. 2015). Given that angiotensin 1 (Ang-1) has been shown to promote vascular stability whereas angiotensin 2 (Ang-2) destabilizes blood vessels via the Tie2/TEK receptor during angiogenic processes, Ang-1 activation reduces vessel leakage in stroke and glioblastoma (Gurnik et al. 2016). In the NVU Ang-1 is mainly expressed in non-endothelial cells such as SMCs, ACs, FBs and to some extent in PCs (Vanlandewijck et al. 2018). How Ang-1 is regulated at the molecular level is not entirely known, but it was shown that the src-suppressed C-kinase substrate (SSeCKS) leads to upregulation of Ang-1 in ACs (Lee et al. 2003). Although Ang-1, through the activation of Tie2/TEK, supports the expression of TJ genes such as ZO-1, additional investigations are required to understand the function of the angiotensin/Tie2 pathway at the NVU in more detail.

More recently, the reelin signalling via the apolipoprotein E receptor 2 (ApoER2), VEGFR2 (vascular endothelial growth factor receptor 2) and downstream Dab1 activation was shown to mediate proangiogenic function in brain ECs and to influence microvessel stability and BBB function in mice (Segarra et al. 2018). Specifically, Dab1 signalling appeared to regulate the attachment of astrocytic endfeet via the endothelial deposition of laminin- α 4 that is recognized by AC-expressed integrin- β 1. The endothelial barrier properties appear to be decreased with regard to both, their paracellular and their transcellular components, suggesting that at least during brain angiogenesis the reelin pathway contributes to the control of NVU formation and stability.

1.5 Transport at the BBB

In the adult BBB, due to the presence of elaborate TJs and the lack of substantial transcellular vesicular transport, essentially all hydrophilic nutrients, fatty and amino acids need to be transported across the endothelial wall at the level of CNS

capillaries. To accomplish this, CNS ECs express a plethora of transporters that can be categorized into efflux solute carrier (Slc) transporters. The efflux transporters belong to the ATP-binding cassette (ABC) family, of which MDR1/P-glycoprotein, (Abcb1, Pgp) and breast cancer resistance protein (Abcg2, BCRP) are highly expressed at the BBB. On the other hand, solute carriers transporting amino acids and glucose transporters are most prominently represented by the large neutral amino acid transporter 1 (LAT1) – a heterodimer composed of Slc7a5 and CD98 – the glucose transporter 1 (Glut-1, Slc1a1) and monocarboxylate transporter 1 (Slc16a1, MCT1), respectively (Abbott et al. 2010; Barar et al. 2017; Duelli et al. 2000). Ions like iron and large molecular compounds like apolipoprotein E (ApoE) are transported via receptor-mediated transcytosis by the transferrin receptor (TfR) and low-density lipoprotein receptor-related protein 1 (Lrp1), respectively (Abbott et al. 2010). While TfR is frequently used as a Trojan horse to carry drugs across the BBB, Lrp1 has been implicated in the clearance of amyloid β , the major component of plaques in Alzheimer's disease (Niewoehner et al. 2014; Dennis and Watts 2012).

Moreover, essential fatty acids required for neuronal function need to be transported into the CNS. Specifically, the Major Facilitator Superfamily Domain Containing 2a (Mfsd2a) was shown to convey essential omega-3 fatty acids and to control transcellular vesicular transport in murine CNS ECs (Ben-Zvi et al. 2014; Nguyen et al. 2014). Recent evidence also points to a therapeutic value of Mfsd2a, as it appears to have beneficial effects in conditions of intracerebral haemorrhage by suppressing transendothelial vesicular transport (Yang et al. 2017).

Interestingly, in the last couple of years growing evidence points to a regulation of BCEC permeability on both the paracellular and the transcellular level during BBB development, maintenance as well as under pathological conditions. Specifically, the paracellular barrier is the first to be established during development and is the last to break down in disease. Transcellular transport instead appears to regulate a wide range of permeability levels from highly leaky to highly tight. This working model has major consequences for our understanding of the BBB, which currently shifts from a “barrier yes-or-no” to a more “graded” barrier concept. Probably the best indication for different “flavours” and functional levels of the BBB comes from work describing barrier heterogeneity within the CNS (Fig. 1a).

2 Vessel and BBB Heterogeneity in the CNS

As mentioned above, the discrimination between impermeable BBB and leaky non-BBB vessels is a highly simplified view. Even within the CNS there is local heterogeneity in barrier composition and structure between different compartments and regions, resulting in diverse levels of permeability (Wilhelm et al. 2016). Probably the most prominent regions harbouring leaky vessels in the brain are the choroid plexus (CP) and the circumventricular organs (CVOs). But also other regions of the CNS, such as the retina, spinal cord and cerebellum with – at first glance – no clear-cut differences in vessel permeability, show considerable site

specificity. In the following, local barrier heterogeneity within the CNS is discussed, focusing on CVOs and their morphological and molecular peculiarities, as well as their functional roles.

2.1 Circumventricular Organs (CVOs)

The CVOs, currently attracting increasing interest in research, are small specialized midline structures localized around the third and the fourth ventricle of the brain. Their capillary plexus is characterized by a high density of fenestrated vessels. Although knowledge of the CVO development is limited, there is no uniform developmental theme, suggesting that the denotation “CVO” comprises a group of structures with similar features but different origin (Kiecker 2017). The comparison of CVOs in different species (fish to human) points to the assumption that the presence of CVOs is highly conserved in the chordate phylum, while their appearance might be diverse (Kiecker 2017; García-Lecea et al. 2017; Benz et al. 2019). The permeable vessel phenotype is the physiological state in these restricted brain regions (Sisó et al. 2010). Permeability does not only mean the absence of endothelial TJs, characterized by the lack of TJ proteins like Occludin and Cldn5, but also the loosened interendothelial adherens junctions and presence of fenestrations compared to parenchymal BBB vessels. The special feature of a leaky vascular phenotype in the CVOs coined the term “windows of the brain”. The peculiarity is used for informational exchange from the CNS milieu to the blood and vice versa. According to their function, the CVOs either sense molecules from the blood (sensory CVOs) or facilitate the secretion of molecular cues (secretory CVOs). While the vascular organ of the *lamina terminalis* (VOLT; synonym: *organum vasculosum of the lamina terminalis*, OVLT), the subfornical organ (SFO) and the area postrema (AP) are classified as sensory CVOs, the median eminence (ME), the neurohypophysis (posterior pituitary, PP), the pineal gland (PI) and the subcommissural organ (SCO) are determined as secretory CVOs. Even though the complete functions are not well understood yet, the AP is described as the vomiting centre, whereas the PI releases melatonin into the blood stream, which is involved in the control of circadian physiology. During the last couple of years, the OVLT, the SFO and the PP together with the median preoptic nucleus (MnPo) were shown to be important for the regulation of thirst and drinking behaviour (Augustine et al. 2018) (Fig. 1c, d).

2.1.1 Common Features of CVOs

Endothelial Cells

The specialized leaky vasculature within the CVOs is atypical in the CNS (Behnson 1927; Wislocki and Leduc 1952). The leakiness was surprising for Price and colleagues as they observed that intravenously or subcutaneously injected tracer molecules were not retained in blood vessels, but accumulated in the CVO parenchyma (Price et al. 1981, 1984). While interendothelial junctions in the CVOs are

less elaborate and the majority of vessels lacks TJ proteins such as Cldn5 and Ocldn, the paracellular transport is facilitated (Morita et al. 2016; Mark and Farmer 1984). The permeability marker Meca32/Plvap is a basic component of diaphragms in fenestrations, caveolae and transendothelial channels (Guo et al. 2016). It is especially expressed in leaky vessels and therefore labels CVO ECs, which are highly fenestrated (Hallmann et al. 1995). While the endothelial expression of Meca32/Plvap is ubiquitous at the beginning of murine embryonic development, the labelling disappears upon onset of BBB functionality at E15 in the CNS vasculature (Liebner et al. 2008; Hallmann et al. 1995). Instead, the permeable phenotype in the CVO vessels is stable throughout life, and vessels retain a high number of fenestrations that are important for their homeostatic function (Hallmann et al. 1995). Morita and colleagues identified the sensory CVOs as sites of VEGF-dependent dynamic vascular plasticity by the presence of endothelial apoptosis as well as sprouting (Morita et al. 2015).

Organization of the NVU

As described above, the BBB function relies not only on the endothelial barrier character, but also on the interaction of all cell types in the NVU, particularly PCs and ACs. As there is a lack of BBB properties within the CVOs it is also interesting to have a closer look at the NVU in these regions. Conspicuously, the perivascular space is notably broad and displays a zone for communication between the blood and the CNS compartment. However, the perivascular space in the sensory CVOs also functions as a diffusion barrier, as high-molecular-weight tracers do not enter into the surrounding tissue, but accumulate in the perivascular region which is surrounded by glial cells (Morita and Miyata 2012). PCs are present in CVOs and express the pericytic markers NG2, N-aminopeptidase (CD13) and platelet-derived growth factor receptor beta (PDGFR β), outlining leaky vessels (Benz et al. 2019; Morita et al. 2014).

As already mentioned above, the CVOs play a crucial role in the regulation of body fluid homeostasis. Experiments with the chronic application of a salt solution instead of drinking water revealed elevated expression of PDGFB in the SFO, OVLT and AP and increased levels of NG2 in the OVLT and the SFO (Morita et al. 2014). These changes resulted in elevated leakage of a low-molecular-mass tracer in the OVLT and the SFO (Morita et al. 2014). Additionally, the typical polarized AC endfeet appearance is missing in the CVOs whereas several layers of astrocytic processes could be evidenced by electron microscopy (Benz et al. 2019; Morita et al. 2016). Accordingly, ACs do not show the polarity markers commonly known from AC endfeet at BBB microvessels. This is supported by the finding that Aqp4, known to be localized at BBB AC endfeet processes, does not show a polarized localization at CVO vessels (Rash et al. 1998; Wolburg et al. 2009). Reallocation of these markers from the endfeet to the whole AC cell membrane implies BBB disruption in different pathological conditions (Warth et al. 2005; Rascher et al. 2002; Wolburg-Buchholz et al. 2009). In the CVOs, labelling of the transmembrane subunit β -dystroglycan, which links the extracellular α -dystroglycan subunit to the membrane, outlines the vascular structure in the SFO (Pócsai and Kálmán 2015),

which is in line with the astrocytic laminin $\alpha 2$ (Lama2) that broadly demarcates the vessel shape (Benz et al. 2019). α -Dystroglycan instead is diffusely distributed in the whole SFO parenchyma. In contrast, the glial fibrillary acidic protein (GFAP) and Kir4.1 represent a tanycyte-like staining pattern (Benz et al. 2019; Pócsai and Kálmán 2015). These findings support the conclusion that ACs are present in the CVOs but are differently organized compared to their appearance in typical BBB vessels. This difference leads to unpolarized AC endfeet that surround the vasculature not as tightly as in the cortex, likely facilitating the communication between the ECs and the surrounding cell types, e.g. neuronal endings (Fig. 1c, d).

Barrier Heterogeneity Within the CVOs

Besides the difference of barrier properties of the CVOs and the cortex, there is further barrier heterogeneity within the CVO vasculature. The CVOs are divided into central parts, which are called “capillary plexus” for the OVLT, “ventromedial core” for the SFO and “central zone” for the AP (Sisó et al. 2010; Morita et al. 2016). The centre regions demonstrate the peak of permeability confirmed by low-molecular-weight tracer assays (Morita et al. 2016). Tracer leakage is reduced in distal zones (“lateral zone” in the OVLT, “outer shell” in the SFO and the “perivascular zone” together with the “lateral zone” in the AP). In the central parts of the CVOs, the expression of Meca32/Plvap is at its maximum, whereas the level of endothelial junction proteins such as Cldn5, Occludin and ZO-1 is nearly completely absent (Benz et al. 2019; Morita et al. 2016). The balance of this negative correlation of permeability- to TJ-related proteins is shifted towards a tighter appearance in the proximal zones indicated by the result of less tracer leakage. Additionally, a further type of barrier heterogeneity is evident along the longitudinal axis of the SFO (Benz et al. 2019). Whereas the caudal tissue harbours mainly leaky vessels, the rostral tip shows a mixture of a leaky and tight endothelial phenotypes on a cellular level, meaning a single Meca32/Plvap-positive and Cldn5-negative EC can be located between two Cldn5-positive and Meca32/Plvap-negative cells. This result leads to the assumption that the molecular cues inducing endothelial barrier properties act on a single-cell level and thereby creating a special transition zone of intermingling tight and leaky vessels at the rostral SFO tip (Benz et al. 2019). How the regulation of barrier properties is achieved on a cell-to-cell resolution is currently not known and requires further investigation.

2.1.2 Fluid and Sodium Homeostasis

One of the basic physiological needs of all organisms is the uptake of water to prevent dehydration as a consequence of urination, sweating and evaporation (Leib et al. 2016). As already mentioned above, the thirst-associated CVOs, the SFO, the OVLT and the PP also take part in fine-tuning regulatory processes for the maintenance of body fluid homeostasis. Therefore, little changes in the blood osmolarity, pressure or volume have to be immediately recognized and counteractions need to be induced, resulting in the sensation of thirst and finally in drinking behaviour. Although the complex signalling network is not completely understood, several recent studies point to the sensory OVLT and the SFO as the predominant sensors

for salt imbalance (Grobe et al. 2011). The monitoring of blood osmolarity is located in the SFO and the OVLT. ACs, glial and ependymal cells express atypical Na (x) channels in both, the SFO and the OVLT (Watanabe et al. 2006; Sakuta et al. 2019; Nomura et al. 2019; Guyenet 2019). Furthermore, the epithelial sodium channel (ENaC) was identified in neurons of sensory CVOs, suggesting a comparable role in regulating sodium reuptake as in the kidney epithelium (Miller et al. 2013). Moreover, angiotensin II (Ang II), a major regulator of blood pressure, may regulate ENaC expression in CVO neurons as it does in the kidney, where it initiates salt and water intake, vasoconstriction and fluid reuptake. Both, the precursor angiotensinogen and the enzyme renin, are synthesized in neuronal and glial cells directly in the SFO (Lavoie et al. 2004).

With optogenetic methods the presence of two distinct neuronal populations within the SFO mediating a “thirst-on” and a “thirst-off” signal were characterized by ETV1 and Vgat expression, respectively (Oka et al. 2015). The lamina terminalis, consisting of the OVLT, the SFO and the MnPo which is located between the two CVOs, was identified as a main actor in thirst regulation and to operate in anticipatory regulation of fluid homeostasis (Augustine et al. 2018). This is particularly important to coordinate body fluid parameters over the entire day–night cycle, specifically during the sleep phases that intercept water consumption. To overcome this problem, mice show an anticipatory drinking behaviour in a short period before resting (Gizowski et al. 2016, 2018). In this context, the drinking behaviour is not motivated by thirst but induced by a circadian rhythm driven by the mammalian master clock accommodated in the supraoptic nucleus (SCN). The latter functions as a pacemaker to induce the individual rhythm on a cellular level by the oscillation of transcription factors circadian locomotor output cycles kaput protein (Clock), and brain and muscle ARNT-like 1 (Bmal1) (Darlington et al. 1998; Emery et al. 1998; Thresher et al. 1998; Gekakis et al. 1998). SCN neurons project into the OVLT, where they activate thirst neurons by the release of the neurotransmitter vasopressin in a clock-dependent manner. These findings bring up the question, if and how vessels in the CVOs may participate in the highly complex regulation of body fluids in a circadian fashion.

2.2 Barrier Properties in the Choroid Plexus

The CP has an outstanding appearance in the CNS. It is a floating structure in the ventricles composed of a capillary plexus of fenestrated blood vessels, which are surrounded by a specialized epithelial lining that is continuous with the ependymal epithelium (Maxwell and Pease 1956). The permeable phenotype of ECs was proved in rat by intravenous injection of tracer molecule cytochrome c, which passes the endothelial wall and accumulates in the CP epithelium (Davis and Milhorat 1975). The authors also noted that there was no tracer found in the cerebral ventricle (Davis and Milhorat 1975). After analysing freeze fracture samples of the rat CP endothelial TJs are described as “... a loose discontinuous arrangement of isolated, often branched junctional elements ...” (van Deurs 1979). The unique role of the CP epithelium was already indicated in the middle of the last century when the authors

described the apical shape occupied with an enormous number of small protrusions and simultaneously suggested a correlation to functions of other epithelia, where surface extensions are associated with water transport (Maxwell and Pease 1956). On the one hand, the cerebro-spinal fluid (CSF) contains nutrients for the brain parenchyma, but also absorbs waste products from the CNS tissue on the other. Therefore, the CP epithelium is responsible for the removal of metabolites and toxic molecules and for the secretion of the CSF. In young adults the turnover of the entire CSF volume takes place four to five times in 1 day and night cycle (Sakka et al. 2011). Recently, neuronal activity and dynamic CSF flow was linked to brain recovery during human sleep phases (Fultz et al. 2019). In the process of aging this renewal of CSF is reduced and thereby yields in the accumulation of waste products as seen for neurodegenerative pathologies (Sakka et al. 2011; Glerup et al. 2019). Research focusses on identifying molecules that show a pattern of expression connected to neurodegenerative diseases such as Alzheimer's dementia (Glerup et al. 2019; Olsson et al. 2016). CSF secretion by the CP is an active and selective process that is highly controlled by the so-called blood–CSF barrier (BCSFB) (Lun et al. 2015)). Therefore, the cell bodies of the epithelial lining are tightly connected similarly to the ependyma of the CVOs. The TJs of the BBB differ from those in the BCSFB by their composition of different claudins. While the predominant claudin in the BBB is Cldn5 the epithelial cells in the adult rat CP are mainly connected by the Cldn1, Cldn2, Cldn3, Cldn9 and Cldn19 (Kratzer et al. 2012; Lippoldt et al. 2000). As already mentioned, the presence of Cldn3 is controversially discussed as a recent study shows that Cldn3-deficient mice represent intact brain barriers (Castro Dias et al. 2019). Interestingly, although Cldn3 appears to be present at the BCSFB, Cldn3 deletion does also not lead to an impairment of the BCSFB (Castro Dias et al. 2019). The differential composition of the TJs probably contributes to the level of tightness, given that evidence points to a more selective barrier function of the BBB compared to the BCSFB, which displays a higher level of leakiness (Strazielle and Ghersi-Egea 2013). Studies of intravenous and intraventricular injections in *Monodelphis domestica* showed that the transport of proteins and hydrophilic molecules is unidirectional from the blood into the CSF, whereas dextran is exchanged bidirectionally (Liddelow et al. 2009). These experiments often do not consider other permeable CNS regions, such as the CVOs or the SVZ, where intravenously injected tracers also accumulate and may potentially reach the CSF by crossing the ependymal wall. The CSF also fills the subarachnoid space, which harbours a leaky vasculature, located between the dura mater and the brain-enwrapping pia mater (reviewed in (Liddelow 2011)). Therefore, cells of the arachnoid membrane directed to the subarachnoid space represent the CSF–brain barrier by the expression of TJs.

2.3 Barrier Properties in the Cerebellum vs. Cortex

The BBB fulfils a protective function to avoid damage to the CNS. This can be negatively influenced, resulting in increased permeability during emotional states or stress (Belova and Jonsson 1982; Skultéryová et al. 1998). Even in a healthy

physiological condition, there are differences in the degree of barrier tightness. While most ECs in the CVOs and the CP lack a barrier phenotype, there are also regional variations of barrier function within the entirety of BBB vessels. This has been shown experimentally using intravenously administered tracers. Yen and colleagues have used Evans blue, which has a molecular size of 981 Da but binds to serum albumin and finally displays a high-molecular-weight tracer (~70 kDa), as well as the low-molecular-weight tracer sodium fluorescein (376 Da) (Yen et al. 2013). Interestingly, they did not reveal an overlaying staining pattern within the brain. While Evans blue was rarely found in the striatal tissue but showed maximal leakage into the prefrontal cortex and the cerebellum, sodium fluorescein was detected at similar levels in the entire brain (Yen et al. 2013). The authors propose different routes of BBB crossing as reason for these regional variations. Furthermore, a molecular heterogeneity in different brain regions was discovered by deleting LRP5 or Fzd4 in ECs, which lead to massive permeability for Evans blue in the olfactory bulb and the cerebellum, but not in the cortex (Zhou et al. 2014). Dominant activation of Wnt/ β -catenin signalling in ECs was able to rescue the Fzd4-knockout phenotype leading to the conclusion of a regional Wnt/ β -catenin dependency within the brain.

As already described, PCs are also known to contribute to BBB functionality (Armulik et al. 2010; Daneman et al. 2010a). However, Villaseñor and colleagues showed in *pdgf-b^{ret/ret}* mice, harbouring 80% loss of PC coverage at capillaries in all brain regions (Moura et al. 2017; Lindblom et al. 2003), that the depletion of PCs did not correlate to the heterogeneity of blood vessel tightness (Villaseñor et al. 2017). Increased leakiness for immunoglobulins and Evan's blue was shown in the cortex, hippocampus and striatum, whereas tracer leakage was restricted in the inter- and midbrain of *pdgf-b^{ret/ret}* mice. As the TJs were not affected by this phenotype the authors suggest a regional-dependent change in EC transcytosis as answer to a brain area-independent PC loss.

2.4 Blood–Spinal Cord Barrier

As discussed above, different CNS regions and structures exhibit different levels of barrier tightness comprising para- and transcellular tightness. While the CVOs and the CP are the leakiest blood–brain interfaces, the cortical vessels, which are commonly studied in the BBB context, present the tightest phenotype. In this regard, the CNS also comprises the spinal cord showing specific barrier properties that will be compared to the cortical BBB below. The BBB in the cortex and the spinal cord share the same building blocks of unfenestrated ECs connected by TJs and AJs, surrounded by PCs, a basement membrane and astrocytic endfeet. Yet, there are subtle differences in the organization of these components. The level of TJ proteins such as Occludin and ZO-1 is lower in the blood–spinal cord barrier (BSCB) in contrast to the BBB, while there is no difference in the expression of Cldn5 (Ge and Pachter 2006). As ZO-1 can additionally be a part of AJs (Tornavaca et al. 2015), it is in line with the observation that also typical AJ proteins, such as VE-cadherin, and the

anchor protein of the AJs to the intracellular actin cytoskeleton β -catenin, are reduced in the spinal cord (Ge and Pachter 2006). These findings suggest a decreased ability to restrict the paracellular transport of blood-borne molecules into the brain, hence increased permeability, which could be confirmed by differently sized tracers (Daniel et al. 1985). Interestingly, the passage of cytokines also shows barrier region-specific properties, as for example the extravasation of interferons is higher across the BSCB than the BBB (Pan et al. 1997). While the amount of all tagged cytokines was lowest in the brain, followed by the thoracic and lumbosacral spinal cord, it peaked in the cervical spinal cord. Particularly, the permeability for IFN α was increased in the cervical spinal cord, whereas IFN α and IFN showed comparable levels of leakage. Further investigations revealed that TNF α was saturated in the distal part of the spinal cord. The authors suggest a correlation between the high baseline passage of inflammatory cytokines with the observation that chronic inflammatory diseases such as multiple sclerosis (MS) or its murine model of experimental auto-immune encephalomyelitis (EAE) often manifest in the cervical spinal cord region.

2.5 BBB at Sites of Adult Neurogenesis

The term “adult neurogenesis” defines the differentiation – after developmental neurogenesis is complete – from neural stem or progenitor cells (NSCs) into neurons, which finally are incorporated into already existing neurons. There are two predominant regions described to be the origin of adult neuronal stem cells (NSCs): the subventricular zone (SVZ) in the wall of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Doetsch 2003; Doetsch et al. 1999). In the SVZ the B1 cells, also called stem cell astrocytes, have astroglial attributes and represent the major NSCs (Obenier and Alvarez-Buylla 2019). Their basal ending surrounds the vasculature and contacts ECs directly, whereas their apical site has a ciliated protrusion into the ependymal lining to facilitate the contact to the CSF. The B1 cells can give rise either to proliferating C cells, which finally differentiate to neuroblasts (A cells) that migrate to the olfactory bulb, or to B2 cells, which are similar to B1 cells but do not enter the ependymal wall. The B1 cells receive signals from young neurons (Fernando et al. 2011; Liu et al. 2005), from C cells (Alfonso et al. 2012), from epithelial cells (Lim et al. 2000) as well as from the vasculature (Shen et al. 2008; Ottone et al. 2014). By the contact to the CSF, the B1 cells have also the opportunity to sense blood-borne molecules entering the CSF by the CP or to signals secreted by the latter (Obenier and Alvarez-Buylla 2019; Planques et al. 2019). Additionally, the B1 cells have also excess to blood-derived molecules as tracer experiments with isothiocyanate confirmed a permeable vascular phenotype in the SVZ (Tavazoie et al. 2008). Detailed investigation of the barrier phenotype in the SVZ revealed that there are some regions lacking PC and AC coverage. Interestingly, in these areas transient-amplifying C-cells and stem cells are often in close contact to the EC lining of vessels. In general, brain injury is associated with an increase of vascular permeability. This is also true for the SVZ, where the

degree of leakiness is elevated under ischaemic stroke (Lin et al. 2018). This change leads to a better accessibility of blood-borne molecules to the stem cell niche. Although the SVZ vascular beds appear to be quiescent as there was no EC division or sprouting detected (Tavazoie et al. 2008), it was shown that systemic VEGF may indirectly act on the neurogenic niche by binding to VEGFR2 expressed by SVZ ECs (Lin et al. 2018). Moreover, VEGF may directly bind to NSCs to activate DLL4-dependent Notch signalling, thereby triggering NSC proliferation and differentiation after stroke (Lin et al. 2018). Such an effect could not be detected in the SGZ of the hippocampus, leading to the conclusion by the authors that the tight vascular barrier in the SGZ inhibits VEGF leakage (Lin et al. 2018), although in the SGZ EC proliferation is frequently observed (Palmer et al. 2000). Clusters of proliferating NPCs are found in close proximity to proliferating cells that are positive for endothelial markers. The result that angiogenesis accompanies neurogenesis indicates that both cellular systems share molecular signals or exhibit a co-dependency on VEGF (Palmer et al. 2000). Another central task of the vasculature is the guidance of neuroblasts from the SVZ to the olfactory bulb (Fujioka et al. 2019). Although there are already many factors known to be involved in the communication between the vasculature, the systemic circulation and the NSCs within the niches (reviewed in Obernier and Alvarez-Buylla (2019), Goldberg and Hirschi (2009)), investigating the heterogeneity of these highly specialized vascular beds is key for understanding the therapeutic potential, for example, to trigger adult neurogenesis in disease states.

The SVZ and the CVOs do not only share the feature of a permeable vessel phenotype, but both are also described as stem cell niches. Besides the SVZ and the SGZ, recent reports identify the CVOs as a source of adult NSCs. The CVOs harbour a lining of specialized ependymal cells, called tanycytes that contain long processes intruding the CVO parenchyma and making contact to the fenestrated vasculature in the central part (Langlet et al. 2013; Mullier et al. 2010). Their cell bodies are closely connected by TJs, marked by ZO-1, Occludin and Cldn5. The tanycytes were also shown to express the cell cycle protein Ki67, suggesting continuous renewal of these cells (Bennett et al. 2009). Bennett and colleagues were able to isolate these cells and differentiate them in culture into either Doublecortin (DCX)- and -Tubulin III-positive neurospheres or into S100-, GFAP- and RIP-expressing glial cells (Bennett et al. 2009). Assays in rats and mice revealed proliferation as well as neuro- and gliogenesis in CVOs. Besides the tanycyte-like NSCs in the AP, the OVL and the SFO were also confirmed to harbour astrocyte-like cells representing a similar protein expression pattern found in NSCs of the SVZ and the SGZ (Furube et al. 2015). NSCs of the ME are able to differentiate into ACs, oligodendrocytes and neurons (Hourai and Miyata 2013). Finally, CVOs were confirmed in humans as a source of NSCs and being associated with recruiting progenitor cells to regions of brain damage and therefore displaying an additional site of adult neurogenesis and brain plasticity (Sanin et al. 2013). Although the vasculature as a rich source of growth and differentiation factors, summarized under the term “angiocrine function” of the vasculature (Rafii et al. 2016), has gained increasing scientific interest, the

question of the vital role of the leaky vasculature for NCSs and adult neurogenesis remains largely unresolved.

3 Neurovascular Coupling

Neurovascular coupling describes the communication and the cooperation between the vascular compartment (predominantly the ECs or blood-derived molecules) with neurons and glial cells. Initially, the subject of “neurovascular coupling” was understood as a unidirectional communication of signals from the neuronal site to the endothelium (Chen et al. 2014; Longden et al. 2017; Hogan-Cann et al. 2019). Newer reports favour the view that the linkage is not a one-way route but rather bidirectional. Interference of these complex mechanisms, guaranteeing a regulated and perfectly adjusted blood flow within the brain, is associated with neuronal impairment, e.g. in neurodegenerative pathological conditions (Iadecola 2017). Specifically, the relationship of cerebral blood flow (CBF) and local neuronal activity evaluated by positron-emission tomography (PET) can be described by the blood oxygenation level-dependent (BOLD) effect. The BOLD effect reflects excess CBF delivery relative to local oxygen consumption as a possibility to non-invasively detected activity-dependent hemodynamic signals (Raichle and Mintun 2006). Additionally, the functional interaction of neuronal activity and vascular perfusion in the developing and adult brain likely coevolve. Support for this has been provided in the rodent barrel cortex, in which during early postnatal development neuronal activity determines vascular density and vice versa (Lacoste et al. 2014). Most of the current literature discusses the neurovascular interaction in the context of CBF and the resulting oxygen and nutrient supply. More recently however, evidence emerged in literature that neurons may take part in locally controlling BBB function (Nishijima et al. 2010). Nevertheless, in-depth analysis of neuronal control of BBB function has not been performed also with respect to different brain regions and therefore, may open novel fields of BBB research.

3.1 Neuronal Activity

The level of neuronal activity demands a high amount of energy (Kasischke et al. 2004). The CNS has no energy deposits and therefore, nutrients and oxygen have to be continuously provided. However, neuronal firing requires energy equivalents and produces metabolites, resulting in the change of several parameters in the surrounding milieu, such as partial oxygen pressure, ion concentration and composition, pH, temperature, neuropeptides and hormones (Ances et al. 2001). These activity-related fluctuations have to be quickly reset to optimal conditions, which is partially achieved by the adaptation of blood pressure and blood flow through vasoconstriction or vasodilation. In the last century Malonek and Grinvald ascertained the immediate and regional specific vascular responses to parenchymal hypoxia, initiated by cortical stimulation (Malonek and Grinvald 1996). Although

the complex signalling cascade is not yet completely understood, ACs were demonstrated to influence neuronal activation by their spontaneous activity, due to their ability to sense and respond to neurotransmitters, and to act on blood vessels dilation or constriction (Gordon et al. 2008; Metea and Newman 2006; Zonta et al. 2003; Parri and Crunelli 2002; Pelligrino et al. 2011; Macvicar and Newman 2015). Neuronal firing consumes oxygen, resulting in decreased oxygen partial pressure and oxyhaemoglobin (Vanzetta and Grinvald 1999; Malonek et al. 1997). This leads to lactate and downstream NO production in ACs, resulting in vasodilation of retinal arterioles, as another site of the CNS vasculature (Kasischke et al. 2004; Pellerin and Magistretti 1994; Hein et al. 2006). However, astrocytic-dependent vasoconstriction, initiated by intracellular calcium increase, leads to the production of the intermediate metabolite arachidonic acid. The latter can then be enzymatically converted to either 20-hydroxyeicosatetraenoic, which constricts vascular smooth muscle cells, or prostaglandin E₂ (PGE₂) and epoxyeicosatrienoic acids (EETs), eliciting vasoconstriction and vasodilation, respectively (Zonta et al. 2003; Lange et al. 1997; Gordon et al. 2011). As mentioned above, so far, the adaption of the vessel diameter in response to neuronal activity has predominantly been investigated. However, signals derived from the vascular compartment may impact on neuronal function and therefore are increasingly considered in neurovascular coupling. It has been demonstrated in Cre reporter mice that extracellular vesicles derived from the haematopoietic system and containing Cre mRNA were able to pass the BBB and to induce recombination in Purkinje neurons (Ridder et al. 2015). The authors comment that under physiological conditions these events were detectable but very rare. However, in the case of inflammatory conditions, the recombination in Purkinje cells and other neurons in several brain regions such as the cortex, the substantia nigra and the hippocampus was increased.

Moreover, in the SFO, as a sensory CVO involved in body water homeostasis, initial evidence was provided that changes in endothelial barrier properties by dominant-active Wnt/ β -catenin signalling influence neuronal activity in water-restricted mice indicated by elevated c-fos staining (Benz et al. 2019). These data suggest that the endothelial tightness properties may play a role in neuronal firing, supporting the concept of a bidirectional neurovascular coupling.

4 Outlook

The BBB describes a highly specific, differentiation state of the vascular endothelium in the CNS that is vital for neuronal function. In the past decade, considerable advances have been made in understanding the formation, maintenance and structural as well as molecular identity of the BBB. Moreover, first evidence for regional differences in the shaping of the BBB and the NVU has emerged. To understand the molecular signature of regional variations of the BBB in the CNS is currently one of the hot topics in the field of BBB research. However, the ultimate goal certainly is to understand the functional consequences of peculiar BBB flavours, particularly to neuronal functionality. The concept might be exemplified by the leaky or partially

leaky barrier in the CVOs and the SVZ, respectively. In these regions of the CNS, the vascular barrier function appears to participate in the regulation of body homeostasis and adult neurogenesis.

Finally, the comprehensive understanding of the BBB function in general, and of its regional heterogeneity in particular, will likely help to understand the role of the BBB in brain pathologies and the potential benefit of targeting the specific BBB properties for therapeutic intervention.

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New Insights in the Complexity and Functionality of the Neurovascular Unit

Johanna Schaffenrath and Annika Keller

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Abstract

The neurovascular unit (NVU) encompasses all brain cells and underlines that neurons, glia and brain vasculature are in intimate physical and functional association. Brain function is dependent on blood flow and local increases in blood flow in response to neural activity – functional hyperaemia takes place at the NVU. Although this is a vital function of the NVU, many studies have demonstrated that the NVU also performs other tasks. Blood vessels in the brain, which are composed of multiple cell types, are essential for correct brain

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development. They constitute the niche for brain stem cells, sense the environment and communicate changes to neural tissue, and control the immune quiescence of the CNS. In this brief chapter we will discuss new insights into the biology of NVU, which have further revealed the heterogeneity and complexity of the vascular tree and its neurovascular associations.

Keywords

Astrocyte · Endothelium · Microglia · Neurovascular unit · Pericyte · Perivascular fibroblast-like cell · Perivascular macrophage · Structural diversity · Vascular smooth muscle cell

1 Introduction

The concept of the neurovascular unit (NVU) was introduced in 2001 by the Stroke Progress Review Group meeting of the National Institute of Neurological Disorders and Stroke (US National Institutes of Health). The report, published in 2002, recognized that extensive studies on the ischaemic injury to neurons and glia have not led to effective treatment of stroke. It was stated – “At a fundamental level, there is an important need to better define the molecular influences and cell-signalling mechanisms that characterize the interactions between circulating blood elements and the blood vessel wall, extracellular matrix, glia and neurons (together, *the neurovascular unit*) during ischemic and haemorrhagic stroke” (https://www.stroke.nih.gov/documents/SPRG_report_042002_508C.pdf). Thus, the concept of the NVU connects all brain cells and underlines that neurons, glia and brain vasculature are in intimate physical and functional association. The report also highlighted barriers, which might make it difficult to study brain vessels specifically – such as the neuron-centric view on the brain and the reluctance to apply basic approaches of vascular biology to brain vessels in order to avoid simple replication of findings. Nevertheless, the small vascular biology community has over the last 10 years made a tremendous step forward in understanding the development and differentiation of brain blood vessels using different animal models (e.g. mouse, zebrafish) and forward and reverse genetic approaches. Also, neuroscientists have joined the endeavour and have greatly contributed to the understanding of how neural cells regulate the development of brain vessels and vice versa. In addition to the molecular regulation of NVU development, there is a better understanding about the structural diversity of the neurovascular unit. Although this might be surprising – new cell types at the NVU have been identified, or rather, they have been rediscovered using new technologies such as single cell RNA sequencing. Hopefully, a deeper characterization of the molecular signature of cells constituting the NVU will lead to the better understanding of the biology of this complex cellular structure that is central for the well-being of the brain. Here, we review recent advances in understanding the functions of individual cells constituting the NVU, the discovery of new cell types and increasing evidence of cross-talk between brain blood vessels and periphery.

2 Intricate Interplay Between Blood Vessels and Brain Parenchyma During Brain Development

In recent years we have witnessed an explosion of studies that have focused on the biology of the NVU. There are new insights on the development of the brain, which is a result of an elegant interplay between vascular and neural cells. Several molecules, either expressed or secreted by the neuroepithelium, stimulate brain vessel development and induce the blood–brain barrier (BBB) properties in brain endothelial cells (e.g. VEGF-A, Wnt ligands 7a/7b, presenilin-1, retinoic acid, reelin) (Daneman et al. 2009; Stenman et al. 2008; Mizze et al. 2013; Wen et al. 2005; Segarra et al. 2018; Haigh et al. 2003). Likewise, vascularization of the developing brain directly influences differentiation of neuronal progenitors by secreting proteins or indirectly by reducing hypoxia levels (reviewed in Tata and Ruhrberg 2018). Developing vessels serve as anchor points for radial glia progenitors, which has been shown to be important in regulation of progenitor proliferation. Loss of the association of radial glia with periventricular vessels in the ventral telencephalon, which is normally mediated via beta 1 integrins, leads to a decreased neuronal progenitor division and this in turn reduced the formation of parvalbumin and somatostatin positive interneurons and defective synaptic inhibition (Tan et al. 2016). A recent study showed the importance of vascular reelin signalling via endothelial Dab1 for the attachment of radial glia to pial vessel surface and proper cortical layering (Segarra et al. 2018). In addition, blood vessels are an important scaffold and inducer for neuronal migration, both during embryogenesis and in adults (Won et al. 2013; Segarra et al. 2015). Developing blood vessels attract oligodendrocyte precursors (OPCs) positive for CXCR4 via CXCL12 and provide a migration scaffold for OPCs in the developing brain (Tsai et al. 2016). In addition, the association between OPCs and blood vessels is important for keeping the OPCs undifferentiated during migration (Tsai et al. 2016).

There are indications that the brain vasculature may possess intrinsic regional differences, which are dictated by the region-specific transcriptional programs of neuronal progenitors (Vasudevan et al. 2008), and that different molecular mechanisms guide brain vessel development in different brain regions during embryogenesis (Cullen et al. 2011; Anderson et al. 2011; Kuhnert et al. 2010). Blood vessels constitute the niche for neural stem cells (NSC) in neurogenic zones, which reside in the subventricular zone of lateral ventricles and in the subgranular zone in the hippocampus. NSC reside near and are in direct contact with capillaries. In addition, blood vessels in these zones display variable permeability (reviewed in Karakatsani et al. 2019). Angiocrine signals, such as EphrinB2 and Jagged, suppress cell cycle entry of NSC. However, in the case of regeneration, angiocrine factors secreted by blood vessels orchestrate proliferation and differentiation of both quiescent and activated NSCs into transit amplifying cells and neuroblasts (reviewed in Rafii et al. 2016).

The induction and maintenance of BBB characteristics is achieved by signals emanating from other cell types surrounding the endothelium, as will be exemplified

below, and this could explain why brain pathologies are associated with BBB breakdown (Neuwelt et al. 2011).

3 Structural Diversity of the Neurovascular Unit

The vasculature is divided into distinct zones, each having a different cellular and acellular content and specialized structure and function (Fig. 1). Penetrating arterioles are composed of lumen forming endothelial cells separated from smooth muscle cells by a basement membrane. Direct contacts called “myoendothelial projections” between endothelial cells and VSMC have been demonstrated in human brain arterioles (Aydin et al. 1991). No direct cell–cell contact has been demonstrated between astrocyte end-feet and endothelial cells or mural cells.

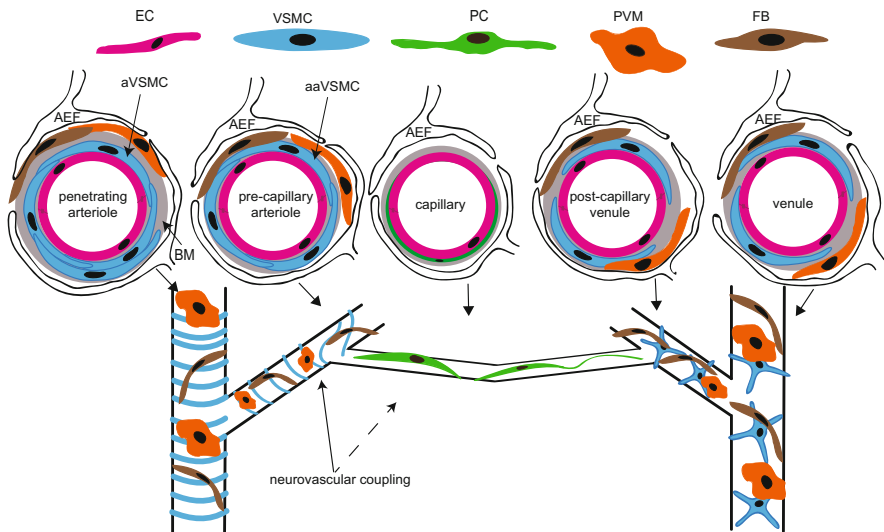


Fig. 1 Structural heterogeneity of the neurovascular unit. In penetrating arterioles, lumen forming endothelial cells (EC) are separated from vascular smooth muscle cells (aVSMC), which are wrapped around endothelium in the circumferential direction, by a basement membrane (BM). Occasionally, endothelial cells and VSMC are directly connected via “myoendothelial projections”. Penetrating arterioles give rise to short pre-capillary arterioles and are covered by distinct contractile mural cells – arteriole VSMC (aaVSMC) or terminal VSMC. Pre-capillary arterioles transit into a dense network of capillaries, which are the predominant vessel type in the brain. Capillary endothelial cells are chaperoned by pericytes (PC), mural cells with a prominent cell body and slender processes, which cover less than 1/3 of capillary circumference. Pericytes share the basement membrane with endothelium, are in direct contact with endothelium at contacts called peg-socket contacts. The capillary endothelium transitions into post-capillary venules that run into ascending venules. Venules have an incomplete mural cell coverage. The entire vascular tree in the CNS is covered by astrocyte end-feet (AEF). In the perivascular space between VSMC layer and astrocyte end-feet reside fibroblast-like cells (FB) and perivascular macrophages (PVM), which are positioned along the entire vascular tree with the exception of capillaries. Note that the elaborate branching of the vascular tree is not depicted on the figure

Perivascular space between VSMC layer and astrocytes in mice contains several cell types, including perivascular macrophages (PVMs) and fibroblast-like cells that are positioned along the entire vascular tree, with the exception of capillaries (Fig. 1). Penetrating arterioles give rise to short pre-capillary arterioles and are covered by distinct contractile mural cells – arteriole VSMC (aaVSMC) or terminal VSMC (discussed below). Although this zone presents a very short area of total cerebrovascular length, it seems to be the region where the neurovascular coupling is initiated (Rungta et al. 2018). Pre-capillary arterioles transit into a dense network of capillaries, which are the predominant vessel type in the brain and where the main nutrient and gas exchange takes place (Cipolla 2009). This vast capillary network [approx. 400 miles (Cipolla 2009)] is supplied by relatively few arterioles, each supplying blood to discrete cortical regions. Accordingly, loss of flow to a single penetrating vessel (i.e. artery/arteriole) inevitably leads to cortical damage (Blinder et al. 2010). Recent studies suggest that also capillaries participate in neurovascular coupling although the precise mechanism remains to be determined. In addition to brain capillary pericytes (Peppiatt et al. 2006; Hall et al. 2014), endothelial cells have also been implicated in flow regulation by retrograde hyperpolarization, which is evoked by local potassium changes (Longden et al. 2017). Capillary endothelium is transiting into post-capillary venules that run into ascending venules. Venules have an incomplete mural cell coverage. Thus, the vascular tree and its neurovascular associations are heterogenous, and when also considering the anatomical heterogeneity of the brain and differences in the requirement for metabolic supply (e.g. white vs grey matter) it becomes evident that the current understanding about the function of the NVU is only the tip of the iceberg.

4 Revisiting the Complexity of the Neurovascular Unit: The Cellular Composition and Function

4.1 Endothelial Cells

Endothelial cells, simultaneously with mural cell progenitors, enter the brain parenchyma early during neural tube development. In mouse, this takes place at embryonic day 9.5, starting in the hindbrain region (Ruhrberg and Bautch 2013). Endothelial cells lining the luminal side of blood vessels possess the BBB. The principal features of the vertebrate BBB are closed cell–cell junctions, a low rate of transcytosis and the expression of various solute carriers (SLCs) and ATP-binding cassette (ABC) transporters. In addition, brain endothelial cells restrict the trafficking of leukocytes into the brain parenchyma and contribute to immune privilege of the CNS (reviewed in Ransohoff and Engelhardt 2012) (Fig. 2). Although the developing brain vasculature displays most of the BBB characteristics very early in embryogenesis and is largely impermeable to plasma proteins (Saunders et al. 1999), it should be emphasized that the development of BBB continues into the postnatal period. Of note, the adult BBB is a dynamic interface, which will be briefly

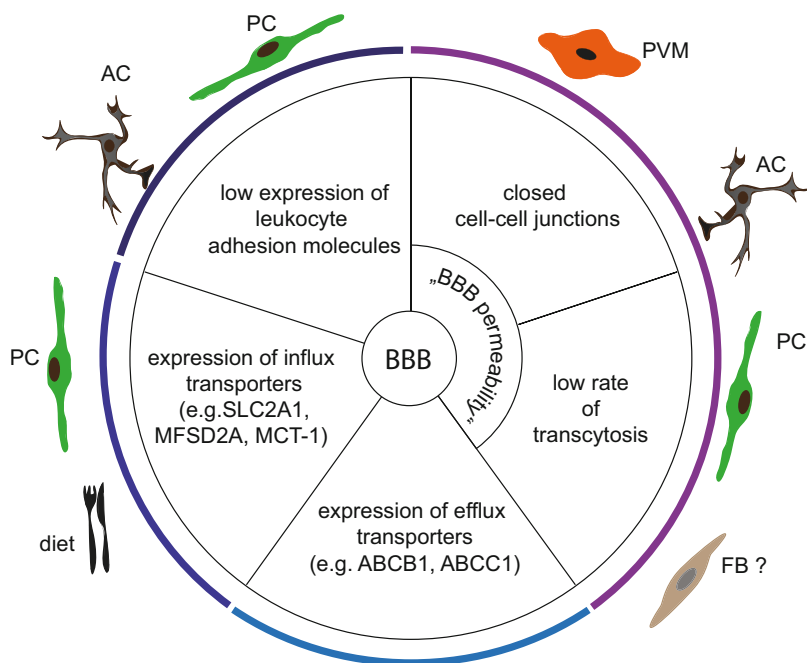


Fig. 2 Components, and cellular and environmental regulators of the BBB. The BBB consists of several components: closed cell–cell junctions, a low rate of transcytosis, the expression of various solute carriers (SLCs), ATP-binding cassette (ABC) transporters, and a low expression of leukocyte adhesion molecules. The so-called BBB permeability to plasma proteins could be the result of an altered endothelial transcytosis rate and/or opening of endothelial cell–cell junctions. AC astrocyte, FB fibroblast-like cell, PC pericyte, PVM perivascular macrophage

discussed below. Many of the identified signalling pathways that induce brain vascularization are also required for the differentiation of brain endothelium (e.g. Wnt, reelin). Less is known about how the barrier properties of the endothelium are regulated in the adult. For example, the canonical Wnt/ β -catenin signalling, which induces brain angiogenesis and barrier properties, is downregulated in adult vasculature and long-lasting high levels of β -catenin signalling in the endothelium can lead to vascular instability and lumen malformation (Corada et al. 2010). A recent study by Corada et al. showed that the transcription factor Sox17, required to induce the arterial fate of endothelial cells (Corada et al. 2013), acts as a rheostat controlling the proper level of Wnt-signalling in brain endothelial cells, which is necessary to maintain BBB characteristics (Corada et al. 2019). In the past, much of BBB research has focused on one characteristic of the BBB – the closed cell–cell junctions between brain endothelial cells, and many excellent reviews that discuss this topic have been published (Dejana et al. 2009; Greene et al. 2019). The consequences of endothelial junctional breakdown in the brain are particularly detrimental because of the extreme vulnerability of the brain to oedema due to the

closed space of the cranium and the release of potentially toxic compounds to neurons after red blood cell disintegration (e.g. haemoglobin, bilirubin).

Since closed endothelial cell–cell junctions prevent any free passage of blood-derived molecules into the brain, the execution of the proper transport function of brain endothelial cells is very important for brain function, and consequently, mutations in SLC transporters expressed by the brain endothelium are associated with neurodevelopmental disorders [e.g. *SLC2A1* – OMIM #606777, #612126; *MFSD2A* – OMIM #616486; *SLC7A5* (Tarlungeanu et al. 2016); *SLC16A2* – OMIM #300523]. Interestingly, a single cell RNA sequencing analysis of brain endothelial cells revealed that transcripts of transporters dominated in capillaries and veins (e.g. *Mfsd2a*), indicating that trans-endothelial transport might be zonal and that brain endothelial cells differ in their function to provide metabolic support depending on the location on the vascular tree (Vanlandewijck et al. 2018). This also has implications for drug delivery. For example, the expression of *SLC36A1* which transports vigabatrin (Thwaites and Anderson 2011), a GABA derivative used to control seizures, is higher on arterial endothelial cells than on capillary endothelial cells (Vanlandewijck et al. 2018).

The presence of efflux transporters (ABC transporters) on brain endothelial cells is a blessing and a curse. Small, lipid-soluble drugs (<400 Da) are able to cross the BBB by lipid-mediated diffusion. However, most prescribed drugs are lipid soluble and substrates for ABC transporters (e.g. P-glycoprotein (Pgp) or multidrug resistance protein) expressed by the brain endothelial cells and thus excluded from the brain. On the other hand, many drugs can be used relatively safely, since they are actively eliminated from the CNS by ABC-transporters. For example, ivermectin, a widely used broad-spectrum antiparasitic agent, is a lethal neurotoxin when administered to Pgp knockout mice (*Abcb1*–/–). Loperamide (Imodium), an opiate used to treat diarrhoea, displays only peripheral opiate-like effects on the gastrointestinal tract, but *Abcb1*–/– mice display opiate effects in the CNS (Hodges et al. 2011). Furthermore, there is significant genetic variation in the genes encoding ABC transporters, which is thought to determine individual differences in drug transport (Sissung et al. 2014).

The BBB is important for the determination of the steady-state composition of the interstitial fluid (ISF) (reviewed in Hladky and Barrand 2016). Ion transport is coupled to cellular metabolic energy and therefore an energy depletion, such as during ischaemic stroke, leads to catastrophic consequences on brain cellular ionic homeostasis (reviewed in Sifat et al. 2019). Thus, understanding the expression and regulation of ion transporters on brain endothelial cells and other cells of the neurovascular unit is crucial in treating various brain diseases and constitutes an important therapeutic target. Unfortunately, identification and spatial location of ion transporters on brain endothelial cells and other brain vascular cells is understudied. Again, a recent single cell RNA sequencing data on brain vascular cells could bring additional insights. For example, although the presence of the Na^+ -pump on the BBB is established, it is interesting that different vascular cells express different subsets of alpha and beta subunits of Na^+ , K^+ – ATPase. For example –*Atp1a1* is expressed by

all vascular cells, including endothelial cells but *Atp1a2*, on the other hand, is not expressed by endothelial cells (Vanlandewijck et al. 2018).

Low rate of transcytosis was described as the second feature of the BBB in the seminal publication by Reese and Karnovsky – “*The second characteristic feature of the endothelium of cerebral vessels is the low frequency of vesicles of the type that have been associated with transport of materials across endothelium elsewhere*” (Reese and Karnovsky 1967). The regulation of low transcytosis rate in the brain endothelium remains to be clarified, however, it has been shown that pericytes suppress endothelial transcytosis in the CNS (discussed below). In addition, studies on a brain endothelial transporter for omega-3 fatty acids – MFSD2A have shown that lipids transported by MFSD2A create a unique lipid composition of brain endothelial cells, which inhibits caveolae-mediated transcytosis (Andreone et al. 2017). An increase in the number of endothelial vesicles is the first change seen in brain endothelium in response to hypoxia and has been described as an early step in endothelial injury preceding alterations in cell–cell junction integrity during many pathological conditions, including ischaemic stroke (Knowland et al. 2014).

Endothelial cells have been suggested to sense circulating molecules in the blood and adapt and respond accordingly. An aged circulatory environment when exposed to young mice leads to microglia activation, reduced neurogenesis and cognitive dysfunction in the hippocampus (Villeda and Wyss-Coray 2013). These effects were shown to be mediated by endothelial VCAM-1 as the genetic deletion of VCAM-1 on brain endothelium prevented the effects of aged plasma on cognitive function in young mice (Yousef et al. 2019). Although it is known that the brain endothelium reacts to peripheral metabolic changes, little is known about specific changes in the endothelium. During fasting in humans and hibernation in animals, adaptive changes occur in the endothelium by upregulation of monocarboxylate transporters (MCT), which results in the uptake of ketone bodies used by brain as an alternative fuel (Andrews et al. 2009). A ketogenic diet has also been shown to improve the symptoms and quality of life among children suffering from the glucose deficiency syndrome (Brockmann 2009).

The endothelial glycocalyx, a polymeric sugar-rich network that covers the apical surface of the endothelial cells, was first described in 1966, in capillaries of rat intestinal mucosa (Dogne et al. 2018). The glycocalyx creates a space between blood and the endothelium; restricts leukocyte and platelet adhesion, acts as a mechanosensor to flow variation, and controls vessel permeability (reviewed in Dogne et al. 2018). The role of the glycocalyx in controlling macromolecule passage was recently demonstrated through in vivo studies on brain blood vessels (Kutuzov et al. 2018). The glycocalyx of cerebral capillaries was described to be more dense compared to continuous lung and heart capillaries (Ando et al. 2018). Loss of the glycocalyx has been shown to occur in cerebral malaria (Hempel et al. 2016) and after brain injury (traumatic brain injury, subarachnoid haemorrhage, blast) (reviewed in Dogne et al. 2018). Repeated blast exposure reduced glycocalyx length and density in various brain regions which was associated with mild memory impairment, and the pre-treatment with hyaluronidase, which increases the thickness

of the glycocalyx, corrected behavioural deficits in rats after repeated blast exposure (Hall et al. 2017).

Endothelial cells can take up myelin debris in vitro and present myelin-derived antigens in MHC-II, to stimulate the trans-endothelial migration of myelin-reactive T-cell (Pinheiro et al. 2016). A recent study by Zhou and colleagues showed that endothelial cells engulf the myelin in vivo upon spinal cord injury and autoimmune neuroinflammation, which leads to endothelial-mesenchymal transformation and tissue fibrosis (Zhou et al. 2019). However, the contribution of endothelial cells to tissue fibrosis is equivocal since no in vivo lineage tracing experiments were performed.

4.2 Mural Cells: Pericytes and Vascular Smooth Muscle Cells

“Mural cells” is a collective term for vascular smooth muscle (VSMC) cells and pericytes, which reside on the abluminal side of the endothelium of blood vessels. However, VSMC and pericytes differ in their anatomical location, the phenotype, gene expression profile and function (Fig. 1). VSMC have elongated morphology and are wrapped around endothelium in the circumferential direction. Depending on the vessel type, they can form multicellular or single cellular layer, and are separated from the endothelium by the basement membrane. VSMC surrounding resistance vessels regulate the vascular tone. Pericytes have a prominent cell body, sending off slender processes along the endothelium, which cover less than 1/3 of the capillary circumference. Pericytes share the basement membrane with the endothelium and are in direct contact with the endothelium at contacts called peg-socket contacts (Armulik et al. 2011). A recent study identified the molecular signature of brain mural cells based on single cell RNA sequencing data in mice. Mural cells can be divided into two main populations: pericytes and venous VSMC, and arterial VSMC (aVSMC) and arteriole VSMC (aaVSMC) (Vanlandewijck et al. 2018). There is a very sharp separation in gene expression profile between aaVSMC and pericytes. In addition to pericyte expression of specific transcripts (e.g. *Abcc9*), pericytes lack the expression of markers for aVSMC/aaVSMC (e.g. *Cnn1*, *Tagln*, *Acta2*). The presence of multiple subtypes of mural cells could partially explain the current controversy on the role of pericytes in blood flow regulation (Hall et al. 2014; Hill et al. 2015; Grutzendler and Nedergaard 2019). As pointed out in a recent review by Berthiaume et al. – these terminal VSMC (aaVSMC) have in the literature been named as pericytes, ensheathing or transitional pericytes and pre-capillary SMC (Berthiaume et al. 2018a). Terminal arterioles surrounded by terminal VSMC (aaVSMC) have a very small diameter, and since the vessel diameter has been often used as a proxy to identify a vessel type and subsequently pericytes, this could have led to an ambiguity in identification of studied cell types. Interestingly, morphologically different mural cells surrounding post-capillary veins and capillaries could not be separated based on transcriptional profiles obtained using the single-cell RNA sequencing. It was suggested that mural cells gradually change their phenotype from pericytes to venous VSMC (Vanlandewijck et al. 2018).

However, it is also plausible that post-capillary mural cells were not sequenced or where not represented within sequenced cells in high enough numbers that would allow their identification using current algorithms. Interestingly, different mural cell subtypes express different set of transporters, further pointing to physiological differences in their function (Vanlandewijck et al. 2018).

Pericytes enter the developing brain tissue together with endothelial cells. CNS pericytes are thought to have a multiple embryonic sources (neural-crest and mesenchyme) (Armulik et al. 2011; Reyahi et al. 2015). Interestingly, a recent study showed that some brain pericytes can also originate from mesoderm-derived myeloid progenitor cells (Yamazaki et al. 2017).

Studies on mice with altered platelet-derived growth factor -B (PDGF-B)/platelet-derived growth factor receptor beta (PDGFRB) signalling, important for pericyte recruitment to the developing vessels, have shown that pericytes regulate several aspects of the neurovascular unit (Armulik et al. 2011). Pericytes were shown to regulate endothelial permeability on the level of transcytosis (Armulik et al. 2010; Daneman et al. 2010) (Fig. 2). Of note, pericyte-deficient endothelium shows reduced expression of *Mfs2da* (Ben-Zvi et al. 2014), however, whether this leads to a reduced uptake of lipids leading to an altered membrane composition favouring transcytosis is unclear (Fig. 2). Although pericyte-deficient mice show permeability to plasma proteins (Armulik et al. 2010), it does not lead to a better delivery of lipid-soluble drugs, indicating an unaltered functionality of transporters (Mihajlica et al. 2018). Interestingly, data obtained from different mouse lines with variable pericyte coverage show that the completeness of pericyte-coverage determines the extent of BBB permeability (Armulik et al. 2010; Daneman et al. 2010; Vanlandewijck et al. 2015; Villasenor et al. 2016). Thus, the brain vasculature can compensate for the loss of pericytes (up to 50–60%) to some extent, and the consequences on the vessel function are seen after a certain threshold is crossed. Pericytes also regulate the inflammatory profile of CNS endothelium (Daneman et al. 2010; Torok et al. 2019; Ogura et al. 2017; Park et al. 2017) and thereby the immune quiescence of the vasculature (Fig. 2). Similar to endothelial permeability, pericyte coverage determines the extent of endothelial inflammation, and determines the localization of neuroinflammatory lesions in a setting of autoimmunity (Torok et al. 2019). There are conflicting results on the role of pericytes on endothelial cell–cell junctions. In some studies, pericytes have shown to regulate the expression of endothelial tight junction molecules (e.g. Cldn5, Zo-1, Occludin) (Bell et al. 2010), however, other studies have not observed the same consequences on the expression of cell–cell adhesion molecules (Armulik et al. 2010; Daneman et al. 2010; Dieguez-Hurtado et al. 2019). Instead, cell–cell junctions between endothelial were reported to have a convoluted appearance but impermeable to plasma proteins (Reyahi et al. 2015; Armulik et al. 2010; Dieguez-Hurtado et al. 2019). It is plausible that in the absence of pericytes endothelial cell–cell junctions are less organized. Pericytes have been suggested to mediate functional hyperaemia (Peppiatt et al. 2006; Hall et al. 2014), and studies on mouse lines expressing low levels or signalling defective PDGFRB, presenting with age-dependent pericyte degeneration, have shown an altered neurovascular coupling and white matter dysfunction (Bell et al. 2010; Kisler et al. 2017; Montagne et al.

2018). Equally dramatic effects were found to take place upon acute pericyte ablation in adult animals – immediate BBB breakdown, severe loss of blood flow, and a rapid neuron loss (Nikolakopoulou et al. 2019). Interestingly, a single pericyte ablation leads to the sustained dilation of the capillary lumen in regions lacking pericyte contact, which persists until processes of neighbouring pericytes cover the exposed territory (Berthiaume et al. 2018b). Thus, pericytes might control the basal vascular tone independently of neuronal activity either mechanically and/or via signalling with endothelium (Berthiaume et al. 2018b). Curiously, the same long-term in vivo imaging study showed that capillary pericytes keep their individual territories by tactile sensing of adjacent pericytes through extending and retracting their terminal processes (Berthiaume et al. 2018b).

In addition to regulating the BBB characteristics of endothelium and functional hyperaemia, pericytes induce the polarization of astrocyte end-feet (Armulik et al. 2010). Defective polarization of astrocyte end-feet (i.e. mis-localization of AQP4) in pericyte-deficient mice was shown to reduce perivascular flow (Munk et al. 2019).

Recent years have brought insights into additional signalling pathways that regulate pericyte development and interaction with endothelium in the CNS. CD146 (MCAM) was shown to be important for the ligand-dependent activation of PDGFRB and subsequent pericyte recruitment to the vessel wall (Chen et al. 2017). Not only the lack of pericytes but also an aberrant communication/association with endothelium and faulty differentiation of pericytes alters endothelial permeability and immune quiescence. Deletion of *Foxf2*, encoding a forkhead transcription factor, leads to hyperplasia and defective differentiation of brain pericytes associated with lower PDGFRB expression (Reyahi et al. 2015). Interestingly, despite a higher number of pericytes, *Foxf2*^{-/-} brains showed a poor endothelial-pericyte association and altered TGF- β -Smad2/3 signalling (Babatz et al. 2018). Many studies have demonstrated a quantitative dependence on PDGFRB for mural cell development. Interestingly, increased PDGFRB signalling also leads to CNS pericyte hyperplasia and differentiation defects (Olson and Soriano 2011). Constitutive deletion of another forkhead transcription factor, *Foxc1*, in pericytes leads to endothelial hyperplasia but no alterations in endothelial-pericyte interaction, nor does this lead to overt BBB defects (Siegenthaler et al. 2013). *Foxc1* deletion in pericytes reduced expression of proteoglycans glypican-3 and asporin (Siegenthaler et al. 2013), and thus the observed defects in endothelial quiescence could be mediated by alteration in the extracellular environment.

Postnatal deletion of *Rbpj* in pericytes, without affecting pericyte numbers, leads to brain haemorrhages, endothelial hyperplasia and alteration in brain pericyte identity in Notch-independent manner that leads to activation of TGF- β signalling (Dieguez-Hurtado et al. 2019).

Pericyte function is not only restricted to vasculature, but also extends to CNS homeostasis and regeneration. Pericytes were recently shown to contribute to remyelination after lysolecithin-induced demyelination injury by supporting oligodendrocyte progenitor cell (OPC) differentiation via LAMA2 secretion (De La Fuente et al. 2017). It has been suggested that pericytes act as multi-progenitor stem cells (reviewed in Armulik et al. 2011). CNS pericytes have been thought to act

as a stem cells and differentiate into multiple cell types in response to an ischaemic insult (e.g. neural cells, vascular cells, microglia) (Ozen et al. 2014; Nakagomi et al. 2015). In addition, pericytes were shown to form scar tissue after spinal cord injury (Goritz et al. 2011). On the other hand, careful lineage tracing experiments using *Tbx18*-CreERT2 line, which was reported to be expressed in pericytes, revealed that in multiple tissues, including brain, pericytes do not significantly contribute to other cell lineages in vivo upon injury (Guimaraes-Camboa et al. 2017). Interestingly, a recent study identified PDGFRB expressing pericytes and fibroblast-like cells on the vessel wall to function as the first line of sensors of systemic inflammation in the brain by expressing CCL2, which elevates excitatory synaptic transmission in neurons (Duan et al. 2018). Of note, deletion of *Rbpj* in postnatal pericytes induced expression of CCL2 (Dieguez-Hurtado et al. 2019).

4.3 Astrocytes

Astrocyte specification in the CNS takes place relatively late during development. In mice this takes place around birth and after neuronal differentiation and initial CNS angiogenesis have taken place (Molofsky et al. 2012). Astrocytes provide another distinct characteristic of brain blood vessels – certain astrocytes ensheath the abluminal side of blood vessels with specialized processes called astrocyte end-feet (Fig. 1). Astrocyte end-feet are slender processes, enriched with water and potassium channels (e.g. AQP4 and KIR 4.1, respectively) that are anchored to the basement membrane of blood vessels via the dystroglycan complex (Wolburg et al. 2009). Mutations in *MLC1* or *HEPACAM*, two membrane proteins also located in astrocyte end-feet and astrocyte–astrocyte contacts, cause megalencephalic leukoencephalopathy with subcortical cysts (MLC), a rare leukodystrophy characterized by chronic white matter oedema (Rodriguez 2013; van der Knaap et al. 2012). MLC1 associates with the $\beta 1$ subunit of Na^+ , K^+ -ATPase, and because of this interaction, MLC1 has been suggested to control intracellular osmotic conditions and volume regulation in astrocytes (Brignone et al. 2011). Localization of MLC1 to astrocyte end-feet is mediated by HEPACAM. In addition, MLC1/HEPACAM complex contains CIC-1 (Jeworutzki et al. 2012), a chloride channel, and mutations in the gene *CLCN2* also cause MLC (Depienne et al. 2013). However, since MLC1/HEPACAM/CIC-1 are expressed also in other glia (oligodendrocytes, Bergman glia) (Hoegg-Beiler et al. 2014), it is not clear to what extent pathogenesis of leukodystrophy is caused by dysfunctional astrocyte end-feet.

Recruitment and maturation of astrocyte end-feet takes place postnatally and is completed 2 weeks after birth in mice (Landis and Reese 1981). Mouse studies have shown that by the end of P15, the formation of MLC1/HEPACAM complex at astrocyte end-feet has taken place (Gilbert et al. 2019) (Fig. 3). In addition, connexin 30 and 43 are enriched at astrocyte end-feet by the end of P15 (Ezan et al. 2012), indicating that gap junction communication between astrocytes is initiated postnatally (Fig. 3). Astrocytes are a heterogeneous population and currently it is not known if astrocytes that ensheath blood vessels have the same characteristics or if

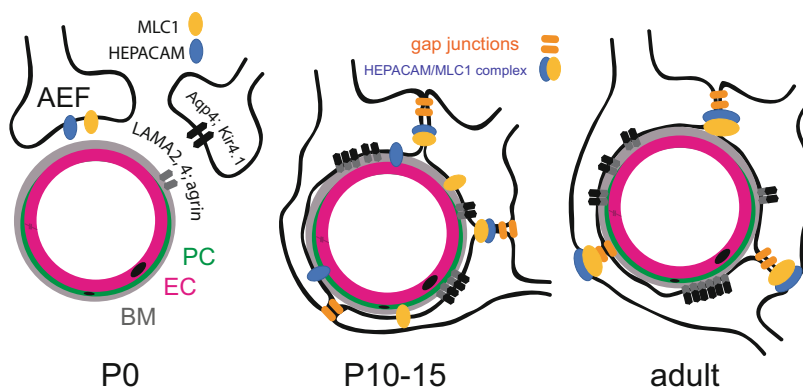


Fig. 3 Development of astrocyte end-feet. Astrocyte specification in mice takes place around birth. AQP4 and KIR4.1 are localized to astrocyte end-feet via binding to dystroglycan complex which connects to extracellular matrix proteins laminins and agrin. At postnatal day ten (P10) until P15, after the AQP4 and KIR4.1 are localized to the AEF, the formation of MLC1/HEPACAM complex at astrocyte end-feet takes place. At the same time gap junctions between astrocytes are formed. AEF astrocyte end-feet, BM basement membrane, EC endothelial cell, PC pericyte

there are differences in molecular composition of astrocyte end-feet dependent on the anatomical location. Astrocyte end-feet have been described to almost entirely surround vessels (Mathiisen et al. 2010) and thus can present a major physical transport barrier between the blood and the brain parenchyma (Fig. 1). However, a recent study showed that the appearance and arrangement of the astrocytic processes was dependent on the tissue preparation method. Cryo-fixed tissue showed a significantly less astrocyte end-feet coverage compared to chemical fixation (Korogod et al. 2015). Thus, further studies are needed to determine the astrocyte end-feet coverage of blood vessels. Development of astrocyte end-feet involves cell-autonomous signalling such as bone morphogenetic protein receptor type 1 (BMPRI1A) signalling in astrocytes, which is necessary for the investment of astrocyte end-feet around vasculature (Araya et al. 2008). As discussed above, pericyte-derived signals subsequently regulate astrocyte end-feet polarization (Armulik et al. 2010). The vascular basement membrane components, agrin and laminins, are important for anchoring astrocyte end-feet proteins Aqp4 and Kir 4.1 via the dystroglycan complex (Wolburg et al. 2009). Thus, pericyte- and endothelial cell-derived basement membrane components such as LAMA2 and LAMA4 might induce the polarization of astrocyte end-feet (Fig. 3). Merosin-deficient congenital muscular dystrophy is an autosomal recessive disorder caused by mutations in the *LAMA2* gene that encodes a laminin alpha 2 chain (Allamand and Guicheney 2002). Magnetic Resonance Imaging analysis of the brain in patients with merosin-deficient congenital muscular dystrophy has shown the presence of vasogenic oedema (Allamand and Guicheney 2002; Sijens et al. 2007). It would be interesting to determine whether the organization of astrocyte end-feet is affected in these patients.

The localization of AQP4 and Kir 4.1, and MLC-1/HEPACAM into astrocyte end-feet are independent from each other (Hoegg-Beiler et al. 2014).

Astrocytes perform short-term control of ionic composition of ISF (Santello et al. 2019) and astrocyte end-feet, in addition to regulating water transport, control blood and lymphatic flow (Jessen et al. 2015). Astrocyte end-feet secrete transmitters that regulate the blood flow at the level of arterioles and thus, mediate the vasomotor responses in functional hyperaemia (Petzold and Murthy 2011). Recent research suggests that astrocyte end-feet at the level of capillaries also regulates blood flow by dilating capillaries (Mishra et al. 2016). The so-called glymphatic pathway (derived from glia and lymphatic) facilitates movement of cerebrospinal fluid (CSF) from the subarachnoid space along paravascular spaces that surround penetrating arterioles. The clearance of ISF occurs along venous drainage pathways (Iliff et al. 2012). The glymphatic function depends on establishment of astrocyte end-feet and their polarized expression of AQP4 during development (Munk et al. 2019). Recently, it was shown that the glymphatic system first develops in mouse hippocampus followed by cortex after P7. The existence of ISF drainage in the brain has been suggested before; however, the drainage pathway was thought to be located only along capillaries and arteries, and the flow of ISF was considered to be opposite to the direction of blood flow (Weller et al. 2009).

Early studies have suggested that astrocytes are important for inducing the BBB properties of brain endothelial cells (Janzer and Raff 1987). However, astrocytes are unlikely to be important in the early induction of BBB properties of brain endothelium during embryonic development. Acute ablation of astrocytes or astrocyte end-feet did not result in an immediate increase in vessel permeability (Kubotera et al. 2019; Schreiner et al. 2015). A careful in vivo two photon study on tracers of various sizes showed the role of extravascular components (basement membrane and astrocyte end-feet) in restricting tracer distribution to brain parenchyma (Kutuzov et al. 2018) (Fig. 2). In addition, there are indications that astrocytes in a pathological setting form a second barrier and astrocyte–astrocyte contacts are crucial in mediating these effects. An induction of tight junction proteins Cldn4, Cldn1 and Jam-A in reactive astrocytes at the glia limitans in models of CNS inflammation – experimental autoimmune encephalitis, results in the formation of a second inducible barrier, which restricts immune cell entry (Hornig et al. 2017) (Fig. 2). Deletion of connexins 43 (*Gja1*) and 30 (*Gja6*) enriched by astrocytes lead to an increased vascular permeability in a setting of stress (e.g. high pressure) (Ezan et al. 2012). In addition to a physical barrier, astrocytes have also merged as regulators of brain endothelial immune quiescence. Astrocyte-derived Sonic hedgehog (Shh) is needed for the active maintenance of endothelial immune quiescence and the Shh activated pathway in endothelium has been shown to be deregulated in multiple sclerosis patients (Alvarez et al. 2011) (Fig. 2). In addition, a recent study implicates astrogliosis and astrocyte end-feet derived CCL19 in development of lymphoma metastasis (O'Connor et al. 2019).

4.4 Microglia

The role of microglia in brain function is increasingly recognized, however, the specific role of microglia in the biology of the neurovascular unit is less explored. Microglia, which colonizes the CNS before angiogenic sprouts enter the CNS (Ginhoux et al. 2010), has been implicated in the morphological shaping of brain vasculature by inducing anastomosis of vascular sprouts (Fantin et al. 2010). In the adult organism, microglia has been shown to send processes that directly contact basement membrane of vessels (Mathiisen et al. 2010). Upon endothelial damage microglia support damaged vessels and facilitate endothelial repair in homeostasis and pathological conditions (Nimmerjahn et al. 2005; Wynn et al. 2013).

Results from our laboratory show that the TREM2-dependent microglial activity is necessary to halt small-vessel calcification in a mouse model of primary familial brain calcification (Zarb et al. 2019). Of note, brain calcification is common in patients with mutations in genes implicated in microglial development and function (e.g. *IRF8*, *CSF1R*) (Rademakers et al. 2011; Bigley et al. 2018). Thus, in addition to clearing myelin, apoptotic cells, and aggregated proteins (e.g. amyloid beta) microglial activity is needed to remove or prevent accumulation of bone-like structures along brain vessels and in the parenchyma.

4.5 Perivascular Macrophages

In addition to microglia, there are so-called border-associated macrophages (BAM) in the brain that reside in perivascular space, meninges and in choroid plexus. Recent studies using methods like single cell RNA sequencing and high dimensional flow cytometry have identified markers for different BAMs and infiltrating monocytes during homeostasis and during neuroinflammation (Van Hove et al. 2019; Mrdjen et al. 2018; Goldmann et al. 2016). BAMs are continuously turned over at relatively slow rate (Mrdjen et al. 2018; Bechmann et al. 2001). PVM are the only BAM subtype that are associated with the blood vessels in the brain parenchyma (Fig. 1). PVM, similarly to microglia and contradictory to previous assumption, originate from yolk sac-derived, PU.1 dependent-erythromyeloid progenitors that are seeded in brain tissue during early embryogenesis (Goldmann et al. 2016). It is not known how PVM are recruited to developing vessels. Before the more precise molecular definition, the presence of PVM in the Virchow-Robin space surrounding arteries and veins was recognized by their ability to phagocytose various intravenously injected tracers (e.g. dextran, horseradish peroxidase) and express CD163. In addition, MHC-II expressing PVMs are present in multiple sclerosis lesions and therefore it was suggested that they act as antigen presenting cells (Fabrick et al. 2005). However, recent studies showed that in a setting of myelin induced neuroinflammation, PVM are not required for the reactivation of encephalitogenic T cells (Mundt et al. 2019; Jordao et al. 2019).

Several studies have demonstrated an important role of PVM for the pathogenesis of major brain diseases. The role of brain PVM has been studied mostly by removing

PVMs with clodronate containing liposomes. Using this approach, PVM have been implicated in amyloid beta catabolism and trafficking, hypertension, bacterial and viral infections, vessel permeability, and vascular stability after ischaemic stroke (Faraco et al. 2017). In an Alzheimer's disease mouse model, PVM were shown to mediate deleterious cerebrovascular effects of A β via CD36-Nox2 signalling pathway and generation of ROS (Faraco et al. 2017). Interestingly, it was revealed that PVM mediate neurovascular dysfunction also in the setting of hypertension and high fat diet. PVM were shown to drive alterations in neurovascular regulation of hypertension by increasing BBB permeability to angiotensin II, which in turn induced production of ROS by NOX-2 by binding to angiotensin type I receptors on PVM (Faraco et al. 2016).

PVM were suggested to participate in metabolic adaptation of the brain endothelial cells in response to long-term changes in the diet. High fat diet, which elicits initially a reduction of endothelial GLUT-1 (*Slc2a1*) and consequently brain glucose uptake, initiates a compensatory increase of vascular-derived growth factor A (VEGF-A) production by PVM. VEGF-A restores GLUT-1 (*Slc2a1*) expression by brain endothelial cells necessary to preserve cerebral glucose metabolism and cognitive function (Jais et al. 2016).

In addition to the above listed functions, PVM could regulate vascular stability and permeability in the brain parenchyma (Fig. 2). In brain circumventricular organs where the endothelial cells lack the BBB characteristics, the PVM form a barrier that restricts entry of molecules of a large molecular weight (Faraco et al. 2017). In addition, PVM take up extravasated blood-derived material also in the retina (Faraco et al. 2017). Similar observations on the role of PVM that limit vessel permeability have been described in the dermis (He et al. 2016). On the other hand, a recent study showed that CD163⁺ BAMs respond to ischaemia by upregulating leukocyte adhesion molecules, and that depletion of BAMs (including PVM) reduces vessel permeability, reduces granulocyte infiltration and improves clinical outcome (Pedragosa et al. 2018).

4.6 Perivascular Fibroblast-Like Cells

Recent molecular profiling studies have identified several subpopulations of the so-called fibroblast-like cells (Vanlandewijck et al. 2018; Saunders et al. 2018) or vascular leptomeningeal cells (Zeisel et al. 2018). These cells were shown to reside between the vessel wall and the AQP4- and GFAP-positive astrocyte end-feet (Vanlandewijck et al. 2018) (Fig. 1). Brain fibroblast-like cells express several extracellular matrix proteins such as laminins, collagens, collagen-modifying enzymes and proteins involved in collagen fibril spacing. Among these, many are fibroblast specific, such as LAMA1. Immunohistochemical staining of LAMA1 allowed anatomical localization of these cells to the wall of arteries, arterioles, veins and venules (Vanlandewijck et al. 2018) (Fig. 1). There is currently very limited knowledge about the function of these cells on the vessel wall. However, a few studies indicate that perivascular fibroblast-like cells contribute to fibrotic scar

formation after CNS insults (Riew et al. 2018; Soderblom et al. 2013; Fernandez-Klett et al. 2013).

It is noteworthy that perivascular brain fibroblast-like cells in adult mice express podoplanin (Vanlandewijck et al. 2018). Podoplanin is the only known ligand for Clec-2 expressed by platelets. Clec-2–podoplanin interaction activates and aggregates platelets and thus is necessary for blood clotting. Mouse knockouts of Clec-2 and podoplanin have shown that Clec-2/podoplanin interaction drives cerebrovascular patterning (Uhrin et al. 2010; Lowe et al. 2015; Finney et al. 2012). Furthermore, Clec-2 knockout embryos present with brain haemorrhaging starting at E10.5. In these embryos, the expression of podoplanin by nestin positive neuronal progenitors but not by endothelial cells was necessary to prevent haemorrhages (Lowe et al. 2015). Platelets are important regulators of vascular integrity in periphery but also in the brain (reviewed in Ho-Tin-Noe et al. 2018). Thrombocytopenia worsens/causes inflammation-induced bleeding in the brain. The function of podoplanin in adult brain has not been addressed, but one could speculate that podoplanin expressing fibroblasts positioned along the vascular tree could form a second line of defence and help to coagulate blood in the case of a basement membrane rupture by recruiting and activating platelets through CLEC-2 (Fig. 2).

5 Gut-Brain Axis Regulating Integrity and Function of Neurovascular Unit

The microbiome, a diverse ecosystem occupying different niches in the human body, is assumed to interact with most, if not all, organs of the host (Kundu et al. 2017). Bacterial toxins and metabolites produced in the gut affect brain function via a complex system including immune and humoral pathways. The gut also affects brain directly via the vagus nerve (Kundu et al. 2017). It is increasingly being recognized that a dysfunctional gut-brain axis contributes to pathology of a wide range of diseases – such as obesity, inflammatory bowel disease but also neurodegenerative diseases such as Parkinsonism.

Several intestinal metabolites are only generated by bacterial microbiota such as short-chain fatty acids (SCF) – acetate, propionate, butyrate (Schonfeld and Wojtczak 2016). These circulating metabolites have been shown to regulate the development of BBB characteristics of brain endothelial cells. Germ-free mice have increased BBB permeability to various intravenously injected tracers due to the reduction in the expression of endothelial tight junction proteins occludin and claudin-5 (Braniste et al. 2014). Administration of SCF, which has been shown to tighten epithelial barrier in the gut, corrected the expression of cell–cell junction proteins and the BBB defect in germ-free mice (Braniste et al. 2014). SCF are transported into cells by monocarboxylate transporters expressed also by brain endothelial cells (e.g. MCT-1) (Kelly et al. 2015), and it would be interesting to study if the effect of SCFs on endothelial cell–cell junction protein expression is endothelial cell-autonomous.

The gut microbiome has been shown to influence the course of familial cerebral cavernous malformations (CCMs) caused by loss-of-function mutations in either of three genes: *CCM1*, *CCM2*, and *CCM3* (Fischer et al. 2013). These genes encode the components of a protein complex that suppresses MEKK3–KLF2/4 signalling in brain endothelial cells (Cuttano et al. 2016). CCMs are vascular lesions appearing like mulberry-like clusters that develop in the venous-capillary bed in the central nervous system. CCM patients suffer from recurrent cerebral bleedings that cause haemorrhages, seizures and neurological impairment. The development of lesions of familial CCM is highly variable, suggesting the existence of genetic and/or environmental disease modifiers. It was recently shown that activation of endothelial TLR4 by Gram-negative bacteria or lipopolysaccharide accelerates CCM formation in mouse models of CCM (Tang et al. 2017). Furthermore, polymorphisms that increase expression of the TLR4 gene are associated with higher CCM lesion burden in humans (Choquet et al. 2014). Thus, increasing evidence points to roles for the microbiome in the pathogenesis of cerebrovascular disease and importantly, these insights offer new strategies for treatment.

6 Conclusion

Our understanding about the developmental, homeostatic and disease aspects of the NVU has grown rapidly over the last few years. The progress described in this chapter is not exhaustive but should give a broad overview on ideas that have been the primary focus so far and directions for future investigations. New functions of neurovascular cell types have been identified, such as endothelial cells engulfing myelin or perivascular macrophages interfering with neurovascular coupling. Neurovascular coupling and its anatomical origin (arteriole vs capillary) has remained a topic of intense discussions. This seems to stem from differences in methodologies and ambiguities in identifying structural components of the vascular tree (e.g. arterioles vs capillaries). Recent advances in RNA sequencing technology on the single cell level have provided insights into the gene expression profile of different vascular cells (e.g. endothelial cells, pericytes) and help to design new tools that would facilitate identification of vascular cell types and vessel segments. Also, given the heterogeneity of the vascular tree and cell types building it, it is necessary for the field to end the semantic confusion, especially around mural cell types, and start to move forward together. Hopefully, future studies will also pay more attention to the development and disease-associated alterations along the vascular tree. Since the vessel architecture and its neurovascular associations are heterogenous and alterations are disease specific, it is therefore unlikely that the mechanism leading to alterations at the NVU is uniform. Whether changes observed in response to injury are detrimental or adaptive and thus beneficial requires deeper investigations. An exciting and somewhat understudied area is the metabolic adaptation of the NVU and its importance in maintaining the healthy brain.

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Brain Endothelial Cells: Metabolic Flux and Energy Metabolism

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Abstract

The neurovascular unit (NVU) consists of multiple cell types including brain endothelial cells, pericytes, astrocytes, and neurons that function collectively to maintain homeostasis within the CNS microenvironment. As the principal barrier-forming component of the NVU, the endothelial cells perform an array of complex functions that require substantial energy resources. The principal metabolic pathways for producing ATP are glycolysis and mitochondrial oxidative phosphorylation. While previous studies have demonstrated that glycolysis is

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a primary pathway for most endothelial cells, details about the energy producing pathways of brain endothelial cells are not fully characterized. The contributions of glycolysis and mitochondrial respiration to energy metabolism are quantifiable using metabolic flux analysis that measures cellular oxygen consumption and acidification (proton production) in a closed microtiter plate format. ATP production rates are then calculated. The bioenergetics of the human brain microvascular endothelial cell line, hCMEC/D3, indicate that these cells exhibit relatively elevated rates of glycolytic flux and glycolytic ATP production, thus confirming their glycolytic nature even in the presence of abundant oxygen. Furthermore, energy producing pathways involving mitochondrial respiration are relatively low, although contributing significantly to total ATP production. Interestingly, the bioenergetics of the hCMEC/D3 cells are relatively similar to those of human primary brain microvascular endothelial cells (hBVECs). These findings allow a quantitative understanding of the bioenergetics of brain endothelial cells in a cultured and proliferative state and also provide a platform for comparative studies of disease states and conditions involving exposures to drugs or metabolic disruptors.

Keywords

Bioenergetics · Endothelial cell · Glycolysis · Neurovascular unit · Respiration

1 Introduction

The entire vascular system of the body is covered on its luminal surface with a layer of cells that is collectively known as the endothelium and is derived from the embryonic mesoderm (Dyer and Patterson 2010). The endothelial cells forming this layer are globally responsible for delivery of metabolic substrates such as oxygen and nutrients and removal of metabolic waste products. In the brain, a unique and coordinated differentiation plan results in endothelial cells with highly specialized structures and physiological functions that include nutrient transport, toxic and xenobiotic compound efflux, interstitial fluid homeostasis, blood flow regulation, angiogenesis, trafficking of leukocytes into the underlying parenchyma, and both innate and adaptive immunity (Aird 2012). Brain function is dependent on these neurovascular properties, which have significant disease implications. If neurovascular dysfunction occurs, then the surrounding neural cells will be dysfunctional and a neuropathology (disease) will result. Conversely, if a disease or condition causes a neurovascular dysfunction, then the altered endothelium becomes collateral damage and the disease consequences are amplified (Neuwelt et al. 2011).

To conduct and sustain the assortment of brain endothelial functions, cells require a metabolic composition that is active, robust, and versatile to supply the necessary metabolic energy and cellular resources. In general, cells are able to utilize various substrates, such as glucose, glutamine, pyruvate, fatty acids, ketone bodies, and amino acids to generate ATP (Corbet and Feron 2017; Dang 2009; DeBerardinis et al. 2008; Diers et al. 2012; Feron 2009; Scalise et al. 2018). It is known that

vascular endothelial cells within each particular tissue or organ environment meet their metabolic demands through the ATP generating pathways of glycolysis via anaerobic metabolism and oxidative phosphorylation via mitochondrial respiration (OXPHOS) (Eelen et al. 2018; Krutzfeldt et al. 1990). However, the detailed metabolic characteristics of endothelial cells existing in the brain and spinal cord are less clear and have not been completely described.

Glycolysis involves the conversion of one glucose molecule into two molecules of pyruvate, which in turn may be converted to lactic acid (Eelen et al. 2018; Jin and Zhou 2019). From this process, a net total of two molecules of ATP are produced from one molecule of glucose (Moreno-Sanchez et al. 2007). The pyruvate formed is converted into lactate, by lactate dehydrogenase, and exported into the extracellular environment, along with a hydrogen ion, via monocarboxylic acid transporters (MCTs) (Beloueche-Babari et al. 2017; Benjamin et al. 2018; Halestrap 2012; Perez-Escuredo et al. 2016). Brain endothelial cells are especially enriched with lactic acid transporters including MCT1 and its activity is highly regulated (Gerhart et al. 1997; Smith and Drewes 2006; Smith et al. 2012).

Alternatively, pyruvate is fully oxidized to CO_2 by the tricarboxylic acid (TCA) cycle of the mitochondria and thereby generates ATP by OXPHOS. In this pathway, pyruvate produced from glycolysis is transported into the mitochondria via the mitochondrial pyruvate carrier (MPC) and converted into acetyl-CoA (Briere et al. 2006; Metcalfe et al. 1988). The TCA cycle, using multiple enzymatic reactions and intermediates, consumes the acetyl-CoA and produces three NADH molecules and one FADH_2 molecule that in turn contribute to the electron transport chain (ETC) to carry and donate electrons and create a proton gradient between the intermembrane space and the mitochondrial matrix (Jafri et al. 2001). The energy of this electrical-chemical gradient is then exploited by ATP synthase to drive synthesis of ~ 33.45 molecules of ATP per glucose molecule (Mookerjee et al. 2017). The electrons participating in the ETC are donated to oxygen as a final electron acceptor producing H_2O .

The metabolic phenotype of a cell is relevant to its physiological status or function. For example, both immune cell and stem cell activation exhibit a change in cellular metabolism when in transition to an activated or quiescent state (Pearce and Pearce 2013; Ryall et al. 2015; Shares et al. 2018; Tan et al. 2015). Extensive studies of diseases, including cancer, have revealed transitions in metabolic pathways during transformation, proliferation, and metastasis that are the focus of strategies for developing novel anti-cancer drugs. Such pathways include glycolysis, lactate export and import, glutamine metabolism, and mitochondrial transport of pyruvate. Targeting glucose metabolism by inhibiting GLUT1, a primary glucose transporter, has been shown to decrease cell proliferation in various cancer types (Wang et al. 2013; Zhao et al. 2016), because many tumors and cancerous cells consume large amounts of glucose to proliferate rapidly (Kim et al. 2017; Vander Heiden et al. 2009; Warburg 1956). Depending on the metabolic state of the cells, targeting lactate and pyruvate metabolism by inhibiting MCTs and MPCs have also shown to be promising treatments for various cancer types (Benjamin et al. 2018; Corbet et al. 2018; Curtis et al. 2017; Diers et al. 2012; Gurrapu et al. 2015; Nelson

et al. 2019; Takada et al. 2016). Such targets have included energy (ATP) generating pathways and agents disrupting either glycolysis or mitochondria OXPHOS (Falkenberg et al. 2019). However, characterizing the metabolic phenotype and studying the effects of inhibiting various pathways have not been studied for brain endothelial cells. Studying the metabolic phenotype may give insights to brain endothelial cell energetic characteristics, as well as provide a platform for comparative studies.

Changes in brain endothelial cell function and integrity may be accompanied by changes in cellular energy demand and metabolism. Altered tight junction integrity, changes in nutrient transporter expression (such as decreased expression or deficiency of GLUT1), inhibition of essential substrate uptake, changes in active efflux of molecules, and disrupted angiogenesis all influence the ATP demand. There have been numerous previous studies that examine structural, transcriptional, and proteomic components of brain endothelial cells involved in endothelial cell integrity and disruption, but few that address the ATP-producing pathways of brain endothelial cells and their plasticity and vulnerabilities.

ATP production and consumption by brain endothelial cells are also relevant to drug delivery to the CNS. It has been estimated that the NVU is impermeable to more than 98% of small-molecule drugs developed for targeting the CNS, making treatment for many neurological diseases, via small-molecule drugs, very challenging (Pardridge 2005). Neurological conditions, including Alzheimer's disease, stroke/ischemia, and glioblastomas involve tight junction break-down and a change in brain endothelial cell integrity and function (Ballabh et al. 2004; Banks 2012; Erdo et al. 2017; Keaney and Campbell 2015; Saraiva et al. 2016; Stanimirovic and Friedman 2012). Furthermore, GLUT1 deficiencies in brain endothelium lead to cognitive impairments and increased permeability of the NVU (Rotstein et al. 2010; Winkler et al. 2015). Decreased expression of GLUT1 in the endothelium is a characteristic of Alzheimer's disease (Abbott et al. 2010). Strokes and ischemia involve the release of pro-inflammatory cytokines, VEGF, and nitric oxide, which leads to tight junction disruption, angiogenesis, and increased NVU permeability (Ballabh et al. 2004). Brain ischemia is also paired with an increase in GLUT1 in brain endothelial cells (McCall et al. 1996), indicating a possible alteration in brain endothelial cell metabolism. Disruption of tight junctions is associated with a decrease in intracellular ATP generation in mouse brain microvascular endothelial cells (Wang et al. 2018). These changes in brain endothelial cell function and integrity in disease states strongly suggest that endothelial cell metabolism is also altered.

In this study the features of hCMEC/D3 cells are extended to cellular bioenergetics and thereby further validate use of this model along with primary human brain endothelial cells potentially applicable to disease states. Significant portions of this chapter were adapted from a Master's degree thesis submitted to the University of Minnesota (McDonald 2019).

2 Materials and Methods

2.1 Cell Culture

Immortalized human brain microvascular cells (hCMEC/D3) were purchased from Millipore[®] (Cat. No. SCC066). Primary human brain microvascular endothelial cells (hBVEC) were purchased from Cell Systems[®] (Cat. No. ACBRI 376). Aliquots (1.0 mL) of cells in growth medium were prepared and stored in liquid nitrogen. Cells were thawed with, and cultured, in growth medium containing 45% Ham's F10 (Gibco, Cat. No. 12390-027), 45% alpha MEM (Gibco, Cat. No. 12561-023), 10% fetal bovine serum, and 1.74 mM L-glutamine (Sigma, Cat. No. G 7513). The growth media was supplemented with 0.5 mL Gentamicin (Gibco, Cat. No. 15710-015) and 5 ng/mL fibroblast growth factor β (ThermoFisher, Cat. No. 13256029). Cells were incubated at 37°C and 5% CO₂ on rat tail collagen type I coated T75 cell culture flasks. Rat tail type I collagen was purchased from Corning (No. 354236). To dissociate cells for passaging, cultures were washed with PBS once, then trypsinized for 3–5 min. Growth media was then added to inactivate the trypsin, cells collected by centrifuged (1,000 rpm, 5 min) and the cell pellet resuspended in fresh growth media. Cell density was determined using a hemocytometer. To avoid culture-induced phenotypic changes culture passage numbers were limited to <20 for immortalized cells and <6 for primary cultures.

2.2 Extracellular Flux Analysis

The quantitation of cellular metabolism was performed using the Seahorse XFe96 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA). Oxygen consumption and proton production are measured, in real time, by using sensitive fiber optic probes in a 96-well culture plate format (Fig. 1). Lowering the sensors into the wells creates sealed compartments containing culture media (~20 μ L) and cells. By repeated measures, at 37°C and normoxia, the rates of oxygen consumption (OCR) and proton production (ECAR, Extracellular Acidification Rate) are determined and the rates of mitochondria respiration and glycolysis, respectively, are calculated. Four injection ports adjacent to each well allow for the programed addition of various substrates, inhibitors or other agents for detailed metabolic analysis. The reagents include oligomycin, trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP), rotenone, antimycin A, and 2-deoxyglucose (2-DG) and are obtained commercially (Agilent Technologies, Santa Clara, CA). The data are normalized to the cellular protein content in each well. Data generated from these conditions enable the calculation of several metabolic characteristics including basal OCR, ATP coupled OCR, maximal respiration rates, non-mitochondrial OCR, ECAR, basal PER (proton efflux rate), glycolytic PER, compensatory glycolysis, glycolytic ATP production rates, and mitochondrial ATP production rates (Mookerjee et al. 2017).

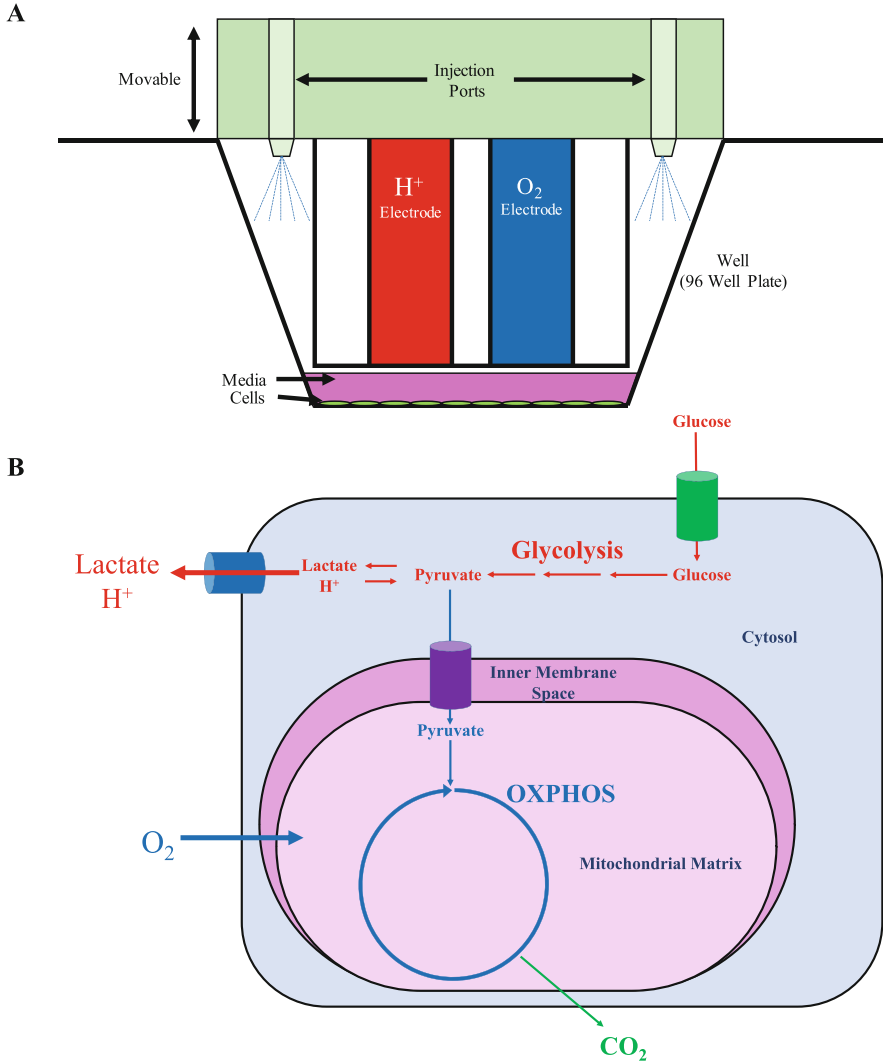


Fig. 1 Extracellular flux analysis configuration and cellular ECAR and OCR measurements. **(a)** Extracellular flux analysis is achieved with sensitive optical probes that measure changes in $[H^+]$ and oxygen in a closed compartment containing culture media and endothelial cells performing glycolysis and mitochondrial respiration. Injection ports surrounding the probes allow timed delivery of various drugs or inhibitors to measure their effects on glycolysis and mitochondrial respiration. **(b)** The primary contributor to ECAR measurements is export of lactic acid formed from pyruvate via glycolysis. Mitochondrial electron transport (TCA cycle and OXPHOS) is detected by the OCR measurements and is based on the oxygen consumption by mitochondria during oxidative phosphorylation and electron transport involving pyruvate or other suitable substrates

2.3 Day Prior to Assay

Cells are grown to 80–100% confluency in a T75 cell culture flask, harvested and seeded at 11.5×10^3 cells per well (0.11 cm^2 surface area) in a rat tail type I collagen coated Seahorse XFe96 cell culture microplate (Agilent, Part No. 101085-004). Cells are then incubated in the microplate for 20–24 h at 37°C and 5% CO_2 . A sensor cartridge is also hydrated according to the manufacturer's instructions.

2.4 Day of Assay

After the overnight incubation of the cell culture plate, growth media is removed by aspiration and cells are washed twice with assay media consisting of Seahorse XF minimal base medium supplemented with 5.83 mM glucose, 1.5 mM glutamine, 1 mM pyruvate, pH adjusted to 7.4 (sterilized by filtration). Assay media (175 μL) is added to each well and the plate incubated for an hour at 37°C in a non- CO_2 incubator. Also, an hour prior to assay, 200 μL XF calibrant is added to the sensor cartridge.

2.5 Mitochondrial Stress Test

The four injection ports in the sensor cartridge are each filled with 25 μL 1.0 μM oligomycin, 0.5 μM FCCP, or 0.5 μM rotenone/antimycin A prepared by dilution using assay media with phenol red. After 1 h of incubation, the 96-well microplate is placed in the Seahorse Flux Analyzer and three basal OCR measurements are made (Fig. 2). Next the contents of each injection port are introduced sequentially and after each addition three additional OCR measurements are recorded (Fig. 2).

Oligomycin inhibits ATP synthase, FCCP uncouples the proton gradient between the intermembrane space and the mitochondrial matrix, and rotenone/antimycin A inhibits complexes I/III of the electron transport chain. The differences in OCR values following the injection of these compounds enable the calculation of ATP coupled OCR, maximal respiration, spare respiratory capacity, proton leak, and non-mitochondrial OCR (Fig. 2).

2.6 Glycolytic Rate Assay

The sensor cartridge is prepared by filling two ports with 25 μL of 50 mM 2-DG (Agilent, No. 103344-100) and 0.5 μM rotenone/antimycin A prepared using assay media without phenol red. Following an hour of incubation, assay media is replaced with fresh media and the plate inserted in the Seahorse Flux Analyzer (Fig. 3). Following three basal ECAR measurements, rotenone/antimycin A to inhibit all electron transport and 2-DG to block glycolysis are sequentially introduced via the injection ports and three ECAR measurements are made following each. The initial

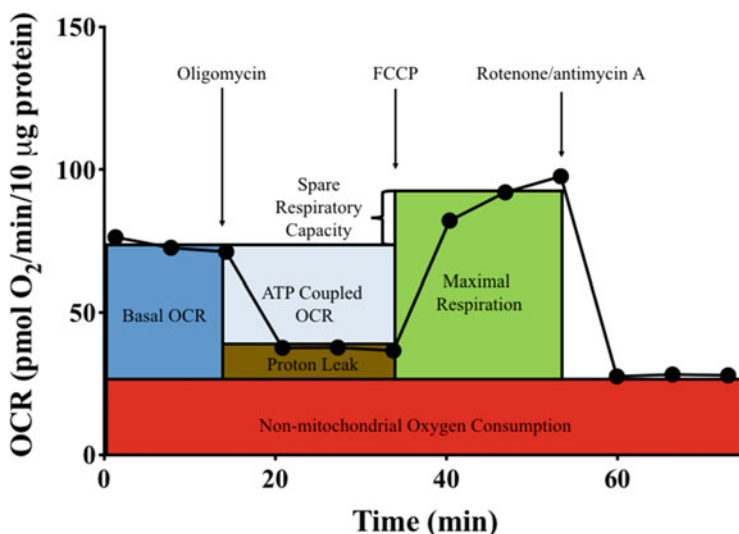


Fig. 2 Mito stress test protocol. A representation of real-time OCR measurements from a mitochondrial stress test is illustrated. Timed injections of oligomycin, FCCP, and rotenone/antimycin A inhibit ATP synthase, uncouple the proton gradient between the inner mitochondrial membrane and the mitochondrial matrix, and inhibit complexes I/III of the mitochondria, respectively. From initial measurements and measurements after timed injections of these compounds, the basal OCR, ATP coupled OCR, maximal respiration, spare respiratory capacity, proton leak, and non-mitochondrial OCR are calculated

basal ECAR measurement, measurements following administration of each inhibitor, and the respective OCR values are determined and used to calculate ECAR, PER from glycolysis (GlycoPER), MitoPER, compensatory glycolysis, and non-glycolytic acidification (Fig. 3).

2.7 ATP Rate Assays

Solutions of 1 µM oligomycin, 0.5 µM FCCP, and 0.5 µM rotenone/antimycin A are prepared in assay media without phenol red and each (25 µL) is added to separate injection ports of the sensor cartridge. Following an hour incubation of the cell culture plate, the assay media is replaced with fresh media and the microplate is placed in the Seahorse Analyzer. Six basal measurements are made followed by three measurements after consecutive injections of oligomycin, FCCP, and rotenone/antimycin A.

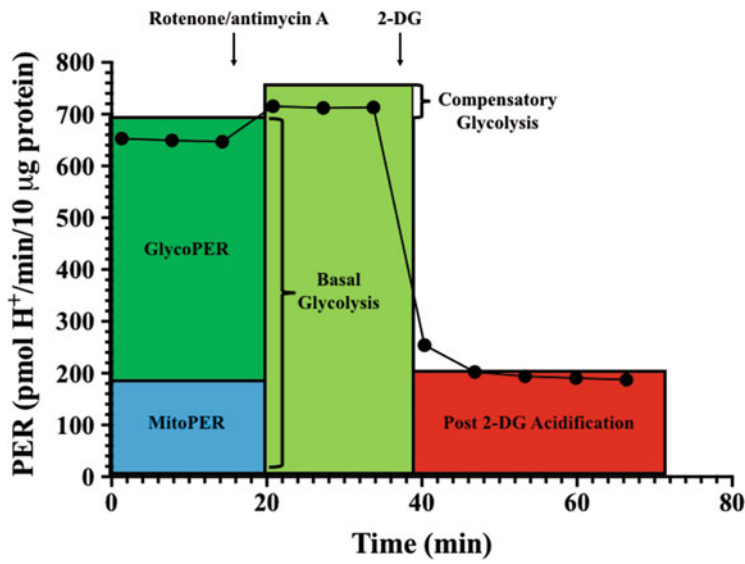


Fig. 3 Glycolytic rate assay protocol. A representation of real-time PER, GlycoPER, and MitoPER measurements of a glycolytic rate assay is illustrated. Timed injections of rotenone/antimycin A and 2-DG inhibit mitochondrial complexes I/III and hexokinase, respectively. From basal real-time measurements, measurements following timed injections of inhibitors, and respective OCR measurements, the basal ECAR, GlycoPER, MitoPER, compensatory glycolysis, and non-glycolytic acidification are quantified

Table 1 Formulas for quantifying metabolic rates of cellular energy metabolism

Equation 1	Glycolytic ATP production rate = GlycoPER
Equation 2	glycoPER = PER – mitoPER
Equation 3	PER = ECAR × buffer Factor ^a × Vol _{XF} microchamber × K _{vol}
Equation 4	mitoPER = mitoOCR × CCF
Equation 5	mitoOCR = OCR _{basal} – OCR _{rot/AA}
Equation 6	OCR _{ATP} = OCR _{basal} – OCR _{oligo}
Equation 7	Mitochondrial ATP production rate = OCR _{ATP} × 2 × P/O

^aBuffer factor is a correction factor used because the assay media is buffered and pH change does not reflect the true number of protons introduced by metabolism. The Buffer Factor is determined by measuring pH changes in the assay media after addition of known acid concentrations

2.8 Calculations

Many cellular processes require ATP to fuel structural changes, metabolism, and signaling pathways. The two primary ATP producing pathways are glycolysis and OXPHOS. OCR and PER measurements can be used to calculate the ATP production rate from the mitochondria or glycolysis, respectively (Divakaruni et al. 2014). Equations 1–7 (Table 1) are used to calculate mitochondrial and glycolytic ATP production rates by the cells being studied. The units for OCR are pmol O₂/min, for

PER are pmol H^+ /min, and for ATP production are pmol/min/10 μ g protein. VolXF represents the geometric volume of the sealed microplate well and is 2.28 μ L. K_{vol} represents the volume scaling factor and is 1.6 for the XFe96. The CO_2 Contribution Factor (CCF) is the amount of acidification that is contributed to ECAR by CO_2 (carbonic acid) that is derived from the mitochondrial TCA cycle. CCF has a value of 0.61 for the XFe96.

The difference in OCR between basal measurements and following addition of oligomycin, an ATP synthase inhibitor, is proportional to the amount of OCR coupled with ATP production. This value is then multiplied by two, to convert to O atoms, and also multiplied by the number of molecules of ATP synthesized per atom of O (P/O value). The P/O ratio may differ slightly based on cell types and metabolite composition. However, a P/O ratio value of 2.75 is most appropriate for hCMEC/D3 and hBVECs based on values for multiple cell types (Divakaruni et al. 2014; Mookerjee et al. 2017). Given this information, a P/O value of 2.75 is used for hCMEC/D3 cells. Glycolytic ATP rates are calculated by using glycoPER values. Glycolysis produces two ATP molecules and two lactate molecules, which are subsequently exported with a proton. Therefore, proton production from glycolysis is directly proportional to glycolytic ATP production.

Measurements of OCR and ECAR under basal conditions and changes following addition of specific metabolic inhibitors enable the calculation of several additional rates using appropriate formulas (Divakaruni et al. 2014) (Table 1).

3 Results

3.1 OCR and ECAR Measurements Reveal Glycolytic Nature of Brain Endothelial Cells

Cells derive ATP, their energy currency for performing work, principally through two pathways, glycolysis and mitochondrial oxidative phosphorylation (OXPHOS). The theoretical basis for measuring these catabolic pathways using extracellular flux analysis has been well documented theoretically and supported experimentally (Divakaruni et al. 2014; Mookerjee et al. 2017).

To characterize the bioenergetics of human brain endothelial cells, *in vitro* cultures were subjected to extracellular flux analysis in order to measure rates of glycolysis and mitochondrial respiration (Figs. 1, 2, and 3). For this purpose, hCMEC/D3 cells, a well-characterized and immortalized human brain endothelial cell line (Poller et al. 2008; Weksler et al. 2013) and primary human brain endothelial cells, hBVEC, were examined.

OXPHOS generates considerably more ATP molecules than glycolysis (~33 versus 2) per glucose molecule. Mitochondrial ATP synthase exploits the electrical-chemical gradient of protons, produced from the electron transport chain (ETC), as a driving force to convert ADP into ATP. This process involves the consumption of oxygen as oxygen serves as a final electron acceptor for the ETC and produces H_2O . The cell Mito Stress Test measures oxygen consumption, in real

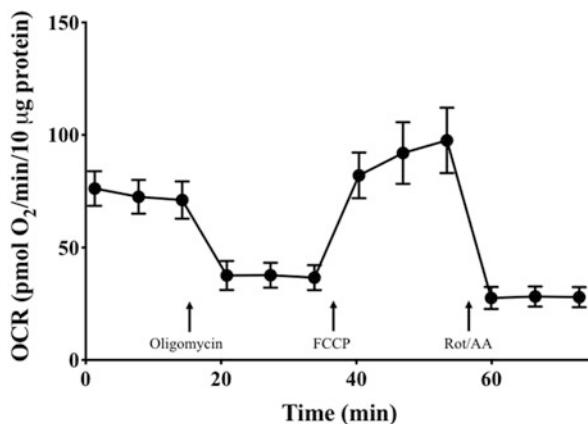


Fig. 4 hCMEC/D3 cells subjected to Mito stress test. OCR measurements were taken in real time in conjunction with stepwise injections of oligomycin, FCCP, and rotenone/antimycin A to characterize mitochondrial respiration by hCMEC/D3 cells. Real-time OCR measurements were used to calculate basal, ATP coupled, maximal respiration, spare respiratory capacity, proton leak, and non-mitochondrial OCR. Values were normalized to 10 µg protein. Each point represents a mean measurement of $n = 3$ independent experiments. Error bars represent the SEM

time, and in conjunction with stepwise additions of oligomycin, FCCP, and Rotenone/Antimycin A, enabling the calculation of various respiratory rates and characteristics (Fig. 4). Using this test, hCMEC/D3 cells exhibited a basal mitochondrial OCR of 44.0 pmol O₂/min/10 µg protein, an ATP-linked OCR of 34.9 pmol O₂/min/10 µg protein, and a proton leak of 9.1 pmol O₂/min/10 µg protein (Fig. 5). These values compare favorably with studies of endothelial cells from other tissues and are relatively low for most proliferating cell types such as a variety of cancers. The Mito Stress Test also determines if mitochondria are operating at maximal capacity to generate ATP or if they have reserve capacity to increase mitochondrial respiration beyond the basal levels. The results indicate that hCMEC/D3 cells are capable of increasing oxygen consumption to 70.8 pmol O₂/min/10 µg protein, a 60.9% increase while maintaining a coupling efficiency of 79.3% (Fig. 5). Non-mitochondrial oxygen consumption was 27.6 pmol O₂/min/10 µg protein.

In contrast, the rate of glycolysis by hCMEC/D3 cells, as determined by measuring extracellular acidification, exhibited a basal PER of 498.9 pmol H⁺/min/10 µg protein and a glycoPER of 483.6 pmol H⁺/min/10 µg protein (Fig. 6). These rates greatly exceed, on a molar basis, the mitochondrial respiration rates (Fig. 5). The glycolytic rate assay also measures the capacity for glycolysis to increase maximally beyond the basal rate. The results indicate a compensatory glycolytic PER of 566.3 pmol H⁺/min/10 µg protein, an increase to 114% of the basal rate (Fig. 6). Furthermore, based on basal PER and glycoPER values, 97.2% of the media acidification (proton production) is attributed to the products of glycolysis as opposed to CO₂ production (Fig. 6). The post 2-DG acidification of 55.0 pmol H⁺/min/10 µg protein.

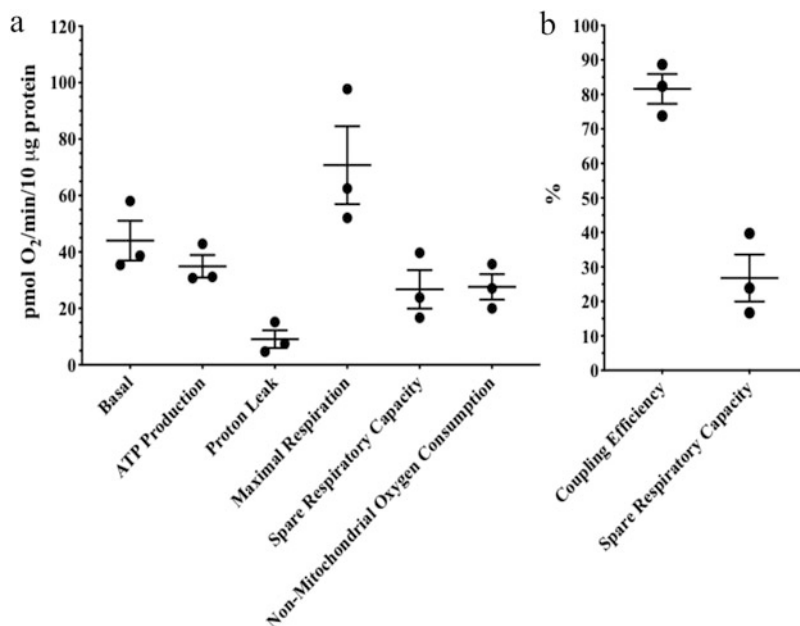


Fig. 5 Oxygen metabolism by hCMEC/D3 cells. **(a)** Cultures of hCMEC/D3 cells were subjected to metabolic flux analysis (Mito Stress Test; Figs. 2 and 4). The basal, ATP coupled, maximal respiration spare respiratory capacity, and non-mitochondrial OCR rates of hCMEC/D3 cells were calculated and graphed in this scatter dot plot. **(b)** The coupling efficiency and spare respiratory capacity percentage were also calculated (right). All measurements were normalized to 10 µg protein. Each point represents one independent assay. Middle bars and error bars represent the mean and SEM of $n = 3$ independent experiments, respectively

Given these metabolic features of mitochondrial respiration and glycolysis, there is still the question of whether hCMEC/D3 cells are a representative model for in vivo brain endothelial cell bioenergetic analysis. To address this question, human primary brain microvascular endothelial cell (hBVEC) bioenergetics were also subjected to metabolic flux analysis. When compared to the OCR measurements of hCMEC/D3 cells, hBVEC rates were very similar to the immortalized cell line for basal rates (Fig. 7). In addition, the maximal respiration, spare respiratory capacity, and non-mitochondrial oxygen consumption were similar. The glycolytic (ECAR) rates for hCMEC/D3 and hBVEC cultures were also compared (Fig. 8). The hBVECs also exhibited similar values for glycolytic measurements and were very consistent in producing other similar glycolytic features. Taken together, these results strongly support the use of hCMEC/D3 for bioenergetic studies of human brain microvascular endothelial cells for in vivo comparisons.

ATP production is a function of glycolysis and mitochondrial respiration. Therefore, rates of ATP formation may be computed using appropriate and validated formulas (Table 1). Using this methodology, OCR and PER measurements of hCMEC/D3 revealed that the ATP production rates from glycolysis and OXPHOS

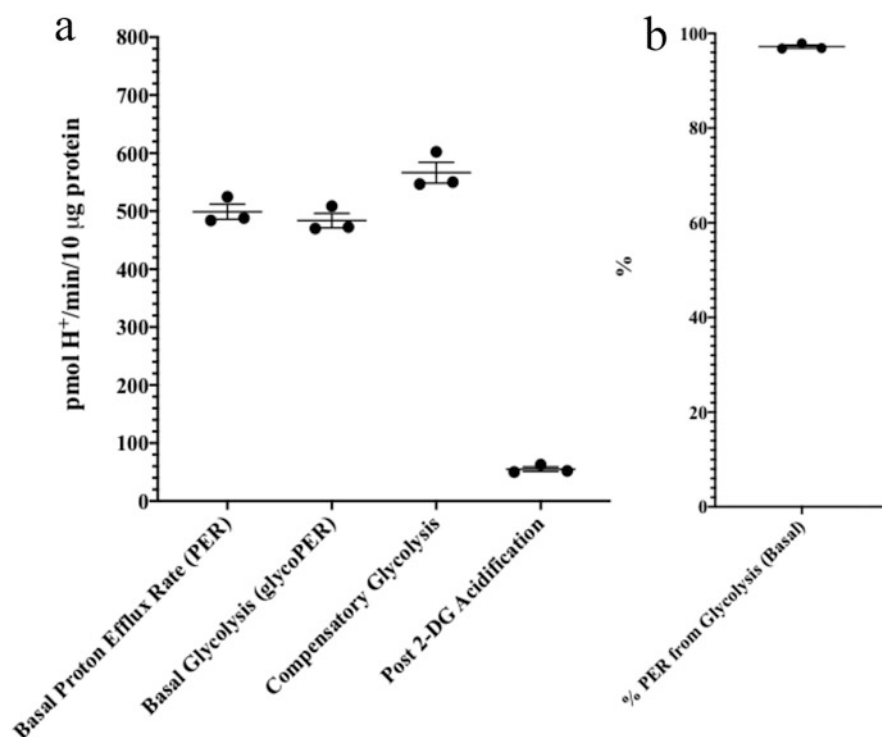


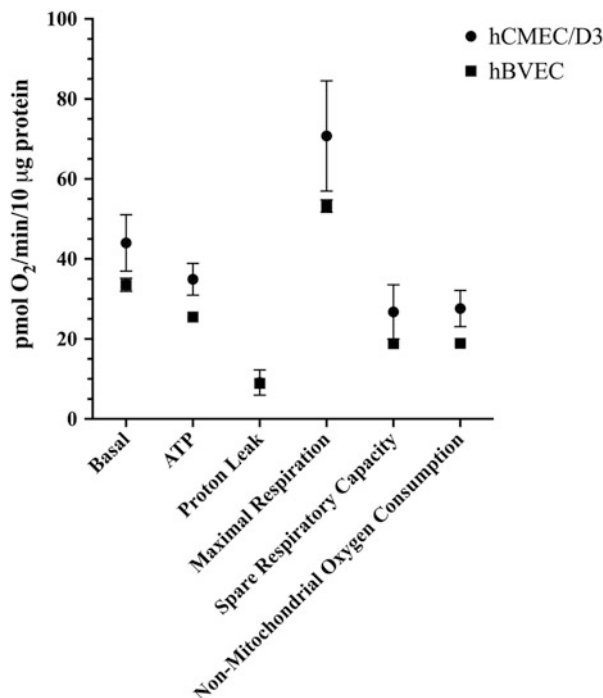
Fig. 6 HCEC/D3 subjected to glycolytic rate assay. **(a)** Real-time measurements of extracellular acidification, in conjunction with sequential injections of rotenone/antimycin A and 2-DG, were used to calculate glycolytic characteristics of hCEC/D3 cells. Basal, glycolytic, compensatory, and post 2-DG acidification PER values are indicated. **(b)** Percent acidification (%PER) was quantified. All measurements were normalized to 10 µg protein. Each point represents one independent assay. Middle bars and error bars represent the mean and SEM of $n = 3$ independent measurements, respectively

are 527.8 and 88.4 pmol ATP/min/10 µg protein, respectively (Fig. 9). Therefore, the total ATP production rate of hCEC/D3 cells is 616.2 pmol ATP/min/10 µg protein (Fig. 9), where 85.7% is contributed from glycolysis and 14.3% is contributed from OXPHOS. These results indicate that the ATP-producing cell metabolism by hCEC/D3 cells is primarily via anaerobic glycolysis and mitochondria play a much smaller, yet significant role.

4 Discussion

Brain endothelial cells are a major component of the neurovascular unit (NVU) and are the cells that form the interface between the blood and brain. In this position it serves as the molecular gateway for all communications that occur between blood-borne nutrients, hormones or other signaling molecules, and the CNS. Therefore, an

Fig. 7 Comparison of hCMEC/D3 versus HBVEC oxygen consumption rates. Basal, ATP coupled, proton leak, maximal respiration, spare respiratory capacity, and non-mitochondrial OCR rates were determined and plotted for comparison of hCMEC/D3 cells and hBVECs values. All measurements were normalized to 10 μg protein. Each point represents the mean value of three experiments. Error bars represent the SEM of $n = 3$ experiments for hCMEC/D3 cells. For hBVEC cells, the error bars represent the SEM of three replicates using one culture preparation

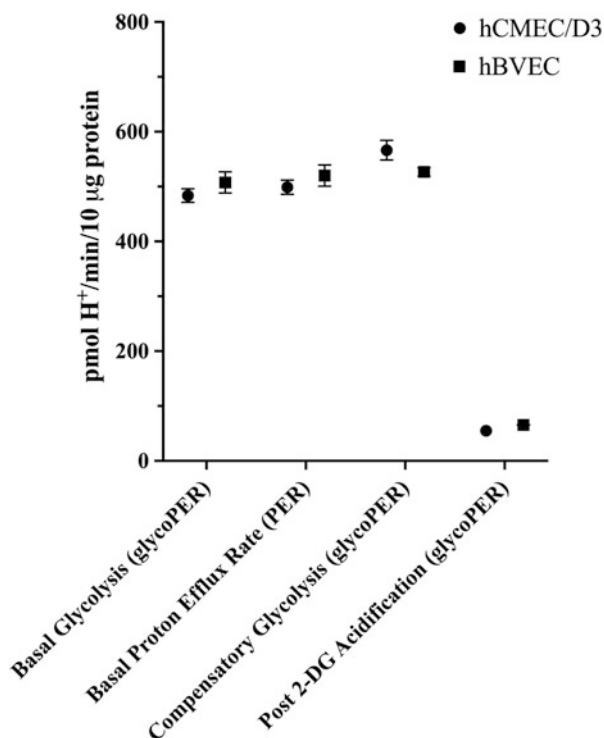


understanding of the metabolic features of brain endothelial cells is important for appreciating the normal functioning NVU and the metabolic disruptions that occur from exposure to disease conditions, toxins, or pharmaceutical agents.

Using real-time measurements of OCR and ECAR, the mitochondrial respiration and glycolytic rates, respectively, of hCMEC/D3 human immortalized microvascular endothelial cells were quantified. Metabolic flux analysis technology enabled determination of many features of brain endothelial cells in addition to OCR including PER and ATP production rates. These experiments revealed that hCMEC/D3 cells exhibit a metabolism driven primarily by glycolysis, as over 97% of protons exported by this cell line were from glycolysis, via lactate production. This is further supported by results from ATP rate assays performed. Furthermore, the assays indicated that brain endothelial cells display an 85.7% glycolytic ATP production rate, while mitochondrial ATP production, although significant, is only 14.3%. Very similar metabolic results were obtained using human primary brain endothelial cell cultures, thus, indicating that the results are not unique to the cell line and that the hCMEC/D3 cells are a valid model for *in vivo* comparisons.

There are advantages to a primarily glycolytic metabolism for brain endothelial cells. Increased glycolytic rates decrease the production of damaging reactive oxygen species (ROS) during mitochondrial electron transport and also allow oxygen to be available primarily to the surrounding tissue (Eelen et al. 2018). Reduced amounts of ROS within the neurovasculature and brain may prevent NVU cell and

Fig. 8 Comparison of HCMEC/D3 versus HBVEC PER values. Basal, glycolytic, compensatory, and post 2-DG PER values were calculated and compared between hCMEC/D3 cells and hBVECs. All measurements were normalized to 10 μ g protein. Each point represents the mean value of three independent experiments. Error bars represent the SEM of $n = 3$ experiments for hCMEC/D3 cells. For hBVEC cells, the error bars represent the SEM of three replicates using one culture preparation

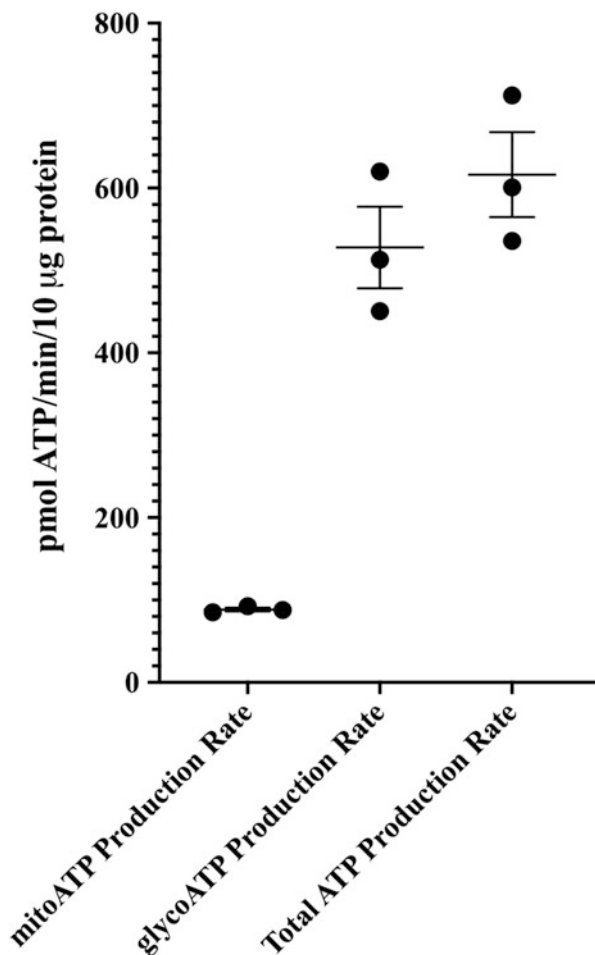


neural cell damage and potential dysfunction. Lactate produced via glycolysis may also act as a signaling molecule for other processes such as active angiogenesis (Hunt et al. 2007; Ruan and Kazlauskas 2013; Sonveaux et al. 2012). Such a response allows adequate vascularization of the brain to produce nutrients, ions, and other molecules easily accessible from the blood. A glycolytic metabolism is, furthermore, believed to precondition endothelial cells to sprout in avascular and hypoxic environments (Gatenby and Gillies 2004). Therefore, it may be beneficial for microvascular endothelial cells to express a highly glycolytic metabolic phenotype to encourage maximal vessel sprouting within the CNS, thereby providing oxygen, nutrients, and other substances for neural function. Finally, a robust glycolytic pathway provides precursors such as ribose for nucleic acids and 3-phosphoglycerate for amino acid and protein synthesis. These are essential for brain endothelial cells in order to respond to signals for growth and proliferation.

Extracellular flux analysis also reveals additional insights into cellular energetic rates. There is supporting evidence that indicates that this approach allows quantification of glycolytic, OXPHOS, and ATP production rates accurately (Mookerjee et al. 2017). Quantifying these metabolic rates can reveal the metabolic phenotypes, such as the extent to which cells utilize glycolytic or mitochondrial respiration for ATP production. Though this indirect method of measuring cellular energetics is well supported and beneficial, it does not provide specific amounts of intracellular

Fig. 9 HCMEC/D3 cell ATP production rates.

Mitochondrial, glycolytic, and total ATP production rates were calculated, using equations provided in Table 1, for hCMEC/D3 cells. All measurements were normalized to 10 μg protein. Each point represents a single independent measurement and error bars represent SEM of $n = 3$ samples



metabolites and ATP. In order to measure these parameters, mass spectrometry, ATP assays, or other tools will be necessary. Actual concentration measurements of these intracellular metabolites and additional glycolytic and TCA cycle intermediates, ADP, AMP, and phosphocreatine would be complementary to the results reported here. Metabolomics is another analytical technology that is gaining acceptance in cancer treatment, stem cell biology, and immunology and is a potential complementary methodology.

The results reported here may contribute to advancing research in CNS disorders or side effects experienced by some therapies. Understanding healthy brain endothelial cell bioenergetics can give investigators a platform to begin studying disease-state alterations in brain endothelial cellular metabolism. A dysfunctional NVU has shown to be associated with various CNS disorders (Yu et al. 2020). This dysfunction may be associated with changes in brain endothelial cell metabolism. Different

disease states may exhibit a specific, altered, metabolic phenotype in brain endothelial cells. Targeting altered metabolic pathways in disease-state brain endothelial cells can provide a non-invasive method of treatment for diseases involved with NVU dysfunction. Furthermore, understanding healthy brain endothelial cell metabolism may provide insight for NVU dysfunction and age-associated pathologies of the NVU. Incorporating a diet or developing supplements that support healthy brain endothelial cell metabolism, in turn, may support healthy CNS function. Adequate levels of glucose, glutamine, pyruvate, and other substances in blood serum can support healthy brain endothelial cell function, and thus healthy CNS function.

Though much improved understanding was acquired from this study, there remain a number of unresolved details regarding the bioenergetics of brain endothelial cells that can be explored. Future studies to investigate the roles of different substrates and metabolites will contribute to details about hCMEC/D3 bioenergetics and function. Such metabolites include fatty acids, lactate, various amino acids, alternative carbohydrates, TCA molecules, and mixed substrate compositions. Other future studies may involve measuring cell bioenergetics in a hypoxic environment, as the partial pressure of oxygen in the CNS vasculature is much lower than atmospheric conditions (Hawryluk et al. 2016). Using a disease-state model to measure differences in energy production between healthy and disease-state will also provide essential insights to changes in metabolism in diseased brain endothelial cells. One example of such a study would be to utilize extracellular flux to measure ATP production rates regarding the importance of ATP driven efflux transporters, like ATP binding cassette (ABC) transporters (i.e., P-glycoprotein, Breast cancer resistance protein). This is important to study as these ATP driven efflux transporters are largely responsible for restricting xenobiotic molecules, or drugs, from entering the CNS environment via the vasculature (Schinkel et al. 1994). Effects of simulating blood flow, co-culture with other cells of the NVU, and in vivo studies can give insights to bioenergetics of brain endothelial cells in a more physiological environment. Metabolic enzyme expression and activity in brain endothelial cells may also reveal important information regarding brain endothelial cell bioenergetics. As with the effect of metabolites and metabolic pathways, changes in brain endothelial cell bioenergetics can very well involve changes in metabolic enzyme or membrane transporter expression or activity. Given the highly glycolytic nature of these cells, it is possible that major changes would involve glycolytic enzymes. This may reveal other targets for diseases involving NVU dysfunction or, conversely, insights to maintaining healthy NVU function. Measuring the bioenergetic properties, using extracellular flux analysis, of hCMEC/D3 cells revealed an abundance of information regarding brain endothelial cell metabolism. These studies provide a glance of brain endothelial cell bioenergetics in vitro and lay the foundation for future studies involving brain endothelial cell and NVU metabolism.

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Part II

Challenges with Findings Drugs to Cross the Blood-Brain Barrier



Considerations When Developing Blood–Brain Barrier Crossing Drug Delivery Technology

Robert D. Bell

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Abstract

Efficient therapeutic transport across the neurovasculature remains a challenge for developing medicine to treat central nervous system (CNS) disorders (Bell and Ehlers, *Neuron* 81:1–3, 2014). This chapter is meant to provide some insight and key considerations for developing and evaluating various technologies and approaches to CNS drug delivery. First, a brief review of various biological barriers, including the immune system, cellular and protein components of the blood–brain barrier (BBB), and clearance mechanisms in peripheral organs is provided. Next, a few examples and learnings from existing BBB-crossing modalities will be reviewed. Insight from “BBBomic” databases and thoughts on basic requirements for successful in vivo validation studies are discussed. Finally, an additional engineering barrier, namely manufacturing and product scalability, is highlighted as it relates to clinical translation and feasibility for

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developing BBB-crossing delivery technologies. A goal of this chapter is to provide an overview of the many barriers to the successful delivery of medicines into the brain. An emphasis will be placed on biotherapeutic and gene therapy applications for the treatment of neurological and neurodegenerative disorders.

Keywords

Blood–brain barrier (BBB) · Central nervous system (CNS) · Drug delivery · Endothelial · Gene therapy · Glycocalyx · Neurotrophic · Pericyte · Transferrin receptor

1 Biological Barriers to CNS Drug Delivery

Several challenges exist in advancing our understanding and ultimately treating brain injury and CNS diseases including heterogeneous patient populations, comorbidities, aging processes, and of course drug metabolism and a limited ability of therapeutics to access the CNS. The BBB is a semi-permeable and highly specialized structure composed of complex cellular and protein interactions (Fig. 1). Many preclinical and clinical studies have demonstrated that the BBB is

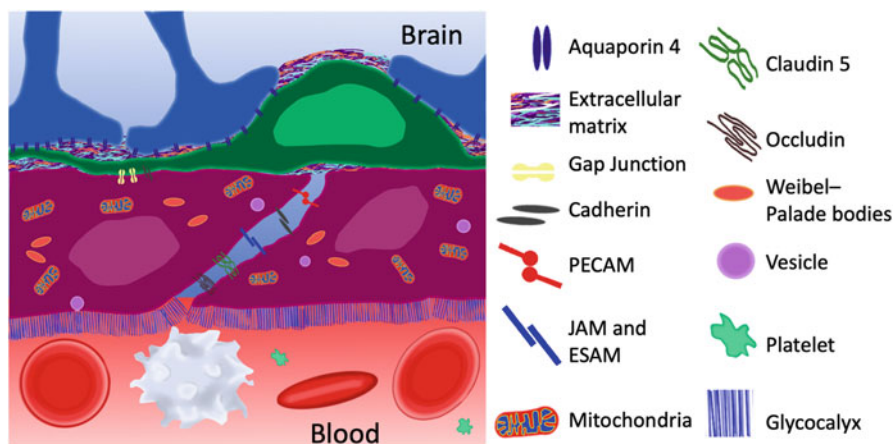


Fig. 1 The Blood–Brain Barrier. Distinct structures and cell types including the glycocalyx, vascular endothelial cells (purple), pericytes (green), the extracellular matrix, and astrocyte end feet (blue) compose the BBB. Pericytes and endothelial cells are embedded in a basement membrane composed of collagen, proteoglycans, laminin, and other matrix proteins. Endothelial cells at the BBB have a high concentration of mitochondria in order to meet energy demands of ATP-dependent transporters and tight junction complexes. Tight junction proteins, including occludin and claudin-5, restrict the paracellular transport of molecules, including small ions, across the BBB. Junctional adhesion molecules (JAM) and endothelial selective adhesion molecule (ESAM) are involved in maintenance of tight junctions. Cadherin and platelet endothelial cell adhesion molecule (PECAM) are among the proteins that form adherens junctions. Pericytes are critical regulators of BBB function and play important roles in the formation and maintenance of the BBB (Bell et al. 2010). (Illustration by Marissa Ilardi, MD)

required to maintain the health and normal functioning of the CNS (Zlokovic 2008). While, on the other hand, the BBB also creates a significant challenge for delivering potentially disease modify medicines to brain and spinal cord tissue. Tight junction proteins sealing the paracellular junctions and limited vesicular transport in the endothelial cells lining the extensive CNS vascular network are often considered primary mechanisms regulating the BBB. However, if one takes a step back it is clear that additional biological barriers exist for an intravenously administered drug to successfully gain access to the brain parenchyma (Fig. 1). For example, the immune system is an important initial barrier to successful CNS drug delivery. Preexisting immunity such as neutralizing antibodies against gene therapy vectors (Mingozzi and High 2013) or adaptive immune responses (e.g., anti-drug antibody formation (Krishna and Nadler 2016)) have an important implication in the overall pharmacokinetic profile and ultimate efficacy of various therapeutic modalities. Next, lining the luminal (blood facing) side of the endothelial cell membrane is a rich glycoprotein and glycolipid covering known as the glycocalyx. While perhaps not as well studied as other cellular and protein components of the BBB, electron microscopy images show the glycocalyx is composed of dense and “brush”-like structures (Ando et al. 2018). Notably, the glycocalyx has also been implicated in the trafficking of macromolecules at the BBB interface (Kutuzov et al. 2018). See Box 1 for additional information on the BBB glycocalyx. Next, as more thoroughly reviewed in chapters “New Insights in the Complexity and Functionality of the Neurovascular Unit”, “Drug Delivery Strategies to Overcome the Blood–Brain Barrier (BBB)”, “The Blood–Brain Barrier in Alzheimer’s Disease”, and “Blood–Brain Barrier Mechanisms in Stroke and Trauma” this book, trafficking through (e.g., transcytosis) or between endothelial cells is required for therapeutics to cross the BBB and begin to gain access to the cells and minimal extracellular spaces found in the brain parenchyma. Once through the endothelium, therapeutics are still faced with additional biological barriers including dense extracellular matrix (ECM) proteins, pericytes, and astrocyte end-feet processes. These biological barriers, from the immune system in circulation through the endothelium and past ECM, pericytes and astrocyte end feet, collectively protect the CNS from potential pathogens and maintain healthy neuronal functioning (Sweeney et al. 2019; Bell and Zlokovic 2009). However, these barriers also clearly create a significant challenge for getting potential disease-modifying therapeutics in the CNS to address serious unmet medical needs. Moreover, the protein, viral vector, or other therapeutic modality must remain stable and intact while encountering these various biological barriers such that when it reaches the final target it can engage the appropriate mechanism of action and/or release the therapeutic payload. Lastly, following the first pass through the BBB rapid drug clearance and metabolism by organs such as liver may create an additional hurdle for therapeutic concentrations to remain high enough in circulation to effectively cross the BBB. While this arduous pathway a drug faces to get into the CNS may discourage researchers from developing strategies to overcome these barriers, others have made triumphant progress and provided key learnings to help guide the development of future BBB-crossing medicines.

Box 1 The BBB Glycocalyx

The negatively charged glycocalyx consists of glycosaminoglycans, proteoglycans, adsorbed plasma proteins, and enzymes at the most luminal surface of the BBB. This extracellular protein brush or mesh-like structure is required for vascular health and function. Disruption of the glycocalyx due to acute injury or chronic disease processes is associated with increased vascular permeability and migration of blood-derived immune cells (Nieuwdorp et al. 2005; Mulivor and Lipowsky 2002). Electron microscopy imaging of cationized ferritin binding on the endothelial surface is the most widely utilized method of visualizing the glycocalyx. Interestingly, reduced cationic ferritin particle binding to the glycocalyx surface has been demonstrated in rats following chronic hypertension-induced BBB damage (Ueno et al. 2004). ELISA analysis of various components of the glycocalyx such as hyaluronic acid in cerebrospinal fluid (CSF) and plasma can also be used to indirectly assess glycocalyx integrity. For example, increased levels of hyaluronic acid in the cerebral spinal fluid in patients with vascular dementia are suggestive of glycocalyx degradation and vascular dysfunction (Nagga et al. 2014). Advancing research and methodologies to further evaluate the glycocalyx in the context of health and disease could have implications in identifying clinical biomarker strategies to assess BBB function and may provide novel therapeutic targets for treating neurovascular dysfunction.

2 Carrier Transport and Receptor-Mediated Transcytosis at the BBB

While small lipid-soluble molecules can enter the CNS through passive transport, specialized carriers and receptors actively mediate transport of small water-soluble, polar molecules and macromolecules across the BBB (Zlokovic 2008; Pardridge 2012). For example, carrier-mediated transport pathways exist for glucose, amino acids, and nucleosides (e.g. adenosine), whereas receptor-mediated transcytosis (RMT) pathways carry macromolecules such as insulin and transferrin into the brain. Transcytosis is a process by which macromolecules are transported within membrane bound vesicles between apical and basolateral domains of polarized cells (Tuma and Hubbard 2003). Amongst the various transcytosis pathways for drug delivery into the CNS, the use of transferrin receptor (TfR) has been particularly well studied. In 1984, Jefferies discovered the abundance of TfR on brain capillaries that functions to deliver the iron-bound transferrin protein into the brain (Jefferies et al. 1984). Subsequent experiments using radiolabeled-tracers demonstrated that anti-TfR antibodies can cross the BBB (Pardridge et al. 1991). Since then several approaches (Fig. 2) have been reported including anti-TfR antibodies carrying therapeutic payloads (Pardridge 2012; Boado et al. 2013), generation of

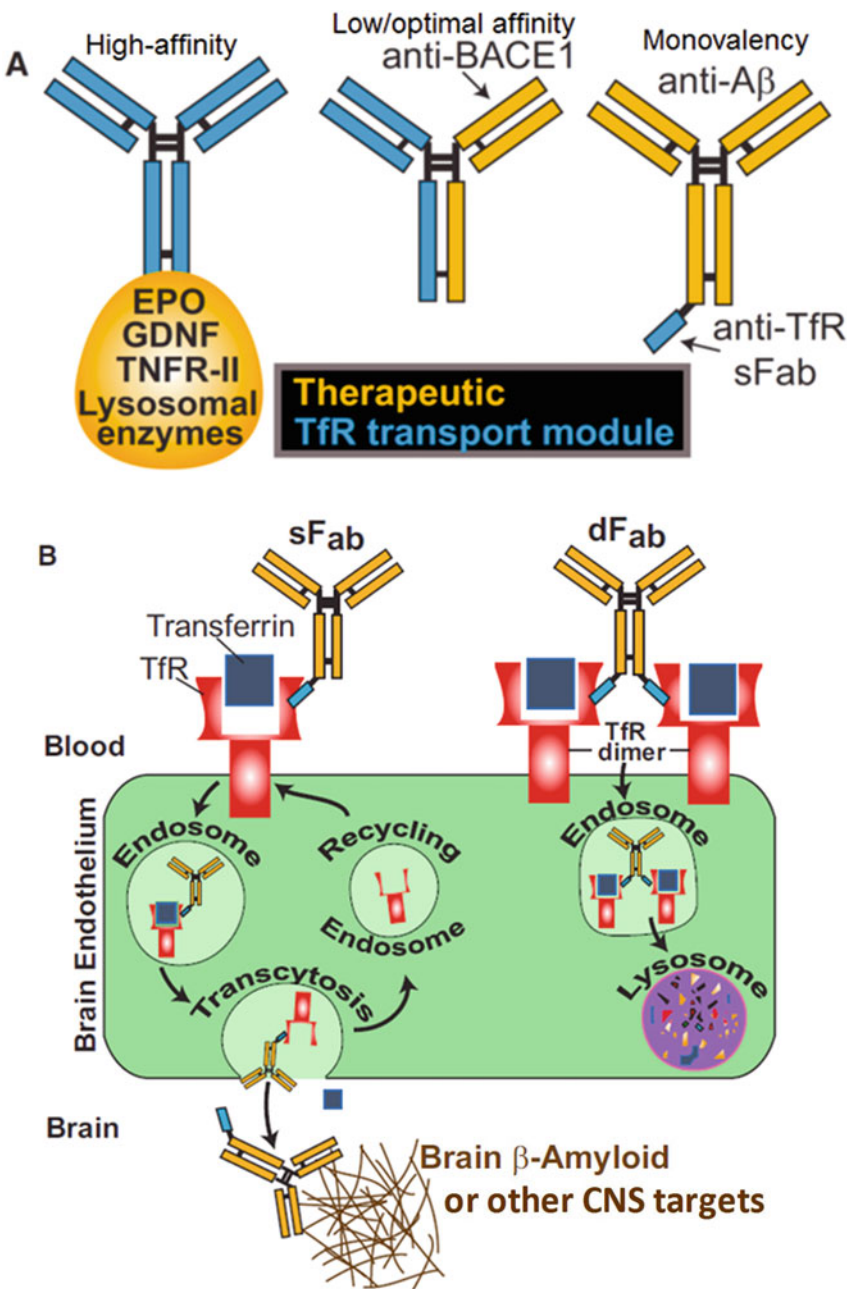


Fig. 2 Anti-TfR vehicles for CNS drug delivery (Bell and Ehlers 2014). (a) TfR-based drug delivery strategies including IgG-based molecular Trojan horse fusion proteins (left) (Pardridge 2012), a bispecific anti-TfR/BACE1 antibody (middle) (Yu et al. 2011), and the anti-TfR sFab Brain Shuttle (right) (Niewoehner et al. 2014). (b) Proposed pathway for differential intracellular sorting of monovalent and bivalent anti-TfR Fab fusions. Whereas monovalent anti-TfR sFab fusions undergo transcytosis across the BBB, bivalent anti-TfR dFab fusions lead to TfR

bi-functional antibodies in which one antibody arm binds TfR and the other a disease-relevant target (Yu et al. 2011) or anti-TfR single F_{ab} fragments fused to the c-terminus of therapeutic monoclonal antibodies (mAb) (Niewoehner et al. 2014). Additional carriers and receptors at the BBB that have demonstrated promising preclinical results related to therapeutic delivery into the CNS include (but are not limited to) the insulin receptor (IR) (Pardridge et al. 1995), GLUT1 glucose receptor, Basigin receptor, and CD98hc (Zuchero et al. 2016). Interestingly, recent evidence using mouse genetic knockout models suggests insulin trafficking at the BBB is actually independent of IR (Rhea et al. 2018). While certain TfR- and IR-targeting modalities have moved into clinical testing (e.g., for the treatment of neurodegenerative and lysosomal storage disorders), none of these approaches have been fully validated or approved for treating human CNS disorders yet. Several other known receptor pathways and molecules discovered through non-biased approaches (e.g., phage-display library screening) are not mentioned here as many of these have not been validated by independent studies and remain controversial in the field.

One key learning from the RMT approaches is the importance of understanding the complete biodistribution profile and potential toxicity liabilities of the therapeutic in peripheral organs. For example, TfR is ubiquitously expressed throughout the body, with very high expression in the bone marrow due to the essential role it has in erythropoiesis (Moura et al. 2015; Sposi et al. 2000). Therefore, while certain TfR targeting strategies may achieve some brain exposure, significant drug accumulation and in some cases clearance/metabolism will occur in most peripheral organs. In fact, researchers have successfully employed TfR targeting mAbs to deliver therapeutic payloads to cancer cells (Chalouni and Doll 2018) as well as cardiac and skeletal muscle (Sugo et al. 2016). Whether therapeutics that utilize TfR targeting modalities are better suited for certain oncology or peripheral indications as opposed to CNS applications remains to be seen. Therefore, while the pharmacokinetics may indeed be suitable for certain applications the mechanism of action(s), therapeutic payload, and the target product profile (dose levels, frequency of administration, and relative safety of the drug) all need to be carefully considered before investing in the development of TfR-based or other RMT-targeting strategy into medicines. Furthermore, in-depth research is likely required to fully understand the complexity and optimize an RMT-targeting mechanism. Understanding optimal binding parameters (e.g., binding site affinity, valency, pH sensitivity), impact of peripheral biodistribution and ensuring normal function of the endogenous pathway is not disrupted leading to adverse effects are just a few examples of important considerations when validating or working towards discovering novel RMT pathways for CNS drug delivery.

3 Lessons from Neurotropic Pathogens

Similar to learnings from endogenous RMT pathways, research into understanding how neurotropic pathogens gain access to the CNS may have the potential to guide novel approaches for drug delivery systems (Salinas et al. 2010). First, several neurotropic viruses including flaviviruses (e.g., West Nile and Tick-Borne Encephalitis Viruses), John Cunningham Virus, Human Immune-Deficiency Virus (HIV), and Herpes Virus are well known to be capable of infecting the CNS and cause significant pathological consequences (Ludlow et al. 2016). These viruses seem to access the CNS via multiple different mechanisms, including carriage by peripheral immune cells into the brain, via peripheral nervous system and axonal transport, breakdown of the BBB by inflammatory mechanisms, and perhaps a direct transcytosis mechanism in the brain endothelium (Salinas et al. 2010). For example, evidence suggests that HIV does not directly cross the endothelial barrier and instead the virus is taken up by peripheral monocytes which are then capable of transmigration across the BBB leading to a further dissemination in the brain (Williams et al. 2015). Whereas, poliovirus, rabies virus, and herpes viruses are among the best characterized pathogenic agents that undergo axonal transport from peripheral nerves into the CNS. Moreover, as these particular pathogens utilize axonal transport they also have the ability to undergo viral replication thereby enhancing their pathological impact. Thus, utilizing peptide fragments such as the HIV transactivator of transcription (TAT) peptide or rabies virus glycoprotein (RVG) peptide may not be the most logical approach for directly engaging and crossing the BBB efficiently for drug delivery applications. Next, certain neuropathic fungus, such as *Cryptococcus* species, particularly *C. neoformans* and *C. gattii* which are the two most common causes of life-threatening fungal meningitis have been suggested to directly cross the BBB. Gelli and colleagues have elucidated a specific role of a secreted fungal metalloprotease (MPR1) and the EphA2-tyrosine kinase receptor in trafficking of *C. neoformans* at the BBB (Aaron et al. 2018; Vu et al. 2014). While additional research is ongoing to better understand pathogen trafficking into the CNS (such as the IM2PACT consortium effort found at www.im2pact.org), the hope is one day some of these mechanisms may be utilized for the delivery of disease-modifying therapeutics.

4 BBB-Crossing Adeno-Associated Virus Capsids

Adeno-associated virus (AAV) has emerged as a promising vector for the delivery of gene therapy to multiple tissues including, for example, the eye (Rodrigues et al. 2018), liver (George et al. 2017), skeletal muscle (Asher et al. 2020), spinal cord and the brain (Deverman et al. 2018). For CNS application most gene therapy programs are utilizing direct injections into the brain parenchyma or intrathecal delivery approaches, though progress is being made to develop novel AAV capsids capable of efficiently crossing the BBB. AAV9 was reported to cross the neonatal mouse BBB (Foust et al. 2009) and this led to the successful development of a gene therapy

(Onasemnogene abeparvovec, sold under the trade name Zolgensma) for infants with spinal muscular atrophy (Mendell et al. 2017). Unfortunately, most capsids (including AAV9) lack the ability to efficiently cross the BBB of adult rodents and primates at clinically feasible doses. The mechanisms regulating the differences between AAV9 CNS exposure in neonatal animals versus adults is still not entirely clear, but speculations have included differences between the BBB and/or immune system between developing neonates versus adults. Regardless, since most AAV subtypes do not have enough BBB-crossing potential for clinical use in adult indications various efforts to modify existing serotypes or develop novel capsids through rational design or non-biased library screening continue to emerge in the field. For example, PHP.B and PHP.eB are AAV9 variants that contain short 7 amino acid peptide insertions into the VP1 domain of the capsid that were discovered to have a remarkable ability to cross the BBB in mice (Deverman et al. 2016; Chan et al. 2017). These insertions allow the capsids to interact with the GPI-anchored protein LY6A (or SCA1) allowing for subsequent transcytosis across the BBB (Huang et al. 2019; Hordeaux et al. 2019). Once inside the CNS parenchyma PHP.B utilizes AAV receptors, such as AAVR, to transduce neurons and astrocytes (Huang et al. 2019). PHP.B and PHP.eB have proven to be incredibly useful AAV capsids in certain rodent species, however LY6A is not expressed at the primate BBB limiting the translational potential of this specific capsid. This highlights that the output of non-biased library approaches may only be relevant in the model system (e.g., in vitro BBB model or rodents) used to perform the screen. Fortunately, with the power of modern genetic tools, deconvolution of the specific targets can allow researchers to gain confidence (or not in the case of PHP.B) in the translational potential of a novel CNS drug delivery technology, such as BBB-crossing AAV capsids. Whether similar GPI-anchored proteins or other targets at the human BBB can be utilized to deliver new AAV capsids and achieve robust CNS transduction following intravenous administration remains to be seen. Promising emerging capsids have been recently reported to cross the BBB of rodents (e.g. AAVF (Hanlon et al. 2019)) and even non-human primates (AAV-HSC (Ellsworth et al. 2019)). However, additional work is needed to uncover the full translational potential of these and other emerging capsids. Interestingly other capsids have been developed to specifically transduce the endothelial cells of BBB, such as AAV2-BR1 (Korbelin et al. 2016). Finally, as briefly mentioned above, an additional aspect to consider in the development of BBB-crossing viral vectors for clinical use is whether preexisting immunity exists against the capsid or particle (Mingozzi and High 2013). For example, since AAV is a naturally occurring virus, a vast majority of the population have been previously exposed and therefore may have existing immunity against certain serotypes.

AAV is a promising vehicle to mediate gene transfer (or knockdown) in humans. Further research is warranted to discover novel AAV capsids for clinical gene therapy applications with improved characteristics including high transduction potency, defined tissue tropism, low neutralization by preexisting anti-AAV antibodies, and an efficient manufacturability profile.

5 Confirming and Identifying New Targets

While understanding various functions of the many proteins expressed at the BBB will be the subject of research for decades to come, there are several useful published databases that can give researchers a sense of mRNA and protein abundance (or whether the target is even present) at the BBB (Kalari et al. 2016; Vanlandewijck et al. 2018; Zhang et al. 2014, 2016; Uchida et al. 2011). Moreover, several of these references have taken advantage of innovative techniques and bioinformatic tools to generate single cell omics datasets from rodents and human BBB samples. It may be obvious, but important to note, that abundance at the BBB alone does not mean a particular protein is optimal for targeting with CNS drug delivery technology. Understanding subcellular localization, complexes with other proteins, changes during development, aging or disease processes are all additional considerations one should think through before pursuing new BBB transport targets. As the tools and datasets become more readily available to better define the molecular and biochemical “BBB atlas” the field will hopefully be able to better understand both transport mechanisms and the role of the BBB in pathological processes.

6 Considerations for In Vivo Validation Studies

In vitro systems are useful to answer many biological questions, however current cell-based models fail to completely recapitulate the physiological complexity of the BBB. Therefore, additional animal studies of BBB-crossing CNS delivery technologies are often required to confirm and gain confidence in each specific approach. Before embarking on animal studies, it is essential to ensure robust and validated methods are in place to measure the pharmacokinetics and biodistribution of the therapeutic. Optimally it should be possible to quantify the drug concentration in blood and tissues at various time points following administration. This allows the researchers to understand how the drug accumulates in various organs including the liver, kidneys, muscle, brain, and spinal cord as a function of the percent of injected dose. The ability to visualize the delivery technology and/or therapeutic payload is also helpful to gain confidence in adequate targeting. For instance, immunohistochemical approaches can help differentiate a drug that has remained within the vasculature versus accumulated in the brain parenchyma. Next, CNS biomarkers and pharmacodynamic responses allow for researchers to confirm if the therapeutic reached the desired target and elicited the appropriate mechanism of action(s). Caution should be taken though in making conclusions based on pharmacodynamic responses that may be due to either peripheral or central nervous system targeting. For example, the use of pain modulators may exert their effect on peripheral nerves or via CNS signaling mechanisms making it somewhat difficult to conclude the exact site of action. Therefore, having a suite of robust, well-validated assays and inclusion of control samples (both positive and negative) is required to gain confidence in novel BBB-crossing therapeutics. Data in animal (and cellular) models is useful for verifying target engagement, evaluating the safety profile, and enabling clinical dose

predictions of a new therapeutic. However, the reality is all models will likely have some limitations and therefore appropriately designed clinical studies are ultimately required to support further development and regulatory approval of a BBB-crossing therapeutic. Confidence in biological translation from *in vitro* and *in vivo* preclinical models to human clinical trials is always associated with some degree of risk and uncertainty in drug development.

7 Manufacturing Considerations and Challenges

Finally, a brief comment on the potential barriers and/or bottlenecks in manufacturing and “scalability” of BBB-crossing therapies, especially those focused on emerging modalities involving nanoparticles, exosomes, and viral-based protein capsules. Even after successful preclinical discovery of a biotherapeutic or gene therapy modality, additional engineering and manufacturing barriers must be considered. For instance, if a novel biotherapeutic protein or AAV-based capsid cannot be manufactured to meet the yields and quality required to support clinical trials, it will be nearly impossible for it to be developed into a successful medicine. Changes in the biophysical characterization of proteins and gene therapy vectors may change when transitioning from small research-grade scale to large clinical manufacturing processes. Therefore, early considerations into the scalability and manufacturing processes in the development of a CNS-delivery technology may help to ensure ultimate translational success and clinical utility.

8 Concluding Remarks

As hopefully now better appreciated there are many barriers to successful delivery and development of CNS therapeutics. Here are a few summary questions to consider when developing blood–brain barrier crossing drug delivery technology:

- Does the drug sufficiently enter the CNS and elicit the intended pharmacological mechanism of action?
- Will the drug exposure and metabolism in peripheral tissues lead to poor efficacy or undesired safety issues?
- Does preexisting immunity or potential immune response to the therapeutic pose a risk in the ultimate clinical translation and efficacy of the therapy?
- Will the preclinical models used in gaining confidence in pharmacokinetics and efficacy actually translate in human clinical trials?
- Can the therapeutic be manufactured at the required clinical scale and quality?

The BBB field has continued to provide important experimental data to fill gaps in our knowledge and provide certain roadmaps that will hopefully allow us to ultimately break through these barriers and successfully address the unmet medical needs associated with CNS disorders.

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Brain Distribution of Drugs: Brain Morphology, Delivery Routes, and Species Differences

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Abstract

Neuropharmacokinetics considers cerebral drug distribution as a critical process for central nervous system drug action as well as for drug penetration through the CNS barriers. Brain distribution of small molecules obeys classical rules of drug partition, permeability, binding to fluid proteins or tissue components, and tissue perfusion. The biodistribution of all drugs, including both small molecules and biologics, may also be influenced by specific brain properties related to brain anatomy and physiological barriers, fluid dynamics, and cellular and biochemical composition, each of which can exhibit significant interspecies differences. All of these properties contribute to select optimal dosing paradigms and routes of drug delivery to reach brain targets for classical small molecule drugs as well as for biologics. The importance of these properties for brain delivery and exposure also highlights the need for efficient new analytical technologies to more comprehensively investigate drug distribution in the CNS, a complex multi-compartmentalized organ system.

Keywords

Brain barriers · Cerebral fluids · Delivery routes · Distribution · Extracellular fluid · Heterogeneity

1 Introduction

The central nervous system (CNS) distribution of drugs obeys similar principles as those that apply for other body tissues. Distribution is dependent on interactive relationships between drug properties, e.g. physicochemical characteristics or the nature of the product (i.e., whether the pharmaceutical is derived from chemical synthesis or biological sources), and tissue properties such as anatomy, composition, fluid perfusion, and vascular characteristics. Nevertheless, brain distribution of drugs can also exhibit tissue-specificity. Several tissue properties of the CNS stand out for their uniqueness when compared to other body systems or organs. For example, brain anatomy, cellular biology and biochemistry, vasculature, and fluid dynamics all exhibit important differences from the rest of the body. These brain specificities make CNS pharmacokinetic-pharmacodynamic relationships also specific, unique, and occasionally quite different from what happens in other body spaces. We will mainly focus this chapter on some of the key factors that continue to make detailed understanding of the biodistribution of CNS drugs challenging and a description of how such factors should be better considered to aid modern CNS drug development.

2 The Prominent Role of Distribution

The conventional definition of pharmacokinetics based on the study of absorption, distribution, and elimination (including metabolism and excretion) (A, D, E) processes is typically applied to the whole body. However, one may also apply these concepts more specifically to the CNS to arrive at a more focused consideration that can be termed neuropharmacokinetics (NPK). NPK can be defined by the qualitative and quantitative study over time of three simultaneous steps following CNS exposure to a medicinal molecule: (1) absorption into the CNS, (2) distribution or intra-CNS partition, and (3) CNS elimination or clearance comprising metabolism and excretion in the complex multi-compartmental system consisting of the brain, spinal cord, blood vessels, and surrounding support tissues.

Unfortunately, the complex relationships encompassed within NPK have not been sufficiently considered by the pharmaceutical industry and most other stakeholders in CNS drug development during the past several decades. Historically, the brain has all too often been kinetically considered as a well-perfused homogeneous and single compartment isolated from the periphery by the blood–brain barrier (BBB) (Fig. 1a). Such a limited consideration resulted in the widespread belief that the CNS active fraction only depended on drug passage across the BBB, but that further CNS distribution and elimination processes typically ignored. When translated to pharmacokinetics for the whole body, this would be akin to imagine that drug PK might be completely understood only by considering the absorption phase, i.e., assuming that the drug effect would not be related to the distribution and elimination phases.

This fragmented and biased approach toward CNS drug PK considerations partly explains why approval rates for new CNS drugs entering clinical development have historically been much lower than for non-CNS therapeutic areas (Pangalos et al. 2007). The complexity associated with the CNS barrier interfaces, primarily the BBB and blood–cerebrospinal fluid barriers, obviously adds a significant PK hurdle but it is important to emphasize that other hurdles also remain beyond these barriers.

The present opinions developed in this chapter are based on considering the brain as a multi-compartment system isolated from the periphery and inside by several barriers (Fig. 1b). Several such biological barriers exist (Saunders et al. 2018): the BBB localized in the brain capillaries; the inner blood–cerebrospinal fluid barrier (BCSFB), a site of CSF fluid secretion associated with the choroid plexus epithelia within the brain's ventricles; the outer BCSFB facing the CSF compartment in the subarachnoid space, associated with the outer arachnoid epithelium component of the meninges; the ependyma separating the CSF from the brain extracellular fluid (ECF); and the neural or glial membranes delimitating the ECF from the brain intracellular fluid (ICF). These barriers help to define brain heterogeneity and intra-CNS partitioning of drugs (see Sect. 3).

Proper consideration of brain A, D, and E processes requires simultaneous investigation using this multi-compartmental concept and an appreciation of the anatomical and biological complexity of the CNS. Absorption to the brain is firmly related to the delivery route for many CNS drugs. In the case of systemic

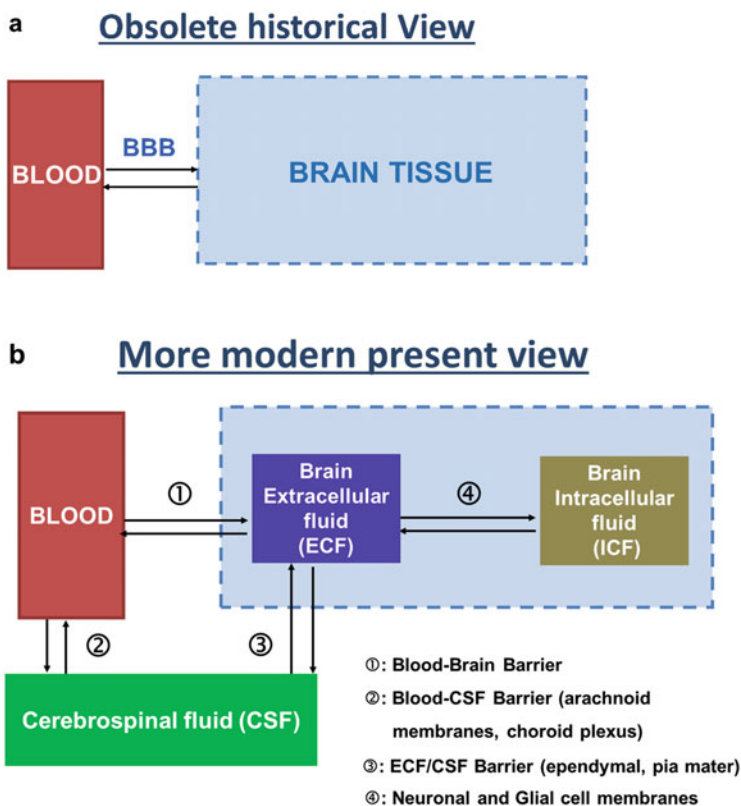


Fig. 1 Obsolete historical (panel **a**) and more modern present (panel **b**) views of the blood-brain compartments. (**b**) Adapted from (Bourasset et al. 2005). Note that brain extracellular fluid (ECF), as shown, and brain interstitial fluid may be used interchangeably

administration, the BBB often represents the main absorption site to the brain whereas for non-systemic intra-CNS routes of administration, absorption to the brain occurs across other sites (Sects. 4 and 6). Regardless, brain distribution and clearance processes will play a fundamental role in determining drug action following the absorption process. A successful distribution means that drug molecules will reach the brain biophase, i.e., the effect site of the drug, at active drug concentrations. Simultaneous brain clearance processes can limit brain exposure, so an appropriate balance between brain distribution and clearance is further required for successful CNS drug exposure.

This NPK complexity is well illustrated by the historical “case example” of two opioids, morphine (M) and its active metabolite, morphine-6 glucuronide (M6G). These two compounds bind the same opioid receptors to mediate analgesia. M6G was observed to be more potent than M *in vivo* despite M6G’s higher hydrophilicity (log P at -2.4 versus -0.2) and poorer BBB permeability (similar to sucrose). In other words, how can it be explained that the poorly BBB permeable M6G can be

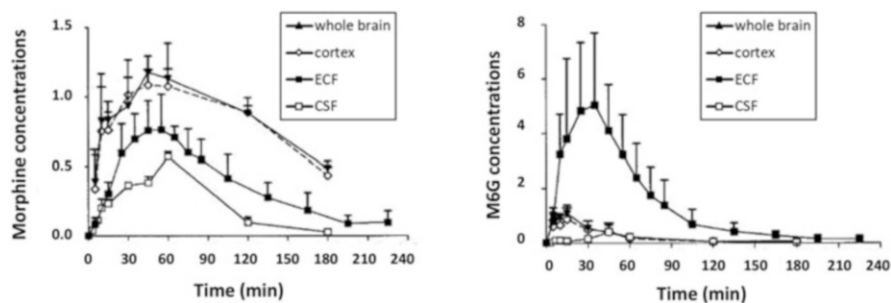


Fig. 2 Plasma and brain morphine and M6G pharmacokinetics after subcutaneous administration in rat of morphine or M6G at a dose of 10 mg/kg. Brain extracellular fluid (ECF) samples were obtained by using the transcortical microdialysis technique. Concentrations were expressed in nmol g⁻¹ in whole brain and cortex and in μmol l⁻¹ in cerebrospinal fluid (CSF) and ECF. Values represent mean ± s.d. (*n* = 5). (Stain-Textier et al. 1999)

more active than M? A detailed pharmacokinetic investigation of both compounds in different brain compartments helped to explain this unexpected observation (Stain-Textier et al. 1999). When examining M and M6G profiles after their subcutaneous administration in different tissues and biofluids of the rat (whole or cortical brain homogenates, ECF and CSF), the levels of each compound were found to be markedly different across the different sampling sites, supporting heterogeneous brain distribution of these two compounds (Fig. 2). Close examination of the ECF levels of M and M6G clarified the enigma of the longer and more potent analgesia mediated by M6G: M6G concentrations were much higher in the ECF compared to the other sites while, in contrast, M concentrations in the ECF were at the low end compared to the other sites; furthermore, direct comparison of M6G to M ECF concentrations revealed M6G levels to be several-fold higher than M. The trapping of M6G in ECF is well illustrated by its ICF/ECF partition ratio of 0.008, compared to 4 for M, suggesting a major extracellular distribution of M6G and intracellular distribution for M. These different patterns in M and M6G brain distribution correlate with the more potent and longer analgesia induced by M6G because opioid receptors are accessible to the ECF space where M6G is trapped. This example emphasizes the importance of the concept of intra-CNS partition, roughly represented by drug partitioning between the aqueous and lipid phases in this complex brain structure. Indeed, more lipophilic compounds than M, e.g. the AMPA-type glutamate receptor modulator S18986 (log *P* = 1.2), can have even higher ICF/ECF ratios. Lipophilic drugs tend to partition towards brain cells, in contrast to hydrophilic drugs which distribute better within the extracellular fluids of the brain (Bourasset et al. 2005). The simple example of M and M6G helps illustrate the multiple scenarios for drug distribution in the CNS, with even greater degrees of complexity possible when considering diverse synthesized chemicals spanning a wide range of molecular and physicochemical properties or biologics such as gene materials, proteins (e.g., antibodies), or other macromolecules and their possible routes of delivery.

The basic principles of drug distribution are commonly distilled down to three main factors having more or less impact according to the properties of the drug under consideration: membrane permeability, tissue perfusion, and binding to proteins or lipids. For macromolecules like antibodies, diffusion and convection are the dominant processes driving biodistribution, whereas a main limiting factor is membrane permeability. For small molecules, tissue perfusion and membrane permeability are often the most determinant factors for biodistribution, however protein binding can also be quite important. The application of such rules to the brain specificities, i.e., to brain barriers and neural or glial membranes, blood and brain fluid flows and brain tissue properties, will be detailed further in the following sections.

3 Brain Heterogeneity

Brain heterogeneity is a common feature across species. For clarity, our considerations will mainly refer to the human brain and other species such as rodents and non-human primates that are among the most highly studied in preclinical CNS drug research and development. We will further limit our coverage of brain heterogeneity to those properties that are more obviously likely to influence the brain distribution of drugs.

3.1 Anatomical Differences and Regional Heterogeneity

The human brain is anatomically sub-divided into three main regions: the forebrain, midbrain, and hindbrain. Each region contains specific sub-regions or structures like the cerebrum containing the four cortical lobes, the thalamus and hypothalamus for the forebrain. Several other structures such as the limbic system containing the hippocampus and the amygdala are illustrative of the complex architectural patchwork apparent in the brain (for complete information on brain structure and functions, see (Bakker et al. 2015; Zupanc 2017)). All these various areas have specific functions and brain disorders or diseases typically related to dysfunctions of one or several connected structures. For example, loss of functional dopaminergic innervation in Parkinson's disease affects basal ganglia circuitry and structures such as the striatum, also implicated in addiction, bipolar and autism disorders. This is the same for epilepsy where cellular dysfunctions observed in various brain areas can induce seizures. Brain cancers simplistically represent a type of brain structure modification, where the infiltration and targeting of anticancer agents to tumor tissue has remained challenging. It follows that an efficient CNS drug must distribute and ideally be targeted as well as possible to the area of clinical and pharmacological interest.

Brain anatomical heterogeneity also depends on its circulating fluid components. The brain is perfused with blood by a large, specialized vascular network where the BBB at the level of brain capillaries provides the primary site for solute exchange between the brain and the periphery. Inside the brain parenchyma, additional fluid

compartments (ECF, ICF, and CSF) are delineated by several physical and physiological barriers which may play a great role in drug distribution heterogeneity. The different barriers between these fluid components express different patterns of permeability, further complicated by the expression of active transport proteins. Indeed, simple predictions of free drug fractions across these barriers at equilibrium for many small molecule drugs based upon pH-partition theory and transport by passive diffusion are frequently limited in NPK due to active transport processes.

Regional brain distribution has been documented for a long time in early preclinical studies by measuring drug levels in homogenates of brain structures following their surgical isolation at one time point. This time and animal consuming method allows for calculation of the percentage of the dose or systemic level reaching the tissue, but it is not able to distinguish ECF drug levels from ICF drug levels and, importantly, unbound from bound drug. Nevertheless, this method was among the first to establish some degree of heterogeneity in CNS drug distribution patterns for many CNS, e.g. in the above example with M and M6G. More sophisticated techniques like quantitative microdialysis allow much greater distinctions with multiple time point analysis in single animals *in vivo* (e.g., as in Fig. 2) (Stain-Textier et al. 1999; Bourasset et al. 2005). Radio-imaging techniques like positron emission tomography (PET) combined or not with anatomical imaging paradigms such as magnetic resonance imaging (MRI) and single photon emission computed tomography (SPECT) are now more frequently used in rodents and primates to quantify both the levels and anatomical distribution of radiolabeled drugs in the brain parenchyma, CSF, and surrounding tissues across time. When combined and co-registered with magnetic resonance imaging (MRI) or classical computed tomography (CT), PET and SPECT allow precise visualization of brain anatomy and dynamically measure drug distribution in nearly any brain sub-structure. Moreover, competition studies are routinely performed with PET and SPECT, allowing CNS activity measurements for drug candidates to be used to elegantly characterize molecular interactions at the brain target, providing a direct relationship between drug concentration and target occupancy. Unfortunately, such methods require heavy and costly equipment as well as expensive and occasionally complex radio-labeling (and subsequent quality control measurements) to yield the desired study compound. More recent label-free methods such as imaging mass spectrometry offer particular advantages, enabling the brain mapping of small chemicals with a high spatial resolution up to the cellular level, coupled with the ability to quantify them (Swales et al. 2015). With the benefit of all these recent and refined technical advances around tissue/fluid sampling and imaging, brain distribution heterogeneity can now be more extensively studied at nearly any level of complexity. Nevertheless, it bears noting that it is often necessary to combine several different techniques in order to gain a complete picture, e.g. to address regional mapping and measurement of free or bound drug fraction. Furthermore, experimental investigations of drug distribution in the brain are today often complemented by sophisticated modeling based on dedicated physiological-pharmacokinetic models allowing further understanding of complex brain distribution processes (Ball et al. 2013; Vendel et al. 2019).

3.2 Different Cellular Types

It is commonly stated that glial cells (astrocytes, microglia, oligodendrocytes, and Schwann cells) outnumber neurons by as much as 2–10 times in the vertebrate forebrain, although more recent work has shown that the neuron:glia ratio varies across species and among different CNS regions (Herculano-Houzel et al. 2015). For example, glia outnumber neurons by 10:1 in the human thalamus and white matter; however, neurons greatly outnumber glia in the human cerebellum. When considering the whole brain, neurons make up approximately 65% of total cells in the mouse (with a total of 68 million neurons), whereas they make up approximately 50% of total cells in humans (with a total of 86 billion neurons). Glia historically were considered to provide predominantly supportive functions for neurons but today they are appreciated to do much more, including prominent roles in blood flow regulation, metabolism, homeostasis, and CNS immunity (Wolak and Thorne 2013). Other different cell types contribute vitally to the various CNS barriers, such as endothelial cells and pericytes of the BBB and epithelial cells of the choroid plexus, ependyma and arachnoid. Molecular targets for most drugs in use today are mostly expressed in neurons but other cells, particularly microglia, are now increasingly recognized as important targets for intervention, e.g. in neurodegenerative diseases. For example, microglial cells (CNS resident immune cells that bear similarities to macrophages and make up about 2% of total brain cells in the mouse brain) can store viruses like HIV and therefore must be targeted for intracellular distribution of antiviral therapy (Dallas et al. 2013). As previously illustrated with the opioid receptors, cell surface receptors are primarily accessible via the ECF fluid space to bind drugs. Intracellular drug delivery has remained a very challenging question, particularly so for biologics and gene materials, as success will depend on the ability to cross several barriers and, with biologics, on their subsequent escape from intracellular organelles such as endosomes, before distribution in the ICF space to reach targets is possible.

Non-specific binding in the brain parenchyma is another critical issue to be considered. Most small molecule CNS drugs are basic molecules with high volumes of distribution that are influenced by both non-specific binding to acidic phospholipids in the plasma membrane and ion-trapping within acidic cellular compartments such as endosomes and lysosomes. Both mechanisms are drug-dependent and drug distribution can be affected by one or both processes. These two features are particularly prominent for the brain, which belongs to tissues sharing among the highest lysosomal trapping activity and phospholipid content.

Another layer of heterogeneity results from lysosomal density and phospholipid content, which also varies across brain areas and cellular types. Lysosomal enzyme activity is reportedly higher in neuron cell bodies (gray matter) than in white matter axons or in astrocytes and oligodendrocytes, providing a possible explanation for why many psychotropics are trapped within neurons more so than in astrocytes or oligodendrocytes (Daniel et al. 2001). Microglia, while present in relatively small numbers in the brain, express a greater range and higher levels of lysosomal enzymes than any other brain cell type.

With respect to small molecule drugs, non-specific phospholipid binding can sometimes become non-reversible and lead to toxic effects like drug-induced phospholipidosis (Shayman and Abe 2013). In contrast, drug trapping within lysosomes is potentially reversible but wash-out times can be much longer than systemic elimination times predicted by the drug half-life. Taking into consideration this last property along with the small intracellular volume of lysosomes (0.01 ml.g brain⁻¹ vs 0.79 for the ICF in rat), significant drug accumulation in lysosomes may under certain conditions play an important role in CNS drug action and toxicity (Loryan et al. 2017).

3.3 Biochemical Heterogeneity

As previously introduced, brain distribution of many psychoactive chemicals is mostly governed by their partition between aqueous and lipid phases. Lipids make up around 50% of the brain's dry weight and are twofold more abundant when compared to other body tissues except adipose. Lipid amount can also vary according to brain areas. For example, myelin has much higher lipid content (approximately 80% of the dry weight) than white matter (~54%) or gray matter (~38%) (Agranoff et al. 1999).

Brain lipidome studies have also shown that each brain region expresses distinct lipidome signatures and that the lipid composition of the brain is markedly different from that of non-neural tissues. Moreover, the largest lipidome divergence between the brain and non-neural tissues has been observed in humans, followed by chimpanzees and macaques, with the smallest found in mice (Bozek et al. 2015). These recent findings highlight not only the high specificity of the brain's lipid organization but also significant differences between species. As lipids are a key component to determine intra-CNS partition, their brain specificity vs other body tissues, variable expression across brain regions, and quantitative importance may ultimately explain various types and extent of drug interactions, thus contributing to heterogeneous drug distribution in brain parenchyma.

3.4 Transporters Heterogeneity

Drug transporters are proteins belonging to two major superfamilies, the ABC (ATP-binding cassette) and SLC (Solute carrier) transporter groups. Altogether, these two superfamilies include more than 400 proteins, with a number involved in drug transport. From an NPK point of view, all these proteins have substrate specificity as well as capacity-limited function. These proteins can transport their substrate either in the direction of the concentration gradient by facilitated diffusion (SLC) or in the opposite direction of the concentration gradient by active transport. In the latter case, transport requires an energy supply, provided either by the hydrolysis of ATP for ABC transporters or mostly by ionic co-transport with the SLC group.

The brain expression of these proteins is ubiquitous: they can be found on all brain interfaces, but with certain quantitative and qualitative specificities. Recent quantitative mass spectrometry analysis has revealed different expression patterns according to the brain barriers and within species (Gomez-Zepeda et al. 2019). For example, P-glycoprotein (P-gp, ABCB1) and breast cancer resistance protein (BCRP, ABCG2) are both expressed on the luminal (blood-facing) side of brain endothelial cells and exhibit species differences: P-gp is more abundant than BCRP in rodents, but less abundant than BCRP in humans and monkeys (Shawahna et al. 2011; Uchida et al. 2011; Hoshi et al. 2013; Al Feteisi et al. 2018). P-gp and BCRP are also expressed on the apical (CSF-facing) side of the outer BCSFB formed by the arachnoid epithelium as well as on the apical (CSF-facing) side of the inner BCSFB formed by the choroid plexus epithelia. The expression of these two transporters has been less clear in parenchymal cells. It has been suggested that BCRP, but not P-gp, is functionally present on microglial cells and astrocytes under physiological conditions. However, P-gp may be expressed in parenchymal cells under pathological conditions such as with epilepsy and certain cancers such as gliomas (Gomez-Zepeda et al. 2019). Transporter expression at each of the brain interfaces can play a crucial role in the brain distribution of drugs. For illustration, we can once again analyze the case of M and its metabolite, M6G, as already discussed above. The heterogeneity of the distribution of M and M6G in the ICF and ECF can be further explained by the involvement of carriers at several brain interfaces. Accordingly, M6G appears to be trapped in the ECF space due to transporters located in the BBB and CSF/ECF interface, while M appears to be trapped in the ICF due to transporters located on brain cell membranes. Older studies by our group demonstrated that these transporters were sensitive to probenecid and hypothesized that they belong to the MRP (MRP3) and OCT (OCT3) families for M6G and M, respectively (Bourasset and Scherrmann 2006). It has now recently been established that M is indeed a substrate of OCT1 and OCT3 (Tzvetkov et al. 2013) and that OCT3 and MRP3 are expressed in brain cells and at the CSF–brain barrier, respectively (Ashraf et al. 2014; Koehn et al. 2019), supporting our initial hypothesis. This example shows how the complex network of brain transporters can determine NPK and direct substrates into a specific brain compartment, even across brain interfaces such as ependymal cells that do not express tight junctions. Currently, this last interface is not considered as a tight physical brain barrier from a strict cellular point of view except during the embryonic period when strap junctions are present between adjacent neuroepithelial cells that later form the ependyma (Saunders et al. 2018). However, the example of M/M6G demonstrates that ependymal transporters can play a role in drug distribution, suggesting the barrier characteristics of this brain–CSF interface may need to be revisited despite their lack of tight junctions. A more recent example also reinforces this notion at a different interface: the brain distribution of a P-gp substrate, erlotinib, was not improved after BBB disruption using the focused ultrasound method, suggesting that P-gp was able to maintain a pharmacologically effective blood–brain barrier despite its physical opening (Goutal et al. 2018). From an NPK perspective, a brain barrier might therefore be more broadly defined as a brain interface in which cellular and/or molecular processes can

potentially limit brain distribution to adjacent fluid compartments, resulting in asymmetric drug distribution.

4 Fluids and Dynamics

NPK considerations require some working understanding of brain fluids, their dynamics, and their relationship to the CNS barriers. Homeostasis of the brain microenvironment depends on its physical separation from the blood, yet at the same time the brain must also accomplish essential exchange of gases and nutrients, as well as the removal of a wide variety and size of waste products. As already discussed above, one key aspect of this exchange is accomplished by the polarized expression of specific transporters at the various CNS barriers. However, another aspect of exchange that has more clearly emerged in recent years concerns the potential for meaningful bidirectional solute transfer between the CSF and brain ECF (Abbott et al. 2018) (note that the terms brain ECF and brain interstitial fluid may be used interchangeably).

CNS tissue is surrounded by CSF, which exerts a buoyancy force upon the brain and spinal cord that allows the tissue to float. The CSF is a complex pseudo-filtrate arising primarily from circulating plasma that is divided unequally between a series of connected cavities inside the brain (the lateral, third, and fourth ventricles) and extraventricular sites (the subarachnoid spaces that typically contain most of the CSF) surrounding the outside of the brain and spinal cord that also contain major surface arteries and veins. A number of studies have suggested that solutes and drugs can potentially distribute from the CSF into the brain along the perivascular space (PVS) associated with penetrating arteries/arterioles. The PVS refers to a vascular connective tissue space that occupies a portion of the vessel outer wall and is separated from the CSF by fibroblast-like leptomeningeal cells that appear to form a CSF-PVS barrier to some solutes (Pizzo et al. 2018). Following fluid/solute distribution into the brain along the PVS, subsequent exchange can occur with the brain ECF adjacent to penetrating vessels and their associated microvessels, with possible clearance out of the brain along the PVS of venules/veins; the whole process appears potentially important for waste clearance and has been variously termed as part of a “glymphatic system” (Iliff et al. 2012, 2013) or “perivascular fluid system” (Abbott et al. 2018; Pizzo et al. 2018) for CSF:ECF exchange. While the precise details of the process and proposed aspects of each hypothetical system remain under debate (Iliff and Simon 2019; Smith and Verkman 2019), it has become clear that PVS transport into the brain from the CSF is an important transport pathway for the distribution of drugs. Further, PVS transport out of the brain may have special significance for central clearance/elimination, particularly for large molecules.

Brain ECF resides within the brain extracellular space (ECS), an interconnected series of gaps surrounded by the cellular processes of neurons, glia, and other brain cells termed the neuropil (Wolak and Thorne 2013; Hrabetova et al. 2018). While the brain ECS occupies ~20% of the total tissue volume in most brain areas of adult

rodents, primates, and other mammals (Sykova and Nicholson 2008), measurements in rats have shown it to be a narrow compartment only 40–60 nm in width under normal conditions (Thorne and Nicholson 2006). Brain ECS is critical for the distribution of neurotransmitters, nutrients, and all drugs. Owing in part to the predicted high hydraulic resistance associated with the extremely narrow width of brain ECS, it has been generally accepted that little if any appreciable bulk fluid flow (convection) is likely to occur in the neuropil of the gray matter under normal conditions. Most transport measurements within brain parenchyma have to date been in excellent agreement with basic diffusion theory, in support of primarily diffusive transport within the neuropil. Indeed, brain capillaries are arranged to take advantage of the short range efficiency of diffusive transport in the surrounding brain ECS. Neurons and other brain cells are typically no further than approximately 10–20 μm from their closest neighboring brain capillaries to allow for the efficient diffusion of O_2 , glucose, and other molecules into the brain across the BBB. There is some evidence that convective transport (also referred to as bulk flow) of brain ECF and CSF can occur along certain preferential pathways within the CNS, e.g. within the PVS, discussed above, and possibly also along axon tracts, but ECF transport within the neuropil ECS of gray matter is likely restricted to diffusion.

Finally, while the composition of the CSF and brain ECF are generally regarded to be similar, this may be strictly correct only quite close to the interface at the ventricular and pial surfaces. The reasons for this are that diffusion greatly limits exchange at greater distances ($>$ a few mm) from these surfaces (Wolak and Thorne 2013) and extracellular matrix components that are abundant within the brain ECS are relatively fixed to cell surfaces (Thorne et al. 2008) and therefore not freely available for exchange with the CSF. Regarding the relative volumes of the various brain fluids, a normal adult human being with a brain and spinal cord weighing 1,300 g will have approximately 150 ml CSF, 260 ml of brain ECF within the extracellular space (i.e., 20% total tissue volume), and 30–70 ml of cerebral blood (i.e., about 2–5% of total tissue volume) (Fig. 3). CSF volumes in rodent species of importance for preclinical drug development range from approximately 300 μl in the rat to approximately 40 μl in the mouse. Finally, CSF flow rates and turnover can be inferred from CSF secretion rates in different species (human, 350–370 mL/min; rat, 2–5 mL/min; mouse, 0.3–0.4 mL/min).

5 Barriers in the CNS

As previously cited, cerebral NPK depends on drug exchange between blood and brain fluids, i.e., blood, extracellular/intracellular brain fluids (ECF and ICF, respectively) and cerebrospinal fluid (CSF) (Fig. 4). These fluids communicate with each other via four brain interfaces: blood–BECF, blood–CSF, ECF–ICF and ECF/CSF. From a strict cellular point of view, the term barrier is given to interfaces with limited paracellular diffusion due to the presence of tight junctions between the cells delimiting the interface. In common practice, only two brain interfaces are therefore typically considered as strict brain barriers: the blood–brain barrier (blood–ECF

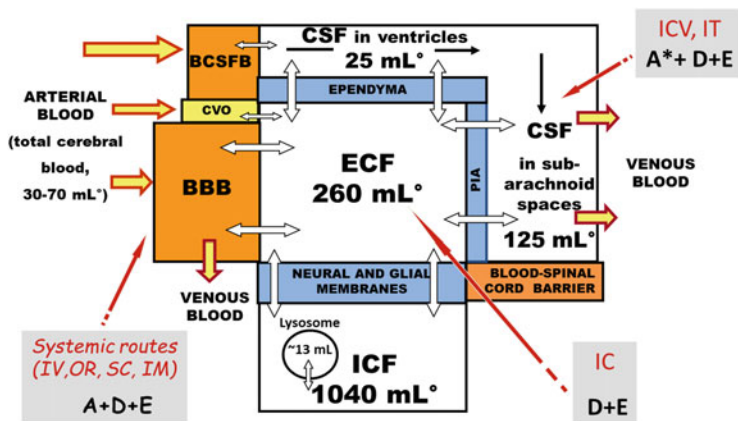


Fig. 3 CNS barriers and compartments: fluid volumes and central administration sites. All cerebral barriers can modulate drug absorption (A), distribution (D), and elimination (E) from outside and inside the CNS. They also influence the selection of the routes of drug administration, i.e., the systemic routes (IV, intravenous; OR, oral; SC, subcutaneous; IM, intramuscular) and the direct routes (ICV, intra-cerebro ventricular; IT, intrathecal; IC, intracerebral). See text for further explanations. Adapted in part from (Scherrmann 2001) with values from (Hammarlund-Udenaes et al. 2014)

interface) and the blood–CSF barriers (blood–CSF interface). However, in light of the expression and function of drug transporters discussed above, this definition may be applied to all brain interfaces. With this understanding of NPK, at least four key brain interfaces can be defined as brain barriers (Fig. 4).

5.1 The Blood–Brain Barrier

As already described in chapter “Structure and Function of the Blood–Brain Barrier” of this book, the presence of several tight junctions between brain capillary endothelial cells (bCECs) leads to the restricted exchange of drugs between the blood and brain ECF, by limiting the paracellular diffusion of molecules. The transcellular pathway is therefore only available for the passive diffusion of small chemicals. In addition to passive diffusion, molecules can be transported from blood to the brain (influx process) or from the brain to the blood (efflux process) via limited-capacity carriers. In the case of biologics, some receptor-mediated trans-endothelial transport can allow access to the brain (Hersom et al. 2016; Toth et al. 2020). Exploitation of potential receptor-mediated transcytosis systems for drug delivery to the brain is currently a highly active area of research, with the most prominent examples targeted to the bCEC-enriched transferrin receptor (e.g., see (Yu et al. 2011)). Therefore, all these active transport processes fully participate in

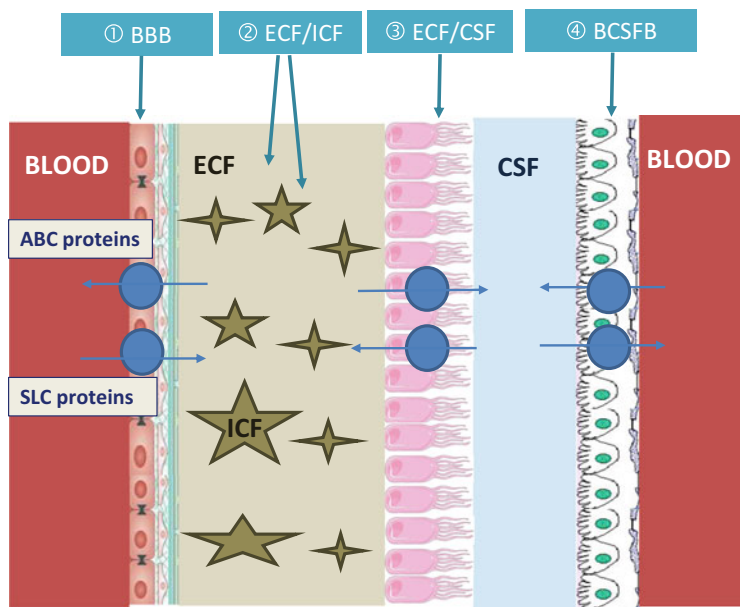


Fig. 4 Schematic representation of 4 key brain interfaces separating the blood and brain fluids. (1) blood–brain barrier (BBB), separating the blood from the brain extracellular fluid (ECF). (2) neuronal and glial cells, separating ECF from the intracellular fluid (ICF). (3) ependymal cells and pia mater separating the cerebrospinal fluid (CSF) and ECF; (4) blood–CSF barrier (BCSFB), separating the CSF from the blood. ABC and SLC transporters are ubiquitously expressed at each brain interface. Adapted from (Bourasset and Scherrmann 2006)

the BBB barrier properties and are of great importance for drug development considerations.

From this molecular point of view, the BBB is easily appreciated to be responsible for a large number of drug exclusions from clinical development. Research is now focused on new strategies to bypass the BBB to allow drugs to reach significant concentrations in the brain biophase. However, it must be emphasized that barrier function provides effective protection for the brain against potentially toxic molecules, both endogenous and exogenous in origin. To cite one example, consider the case of ivermectin, whose LD50 is approximately 100 times lower in P-gp KO mice than in WT mice (Schinkel et al. 1994). It is noteworthy that one substrate can be recognized by several transporters, reinforcing the gatekeeper function of the BBB. In fact, even in the absence or modification of the expression and/or function of one transporter, a molecule can be excluded from the brain by a different transporter. Such situations were first described for common substrates of P-gp and BCRP, including sorafenib and erlotinib, for which a synergistic cooperation was demonstrated (Kodaira et al. 2010; Agarwal and Elmquist 2012). Agarwal and Elmquist showed that P-gp efflux of sorafenib was masked by BCRP efflux because

of higher sorafenib affinity for BCRP than P-gp; nevertheless, P-gp action upon sorafenib was easily revealed in the absence of BCRP (Agarwal and Elmquist 2012). Similarly, our group has demonstrated a cooperation between BCRP, P-gp, and the ABC transporter ABCG4 in the BBB efflux of amyloid-beta peptide ($A\beta$) (Do et al. 2012; Dodacki et al. 2017). In the latter case, a tighter affinity of $A\beta$ for ABCG4 than for P-gp was reported (Dodacki et al. 2017). Taken together, these data emphasize the importance of cooperation between transporters at the BBB and the need to understand substrate capacity and affinity of transporters in detail to best appreciate such cooperation.

From a pathophysiological point of view, there is significant potential for complex BBB dysregulation with the onset and development of neurodegenerative diseases, epilepsy, and metabolic diseases, something that must always be considered (see chapters “The Blood-Brain Barrier in Alzheimer’s Disease”, “Blood-Brain Barrier Mechanisms in Stroke and Trauma”, “Brain Barriers and Multiple Sclerosis: Novel Treatment Approaches from a Brain Barriers Perspective”, “Epilepsy and Alterations of the Blood-Brain Barrier: Cause or Consequence of Epileptic Seizures or Both?” and “Novel Treatment Approaches for Brain Tumour from a BBB Perspective” of this book). One example is GLUT-1 deficiency syndrome where the GLUT-1 glucose transporter is lacking, resulting in severe and resistant epilepsy due to decreased glucose distribution into the brain (Brockmann 2009).

5.2 The Brain ECF-ICF Barrier

This barrier is simply formed by the plasma membranes of neuronal and glial cells. Barrier properties arise from the presence of transporters and receptors on these membranes, leading to asymmetric distribution of exogenous and/or endogenous molecules in ECF and ICF. ICF distribution can be considered to represent the bound brain fraction ($Brain_b$), whereas the brain ECF represents the unbound brain fraction ($Brain_u$) of the molecule (Taccola et al. 2018), in the absence of extracellular protein binding. With significant intracellular accumulation, NPK considerations allow one to see that cells constitute a drug reservoir and thus can enhance the brain volume of distribution ($V_{d_{brain}}$) of several drugs. Recall the case of morphine, where the measured $AUC_{ICF/ECF}$ ratio of 4 may be explained by the transport of morphine from brain ECF to ICF via carrier-mediated transporters located on neuronal and glial membrane cells (Bourasset and Scherrmann 2006). Similarly, the $AUC_{ICF/ECF}$ ratio of S18986 and the $Brain_b/Brain_u$ ratio of PKRinh, two research molecules, were respectively found equal to 4 and 4,700; in these two cases, ICF distribution was explained by both the lipophilicity of the molecules and their affinities for pharmacological targets located on the neuronal cell membranes (Bourasset et al. 2005; Taccola et al. 2018).

Consideration of $Brain_b$ distribution is of major importance since it may sometimes explain a weak unbound brain ECF over plasma ratio, *i.e.* $Brain_u/Plasma_u$, also known as the $K_{pu,u}$ ratio, without the involvement of any efflux transporter at the BBB (Bourasset et al. 2005).

5.3 The CSF-ECF Barrier

The ECF/CSF interfaces consist of ependymal cells lining the periventricular tissue that faces the ventricular CSF and fibroblast-like pial cells making up the pia mater that faces the subarachnoid space CSF. The two fluids (ECF/CSF) are exchanged at this interface, but their renewals are independent and occur at different rates, so there is generally no concentration equilibrium between them (see above Sect. 4 for more discussion). It has long been thought that the ECF/CSF interfaces do not constitute a real physical barrier although this may not strictly apply for macromolecules. Ependymal cells do not have tight junctions in adulthood but may act as a barrier during development by restricting diffusion (Whish et al. 2015). In addition, the expression of carriers such as MRP1, P-gp, ABCA2, Glut-5, URAT1 (SLC22A12), or EAAT1 (SLC1A3) on ependymal cells, as well as the specific NPK of M6G previously described above, suggests that these interfaces could indeed control molecular exchange between the ECF and the CSF in subtle ways that have not been well appreciated, for small molecules as well as for biologics (Zhou et al. 2002; Mercier et al. 2004; Bourasset and Scherrmann 2006; Strazielle and Ghersi-Egea 2013; Tomioka et al. 2013; Bernstein et al. 2014; Ueno et al. 2014; Akanuma et al. 2015).

5.4 The Blood–CSF Barrier

This interface is located both at the level of the outer arachnoid epithelial cells forming the hemato-arachnoid barrier (outer BCSFB) and at the level of the epithelial cells of the choroid plexus (CP; inner BCSFB). Unlike the BBB, the endothelial cells of the CP are fenestrated, while the epithelial cells are connected to each other by tight junctions that give this interface its barrier function. Both BCSFBs express carriers on endothelial and/or epithelial cells, e.g. P-gp and BCRP, that contribute to the barrier function of these two interfaces (Strazielle and Ghersi-Egea 2015; Saunders et al. 2016; Zhang et al. 2018; Gomez-Zepeda et al. 2019).

Since the surface area of the blood–CSF interface is smaller than that of the BBB, its role in drug transport and brain exposure has historically been considered minor from a drug delivery perspective. However, drug exchange between CSF and blood is important and necessary to protect the brain, but also, and perhaps most importantly, to detoxify the brain. Furthermore, as discussed above in Sect. 4, transport into the brain along the PVS pathways has recently emerged as a previously underappreciated route for biodistribution into brain from the CSF; further work is needed to better understand the importance and relevance of the CSF-to-brain pathways for drugs, particularly for antibodies (Pizzo et al. 2018). Finally, it has been suggested that these interfaces may play a role in Alzheimer's disease by participating in the clearance of amyloid-beta from the brain (Matsumoto et al. 2015). Hence, the role of the BCSFBs may be quite important from both a physiological and pathological point of view.

6 Delivery Routes

Many different routes of administration exist for the delivery of drugs to the brain. The most commonly utilized route for small molecule drugs targeted to the CNS has been the oral route, owing to its ease of application, patient compliance, economy, and the strong historical emphasis placed on developing oral drugs by nearly all pharmaceutical companies. With respect to macromolecule drugs and gene therapies, primarily parenteral systemic routes (intravenous, intramuscular, and subcutaneous administration) have been employed to achieve distribution to the brain from the bloodstream. While this approach has to date not yet been successful for CNS delivery of biologics other than for a single adeno-associated virus (AAV)-based gene therapy approval in 2019 (U.S.) for the treatment of spinal muscular atrophy (AAV-9:SMN1; onasemnogene abeparvovec), there are a number of ongoing and soon-to-be initiated clinical trials evaluating brain delivery and efficacy of macromolecule therapeutics targeted to RMT systems at the BBB, a strategy that has already been briefly discussed above. Alternatively, a number of regional routes of administration (intraventricular, intrathecal, intraparenchymal, and intranasal) have also been extensively evaluated for the delivery of biologics directly to CSF and/or brain in order to bypass the BBB or to gain a selective targeting advantage. Other than the intranasal route of administration (Thorne et al. 2004), these routes have the disadvantage of being significantly more invasive. Nevertheless, there are currently three clinically approved regionally administered biologics (U.S. and E.U.), each of which utilizes CSF infusions or injections: (i) intrathecal (lumbar) ziconotide, a small peptide for chronic pain; (ii) intrathecal (lumbar) nusinersen, an antisense oligonucleotide for spinal muscular atrophy; and (iii) intraventricular cerliponase alfa, an enzyme replacement therapy for a form of neuronal ceroid lipofuscinosis (Batten disease). As with the parenteral systemic approaches, numerous clinical trials are ongoing that utilize regional delivery of macromolecule drugs as well as AAV-based gene therapies. Further general description of the factors to be considered for NPK evaluation of biologics delivered to the CNS by the different routes of administration mentioned above can be found elsewhere (Thorne and Frey 2nd 2001; Hammarlund-Udenaes et al. 2014).

7 Neuropharmacokinetic Imaging and Interspecies Differences

As mentioned above, nuclear imaging techniques such as single photon emission tomography (SPECT) and positron emission tomography (PET) are of great interest to study the brain distribution of drugs. The main advantage of these imaging techniques is their non-invasive or minimally-invasive nature. This allows repeating a study on the same living subject, including humans, in identical or comparative conditions at different time points. Using these approaches, translational studies may be easily conducted in animals for proof-of-concept or detailed biodistribution considerations and then subsequently applied to humans to assess the brain distribution and associated receptor occupancy of drugs.

The advantages of PET over SPECT include higher sensitivity (detection efficiency) and better temporal and spatial resolution. PET imaging benefits from straightforward 3D quantifiable recordings. For the delineation of organ substructures on PET images, anatomical information is often required and may be achieved by using integrated systems (PET/CT or PET/MRI) or by performing additional scans for anatomical data on stand-alone CT or MRI with subsequent co-registration of the two imaging modalities.

PET imaging uses radioactive isotopes emitting positrons (β^+ decay), such as fluorine-18 or carbon-11, which collide with electrons in the tissue. Subsequent annihilation of these particles releases two photons, which can be detected and allow calculation of the point where the positron and the electron collided. With the strategy of radioisotopic labeling, it is now possible to synthesize the radioactive analog of the drugs of interest and study their *in vivo* distribution.

Two main parameters may affect the brain distribution of radiolabeled drugs and be responsible for interspecies variability: (i) the xenobiotic transporters expression/activity profile at the BBB and in the other brain barriers, and (ii) the expression/distribution of specific and non-specific brain binding sites for the drugs.

7.1 Variability in Transporter Expression/Activity at the BBB

Species differences have been reported in the substrate recognition and transport efficacy of xenobiotic transporters at many biological interfaces, including the BBB. For instance, while BCRP was shown to be the most abundant ABC transporter at the human BBB, P-gp (mABCB1A) was found to be more abundant at the mouse BBB than mBCRP (Shawahna et al. 2011; Uchida et al. 2011). While mMRP4 was detected as the third most abundant ABC transporter at the mouse BBB, MRP4 expression was found almost 6 times lower in monkeys than in mouse (Ito et al. 2011). These examples of species differences in transporter expression are expected to be among the factors leading to species differences in brain distribution and elimination of drugs. For illustration, Syvänen et al. assessed differences between rats, monkeys, and humans in brain distribution of three radiolabeled P-gp substrates ($[^{11}\text{C}]$ verapamil, $[^{11}\text{C}]$ GR205171 and $[^{18}\text{F}]$ altanserin) and found higher brain-to-plasma ratios of radioactivity in monkeys or humans as compared with rats. They also observed higher increases in brain distribution of these radiotracers following cyclosporin A treatment, a potent P-gp inhibitor, in rats than in monkeys (Syvanen et al. 2009). Similar observation has been made using tariquidar, another potent P-gp inhibitor (Fig. 5).

These studies using PET imaging and drug transporters inhibition strategies highlight and reveal the obvious interspecies differences in brain uptake and distribution of drugs due to variability in the expression or activity of drug transporters at the BBB level or other brain interfaces (Tournier et al. 2018).

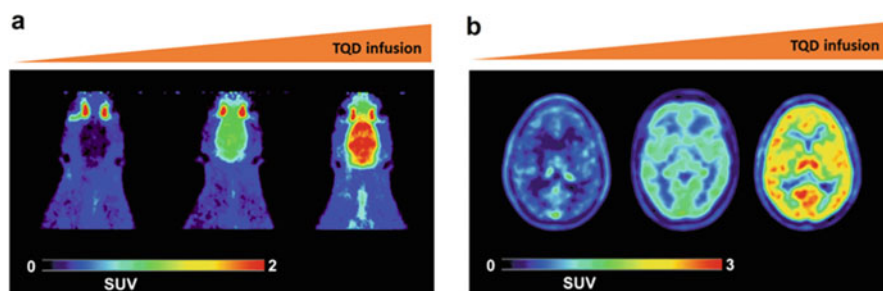


Fig. 5 PET summation images of rat (a) and human (b) brain of (*R*)-[^{11}C]verapamil at baseline and after administration of increasing doses of tariquidar (TQD). Concentration of radioactivity is normalized to injected radioactivity amount per body weight and expressed as standardized uptake value (SUV). Adapted from (Kuntner et al. 2010; Bauer et al. 2012, 2015; Tournier et al. 2018)

7.2 Variability in Brain Target Expression/Distribution

Differences in brain distribution of drugs may also be due to differences in the distribution of brain receptors between species. For example, it is well described in humans that the occipital cortex is devoid of mu opioid receptors and may be used as a reference region for quantification of mu opioid receptor occupancy or availability with PET. In rodents, the brain region that exhibits no specific opioid binding and that is used as a reference region is rather the cerebellum. In contrast, specific opioid binding (mu and/or kappa) was reported in both the cerebellum and the occipital region of non-human primates, making the arterial blood sampling necessary in monkeys to quantify changes in opioid receptor occupancy (Galynker et al. 1996).

In summary, while preclinical experiments may often be very useful in developing and improving methodological aspects and addressing certain questions, small pilot studies in healthy human volunteers with PET imaging are in many cases extremely valuable to assess the brain distribution of drugs *in vivo*.

8 Conclusions

Drug CNS efficacy is not exclusively dependent on the BBB permeability to the drug. Brain distribution can be a limiting step for CNS drug development even when the drug is well absorbed by the brain, but, nevertheless, cannot reach specific cellular targets at the concentrations required. In contrast, a compound with low BBB permeability may have pharmacological interest if a sufficient active fraction can reach the biophase. These two opposite situations emphasize that detailed understanding and consideration of specific CNS barrier and brain tissue properties ranging from anatomy to biology are often necessary to comprehend heterogeneous biodistribution and, ultimately, the drug effects that result. Recent technological advances paired with carefully designed studies are beginning to unravel the complexity of drug distribution in exciting new ways that are expected to result in

significant future progress for the CNS drug research and development area at both the preclinical and clinical level.

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Brain Distribution of Drugs: Pharmacokinetic Considerations

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Abstract

It is crucial to understand the basic principles of drug transport, from the site of delivery to the site of action within the CNS, in order to evaluate the possible utility of a new drug candidate for CNS action, or possible CNS side effects of non-CNS targeting drugs. This includes pharmacokinetic aspects of drug concentration-time profiles in plasma and brain, blood–brain barrier transport and drug distribution within the brain parenchyma as well as elimination processes from the brain. Knowledge of anatomical and physiological aspects connected with drug delivery is crucial in this context. The chapter is intended for professionals working in the field of CNS drug development and summarizes

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key pharmacokinetic principles and state-of-the-art experimental methodologies to assess brain drug disposition. Key parameters, describing the extent of unbound (free) drug across brain barriers, in particular blood–brain and blood–cerebrospinal fluid barriers, are presented along with their application in drug development. Special emphasis is given to brain intracellular pharmacokinetics and its role in evaluating target engagement. Fundamental neuropharmacokinetic differences between small molecular drugs and biologicals are discussed and critical knowledge gaps are outlined.

Keywords

Biologicals · Brain barriers · Brain slice uptake · Free drug hypothesis · Intracellular pharmacokinetics · $K_{p,uu,brain}$ · Neuropharmacokinetics · Small molecules

1 Introduction to CNS Drug Distribution

Most drugs are delivered to the brain via blood after enteral or parenteral administration. This means that the blood–brain barrier (BBB), the wall of brain capillaries, is the interface through which a drug reaches the brain. Thus, BBB interactions with drug molecules by passive transport, active efflux or active uptake, determine how much of the drug is delivered to the brain. The presence of the BBB throughout the brain creates a contact surface for drug delivery to the whole brain parenchyma, as the BBB is characterized by a large surface area and short distances between capillaries. The disadvantage or advantage, depending on the context, is that the BBB restricts the passage of many drugs to the brain through active efflux, mainly by P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), but also by actions of other transporters, thereby decreasing central effects or central side effects (Schinkel et al. 1996; Doran et al. 2005; Polli et al. 2009; Zhao et al. 2009; Kodaira et al. 2011). Due to the low CNS exposure of many marketed drugs and novel drug candidates, experimental routes of administration such as intrathecal, intranasal or even MRI-guided focused ultrasound with microbubbles are under investigation as potential methods to improve brain uptake.

It is crucial in drug development to choose, at an early time point, the right compounds for further development into clinically viable drugs, in order to save resources and improve success rate. Here, early investigations of the amount of drug delivered to the brain are of great importance. As a basis, the unbound (free) concentration at the site of action is the driving force of both pharmacokinetic and pharmacodynamic processes, as only unbound and unionized drugs could pass membranes and interact with targets. This has been primarily supported by the free drug hypothesis (Davson and Matchett 1953; Schanker 1962). Validity of free drug hypothesis has been confirmed in seminal experimental studies related to BBB unbound drug transport and its relation to pharmacological effect (Ekblom et al. 1992b; Wang and Welty 1996; Hammarlund-Udenaes et al. 1997; Xie et al. 1999;

Bouw et al. 2001; Deguchi 2002; Kalvass and Maurer 2002; Mano et al. 2002; Gupta et al. 2006; Bundgaard et al. 2007; Kalvass et al. 2007; Liu et al. 2009b; Watson et al. 2009). Therefore, the methods to estimate drug delivery to the brain should focus on unbound concentrations.

The concepts and methods for studying small molecule drugs that will be described in this chapter were primarily developed in the group of Translational pharmacokinetics and pharmacodynamics at Uppsala University, Sweden. It includes parameters used to quantify partitioning of unbound drug across the BBB, as described by $K_{p,uu,brain}$ (Gupta et al. 2006; Hammarlund-Udenaes et al. 2008), across the brain parenchymal cell membranes, as described by $K_{p,uu,cell}$ (Friden et al. 2007). It also embraces prediction of the transport between intracellular and subcellular acidic organelles, $K_{p,uu,lyso}$ (Friden et al. 2011), and the partitioning between total and unbound drug in brain tissue $V_{u,brain}$ (Friden et al. 2007). Methods to evaluate these parameters are cerebral microdialysis (Ekblom et al. 1992a; Hammarlund-Udenaes et al. 1997; Hammarlund-Udenaes 2017), the brain slice method (Friden et al. 2007, 2009a; Loryan et al. 2013), the brain homogenate method (Kalvass and Maurer 2002; Mano et al. 2002) and the combinatory mapping approach (CMA), combining several methods to obtain an overview of drug properties regarding BBB transport and intra-brain distribution (Loryan et al. 2014, 2016).

The knowledge on distributional factors for large molecules and their action within the brain is lagging far behind (see Sect. 3). Passage of these molecules across the BBB is even more restricted compared with small molecule drugs. Attempts to improve brain uptake of antibodies and protein drugs have been made by various groups. Utilization of endogenous receptors expressed at the BBB that include, among others, the transferrin receptor (TfR), insulin receptor and low-density lipoprotein-receptor related protein has been explored (Stanimirovic et al. 2018). These pathways for receptor-mediated transport have shown some promising results, but are still mainly investigated in preclinical studies (Sehlin et al. 2016; Johnsen et al. 2019).

2 Key Parameters for Characterization of CNS Drug Distribution

There are two main aspects describing drug delivery: the rate and the extent (see the box Key Definitions). The main focus of this chapter is on the extent of BBB transport. Rate of delivery has been studied for a long time in vitro but also in vivo. The rate of transport across the BBB is of great importance for drugs where a very rapid effect is desired, such as for anaesthetics and some analgesics, but in general, it is not crucial for most therapeutic drugs, dosed repeatedly (if permeability across the BBB is within reasonable limits). It is worth mentioning that the high correlation between the brain capillary permeability coefficient (a measure of rate) and the octanol/water partitioning coefficient has historically been influential in driving CNS drug development towards selection of very

lipophilic drugs (Levin 1980) without significant success. As a result, many drugs aimed for action in the CNS exert extensive plasma and brain tissue binding, as well as are substrates for efflux transporters.

The extent of BBB transport will determine the therapeutic outcome. Here, research during the last decade has pointed at the value of using unbound concentration measurement *in vivo* to map the success rate of brain drug delivery (Friden et al. 2007, 2009b; Hammarlund-Udenaes et al. 2008, 2009; Loryan et al. 2014, 2016; Gustafsson et al. 2017). Total brain to plasma concentration ratio ($K_{p, \text{brain}}$) was earlier the default measurement, with the alternative logBB used in *in silico* modelling (Wan et al. 2009). This parameter has, however, been shown to deflect optimal decision making, as both plasma protein binding and brain tissue binding are factors determining $K_{p, \text{brain}}$ together with the BBB transport itself. Remarkably, brain tissue binding can differ between being absent to extensive binding with the bound concentrations being up to 3,000-fold higher than the unbound ones. Equally variable is plasma protein binding with ranges from no binding to very high degree of binding. Thus, a more accurate estimation of brain delivery is made by measuring unbound concentrations ratio across the BBB with the $K_{p, \text{uu}, \text{brain}}$ parameter (see Sect. 2.1.1).

$K_{p, \text{uu}, \text{brain}}$ is determined in order to assess the concentration of unbound drug in the interstitial fluid (ISF) in relation to plasma unbound drug concentration. However, there is a general perception that after crossing the BBB, a drug is unrestrictedly and evenly distributed among the brain parenchymal cells primarily by passive diffusion, not taking into consideration the role of the cellular membrane and subcellular compartments in neuropharmacokinetics (neuroPK). Apparent progression in understanding of the cell proteome and development of specialized atlases (Vanlandewijck et al. 2018) has spread the light into more intricate cellular localization of proteins that may have crucial role in governing drug distribution between and within cells. In spite of the fact that a majority of the currently available small molecular weight CNS drugs have extracellular membrane targets, there are numerous intracellular targets that have important roles in initiation of either therapeutic effects or side effects. Hence, understanding of unbound intracellular concentrations by means of $K_{p, \text{uu}, \text{cell}}$ (discussed in Sect. 2.2.3) will certainly facilitate the establishment of valid exposure–response relationship (Fig. 1).

Key compartments of CNS drug disposition are represented by the blood in brain capillaries, the interstitial and intracellular fluids, including lysosomal fluid, and the CSF. Barriers at the blood–brain (BBB) and the blood–CSF (BCSFB) as well as at the intracellular-to-interstitial (cellular barrier, CB) interfaces are shown as red dashed lines. Unbound (free) and unionized drug present in the bloodstream is subject to transport across these interfaces. Passive and facilitated diffusion, active efflux and influx can take place at the BBB, BCSFB and CB influencing the net flux of the unbound drug transport across the barriers, determined by $K_{p, \text{uu}, \text{brain}}$, $K_{p, \text{uu}, \text{CSF}}$ and $K_{p, \text{uu}, \text{cell}}$, respectively. Physiological pH gradients are indicated by the pH of the respective compartments. According to the existing knowledge, the transport of drug from the brain across the ependyma occurs via passive diffusion of unbound drug. T represents extracellular or intracellular targets. The figure is modified from (Hammarlund-Udenaes et al. 2008) with permission from the publisher.

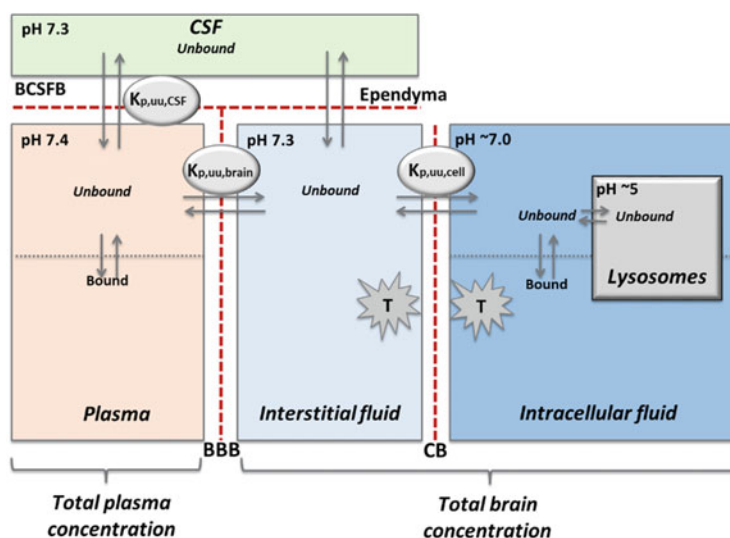


Fig. 1 Schematic illustration of unbound drug disposition in the CNS

Key Definitions

Neuropharmacokinetics (neuroPK) – studies describing the fate of drugs in the CNS, including distribution processes to, within and out of the CNS. Parameters characterizing both the extent and rate of transport are determined

$K_{p,uu,brain}$ – The steady state ratio of brain interstitial (extracellular) fluid to plasma unbound drug concentrations, providing a direct quantitative description of the net BBB drug transport, including passive transport and active influx/efflux

$K_{p,uu,CSF}$ – The steady state ratio of CSF to plasma unbound drug concentrations, providing a direct quantitative description of the net BCSFB drug transport

PS – permeability surface area product described the rate of transport of a drug across the BBB. Influx clearance (K_{in} , CL_{in}) measured in $\mu\text{L min}^{-1} \text{g brain}^{-1}$ or $\mu\text{L s}^{-1} \text{g brain}^{-1}$ is used

$K_{p,uu,cell}$ – The steady state ratio of intracellular to extracellular unbound drug concentrations, describing the drug transport across the cellular membrane (average for all cell types within the brain)

$K_{p,uu,cyto}$ and $K_{p,uu,lyso}$ – Predicted steady state ratios of cytosolic to extracellular, and lysosomal to cytosolic unbound drug concentrations, respectively, describing the unbound drug transport across the cellular and lysosomal membranes driven merely by the pH gradient

2.1 Drug Transport Across CNS Barriers with Focus on BBB and BCSFB

Drug transport, apart from being passive due to favourable physicochemical properties, is regulated by various transporters primarily belonging to the ATP-binding cassette (ABC) family. Within this transporter family, P-gp is the most extensively studied transporter with a myriad of substrates. Other transporters such as BCRP and multidrug resistance-associated proteins (MRPs) are also important for regulation of brain drug delivery. The combined action of several transporters, as well as passive transport, will result in a specific and individual equilibration across the barrier for each drug.

In CNS drug disposition, aside from the BBB, the blood–CSF–barrier (BCSFB), the blood–spinal cord barrier (BSCB), and the blood–arachnoidal barrier (BAB) are active players, although likely to a much smaller degree. The BCSFB has a slightly different transporter profile than the BBB, with varying degrees in expression (Gazzin et al. 2008; Okura et al. 2011; Uchida et al. 2011; Zhang et al. 2018). This leads to drug-specific differences in transport across these barriers reflected in $K_{p,uu}$ values.

2.1.1 Unbound Brain to Plasma Concentration Ratio, $K_{p,uu,brain}$

Underlying Principles

The unbound brain to plasma concentration ratio, $K_{p,uu,brain}$, is a parameter to quantitatively determine the transport capacity of drugs across the BBB, without interference from plasma protein binding or brain tissue binding (Eq. 1). By comparing the unbound brain interstitial fluid concentration that is by definition unbound, with the unbound plasma concentration, a ratio is achieved which can be lower than, equal to or above unity.

$$K_{p,uu,brain} = \frac{C_{u,brain,ss}}{C_{u,plasma,ss}} = \frac{AUC_{u,brain}}{AUC_{u,plasma}} \quad (1)$$

$C_{u,brain,ss}$ is the steady state unbound concentration of a drug in brain ISF, $C_{u,plasma,ss}$ is the steady state unbound concentration in plasma. It is important to note that direct assessment of unbound concentrations in brain and plasma is possible only by using the microdialysis technique (see chapter “In Vivo Studies of Drug BBB Transport: Translational Challenges and the Role of Brain Imaging”). Alternatively, $K_{p,uu,brain}$ can be assessed by calculating the area under the curve (AUC) of an unbound drug concentration – time profile in brain and plasma after single dosing.

The values of $K_{p,uu,brain}$ can range from very low to above unity. Studies on this are mainly made in rodents. Examples are loperamide with a $K_{p,uu,brain}$ of 0.01, meaning that only 1% of the drug equilibrates across the BBB, in this case due to active efflux with P-gp (Friden et al. 2009b). At the other end is oxycodone with a $K_{p,uu,brain}$ of 3, indicating an active uptake at the BBB causing threefold higher unbound brain ISF than plasma concentrations (Bostrom et al. 2006), while

diazepam has a value around unity, describing dominantly passive transport (Friden et al. 2007).

The $K_{p,uu,brain}$ concept was first described in 2006 by Gupta and colleagues (Gupta et al. 2006) and further elaborated, refined and studied by (Bostrom et al. 2006; Gupta et al. 2006; Bundgaard et al. 2007; Friden et al. 2007, 2009b; Hammarlund-Udenaes et al. 2008; Sadiq et al. 2011; Hammarlund-Udenaes 2017). As a part of the development of methods for translational neuroPK, the $K_{p,uu,brain}$ concept was implemented into the field of brain imaging, in particular positron emission tomography (PET) with promising results (Gunn et al. 2012; Schou et al. 2015; Gustafsson et al. 2017). The concept has also been applied to the field of nanotechnology, in particular, to quantitatively evaluate the impact of liposomes on improvement of drug delivery to the brain (Lindqvist et al. 2013; Hu et al. 2017, 2018, 2019a, b).

Current and Emerging Methodologies

The use of microdialysis for the investigation of drug neuroPK has limitations associated not only with being an invasive and labour-intensive procedure, but also with the nonspecific adsorption of lipophilic compounds to the plastic tubing used for calibrator delivery and sampling from the microdialysis probe. This has led to development of alternative methods. The brain partitioning coefficient, $K_{p,brain}$ Eq. (2) has often been used in the pharma industry, and new methods to “dissect” the complex parameter to derive $K_{p,uu,brain}$ was sought for.

$$K_{p,brain} = \frac{C_{tot,brain,ss}}{C_{tot,plasma,ss}} = \frac{AUC_{tot,brain}}{AUC_{tot,plasma}} \quad (2)$$

$C_{tot,brain,ss}$ and $C_{tot,plasma,ss}$ are steady state total concentrations of a drug in brain tissue and plasma, respectively. $AUC_{tot,brain}$ and $AUC_{tot,plasma}$ are AUCs obtained in brain and plasma. By estimating plasma protein binding with the fraction of unbound drug in plasma, $f_{u,plasma}$, it is possible to estimate the unbound concentration in plasma. With the development of methods for evaluation of drug brain tissue binding by means of $f_{u,brain}$ (see details in Sect. 2.2.2) it is feasible to get a proxy to unbound brain concentration. Altogether, this makes estimation of $K_{p,uu,brain}$ possible without the use of microdialysis, as described below.

$$K_{p,uu,brain} \approx \frac{C_{tot,brain,ss}}{C_{tot,plasma,ss}} \frac{f_{u,brain}}{f_{u,plasma}} \quad (3)$$

With the understanding of the intricate details of the process of drug distribution in the brain parenchyma (presented in Sect. 2.2), the limitations of the $f_{u,brain}$ parameter have become obvious. This has led to the development of more advanced methods, e.g. the brain slice assay for high-throughput (Friden et al. 2007, 2009a; Loryan et al. 2013), that can be used instead of, or together with, brain homogenate binding. It estimates $V_{u,brain}$, the unbound volume of distribution in brain, capturing key drug distribution processes in the brain parenchyma (see Sect. 2.2.3). The main

difference between the two methods is that the brain slice has living cells with pH gradients remaining, while the brain homogenate will only describe binding. Hence, Eq. 4 is proposed to assess the extent of BBB unbound drug transport, and is an integral part of the combinatory mapping approach (CMA), presented in detail in Chap. 10 (Frideren et al. 2007; Hammarlund-Udenaes et al. 2008; Loryan et al. 2014). CMA has been subsequently adapted for investigation of BBB transport of several antipsychotics in brain regions of interest (Loryan et al. 2016).

$$K_{p,uu,brain} \approx \frac{K_{p,brain}}{f_{u,plasma} \cdot V_{u,brain}} \quad (4)$$

$K_{p,uu,brain}$ and $K_{p,uu,cell}$ equal to unity is indicated as grey dashed lines (Fig. 2). $K_{p,uu,brain}$ equal to 0.5 is indicated as a red dashed line in A. Compounds exhibiting $K_{p,uu,brain}$ and $K_{p,uu,cell}$ lower than unity are subject to predominant active efflux. Group A: inhibitors of PDE10 (Phosphodiesterase 10); Group B: inhibitors of PDE2 (Phosphodiesterase 2); Group C: modulators of GS (Gamma secretase); Group D: positive allosteric modulators of mGlu2 receptor (Metabotropic glutamate receptor 2); Group E: positive allosteric modulators of mGlu5 receptor (Metabotropic glutamate receptor 5); Group F: positive allosteric modulator of nicotinic alpha 7 receptor (alpha 7 nicotinic receptor); antagonists of D2/D3 (dopamine D2/D3 receptors), and

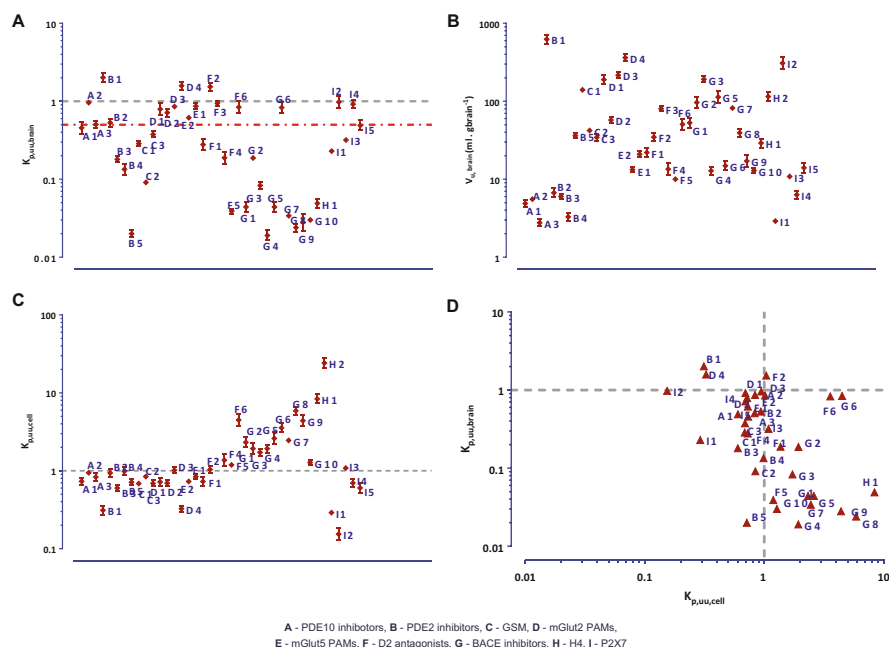


Fig. 2 Key neuroPK parameters estimated by combinatory mapping approach (CMA) for a set of 40 compounds. (a) $K_{p,uu,brain}$, (b) $V_{u,brain}$, (c) $K_{p,uu,cell}$ and (d) the relationship between $K_{p,uu,brain}$ and $K_{p,uu,cell}$

antagonists of 5HT₆/5HT₂/5HT_{2A} (5-hydroxytryptamine 5HT₆/5HT₂/5HT_{2A} receptors); Group G: inhibitors of BACE (beta-site amyloid precursor protein cleaving enzyme); Group H: antagonists of H₄ receptor (histamine H₄ receptor); Group I: antagonists of P2X₇ receptor (P2X purinoceptor 7). Data are taken from (Loryan et al. 2014) with permission from the publisher.

Applications in Drug Development

The $K_{p,uu,brain}$ concept has made a huge impact on shaping how the assessment of CNS exposure is performed in present-day drug development programs. $K_{p,uu,brain}$ is essential for choosing the best compounds for further development and discarding those that have insufficient passage across the BBB to exert effects or those that have a less restricted passage and may cause central side effects. It is to be noted that there is no strict value under which compounds are to be discarded. It is a complex relationship where the potency as well as the type of effect and side effect needs to be included in the evaluation. As an example, paliperidone (F5 in Fig. 2a) has a $K_{p,uu,brain}$ of 0.04 while still being a valuable drug with central action (Loryan et al. 2014). Many pharmaceutical companies and others have worked on developing in silico models for prediction of $K_{p,uu,brain}$ (Friden et al. 2009b; Loryan et al. 2015; Dolgikh et al. 2016; Liu et al. 2018). However these models have little explanatory power, as the BBB transport is mainly influenced by active processes at the BBB.

The $K_{p,uu,brain}$ concept has been translated to BBB cell culture studies by other groups (Culot et al. 2013; Heymans et al. 2018). For cell studies, the issue is always the lack of similarity in expression profiles of efflux and uptake transporters in the BBB cell models to the in vivo situation, requiring further comparative studies and refinement of in vitro protocols (Helms et al. 2016). It is crucial to evaluate the cell type being studied, as non-endothelial cell lines, such as the Caco-2 or MDCK epithelial cell lines often used in drug industry, are far from optimal for prediction of BBB transport (Hakkarainen et al. 2012). It is also crucial to evaluate the extent of transport and not compare the rate of transport with the extent of transport, since these are very different properties of a drug (Cecchelli et al. 2007; Hakkarainen et al. 2012). New BBB in vitro models based on human pluripotent stem cells or haematopoietic stem cells are interesting alternatives, but also here, further studies are required to prove the predictability and comparisons to in vivo findings (Cecchelli et al. 2014; Lippmann et al. 2014).

2.1.2 Unbound CSF to Plasma Concentration Ratio, $K_{p,uu,CSF}$

Underlying Principles

CSF sampling is one of the few currently available methods for assessment of CNS exposure, which is also applicable for humans. The CSF concentration of a drug is regulated by the complex interplay between the barriers of CNS among them BBB, BCSFB and BAB. However, the contributive role of each barrier on achieving a certain level of CSF exposure for a given drug is challenging to investigate. As CSF can be sampled from various anatomical locations such as brain ventricles, cisterna magna, as well as the subarachnoidal space including lumbar space, the site of

sampling becomes crucial for the interpretations (Westerhout et al. 2012; de Lange 2013). In pharmacokinetic animal studies, CSF is often sampled from cisterna magna, while in humans it is collected from the lumbar subarachnoidal space. For evaluation of the extent of BCSFB transport, unbound CSF to plasma concentration ratio, $K_{p,uu,CSF}$ can be assessed as described below.

$$K_{p,uu,CSF} = \frac{C_{u,CSF,ss}}{C_{u,plasma,ss}} = \frac{AUC_{u,CSF}}{AUC_{u,plasma}} \quad (5)$$

$C_{u,CSF,ss}$ is the steady state unbound concentration of a drug in CSF, $C_{u,plasma,ss}$ is the steady state unbound concentration in plasma. $AUC_{u,CSF}$ and $AUC_{u,plasma}$ are AUCs obtained in the CSF and plasma after single or repeated administration of a drug. In the ideal case, sampling could be performed using microdialysis, potentially with parallel placement of the probes at different anatomical sites (Westerhout et al. 2012; Kervezee et al. 2014). Microdialysis allows dynamic collection of unbound drug in blood and various sites of CSF, without interference of the hydrodynamic state of the CSF compartment (e.g., volume, pressure). As this application is often unattainable, unbound concentrations in CSF and plasma should be obtained by correction of total concentrations for drug protein binding in the respective fluid. Often the assumption that measured CSF concentration is merely unbound is relevant, i.e. $C_{u,CSF} = C_{CSF}$. The composition of CSF is strictly regulated which is in part reflected in low protein content. However, there is a dynamic equilibrium between CSF and plasma albumin in normal conditions, which is about a 1/200 ratio in humans and called the albumin quotient (Cutler et al. 1967). Hence, for more precise assessment of $C_{u,CSF}$, especially for highly bound drugs, it is recommended to measure the unbound fraction of the drug in CSF, $f_{u,CSF}$. Due to the limitations related to collecting enough blank CSF, Fridén et al. suggested calculation of $f_{u,CSF}$ based on the albumin quotient and plasma protein binding in respective species (Fridén et al. 2009b). It is important to bear in mind that the state of the BBB and BCSFB in pathological conditions often causes increased protein content in CSF and $f_{u,CSF}$ measurements become more critical.

Current and Emerging Methodologies

Mathematical modelling, in particular physiologically based pharmacokinetic (PBPK) models focused on profiling CSF drug concentration are among emerging methodologies in the field, opening up new perspectives for translational pharmacokinetics (Westerhout et al. 2012; Yamamoto et al. 2017; Vendel et al. 2019). PBPK models supported by extensive experimental data provide insight into intricate mechanisms that may influence CSF pharmacokinetics. For instance, using this approach Kervezee et al. described the presence of a circadian rhythm in P-gp function (Kervezee et al. 2014). In particular, based on plasma, ISF, CSF and total brain concentrations of quinidine after intravenous administration in the active and resting periods of the animals, they concluded that the time of administration significantly affects P-gp mediated transport as well as CSF flux. In particular, they describe higher activity of P-gp-mediated transport from the deep brain

compartment to the plasma compartment during the active period while CSF flux is higher in the resting period compared to during the active period.

Quantitative pharmacoproteomics is also essential for understanding the role of BCSFB in regulation of CSF concentrations and its relation to brain ISF concentrations. There are several developments in the field as presented in some selected manuscripts (Gazzin et al. 2008; Okura et al. 2011; Uchida et al. 2011; Zhang et al. 2018). For instance, the transporters BCRP and P-gp display very low expression levels in choroid plexuses, compared to brain capillaries, while OATP1A2 and MRP4 are found abundantly at the BCSFB (Braun et al. 2017; Zhang et al. 2018).

Applications in Drug Development

There have been several studies focusing on the utility of CSF sampling in drug development (Shen et al. 2004; Liu et al. 2006, 2009a; Lin 2008; Friden et al. 2009b; de Lange 2013). Often the key question is related on the use of CSF as a surrogate for brain ISF. In a study by Fridén et al., the relationship between $K_{p,uu,CSF}$ and $K_{p,uu,brain}$ obtained in rats was described with a coefficient of determination of 0.8 (Friden et al. 2009b). However, the authors describe an overall tendency for CSF data to over-predict $K_{p,uu,brain}$ for drugs with low $K_{p,uu,brain}$ and to under-predict the value for drugs with higher $K_{p,uu,brain}$, something that has also been observed in other independent studies (Shen et al. 2004; Loryan et al. 2014). Hence, CSF utility needs to be evaluated on a case-by-case basis.

Taking into consideration the observed differences in CSF concentration-time profiles depending on the sampling site, the design of experiments should take into account site of sampling and timing (Westerhout et al. 2012; de Lange 2013). A study design with steady state measurements and constant infusion of drugs that have shorter half-lives is recommended for optimal output, if modelling is not an alternative. Also, potential species differences in expression of transporters at both BCSFB and BBB need to be accounted for in translational studies.

2.2 Drug Distribution within the Brain Parenchyma

After passing the BBB, a drug distributes within interstitial space and, if feasible, into brain cells. The process of distribution of drugs within the brain has attained much increased interest lately. Methodologies have been established allowing us not only to investigate the potential mechanisms of drug distribution beyond the BBB, but also to quantify it in both static and dynamic conditions. Substances are distributed within the brain interstitial space mainly by diffusion and convection. It is to be noted that the diffusion distance between brain capillaries and parenchymal cells is rather small. It is currently widely accepted that the diffusion of a molecule is governed by the features of the extracellular space such as width ranging 35–60 nm (Thorne and Nicholson 2006), volume fraction, viscosity, geometry as well as by properties of the molecule. This will determine any potential binding to the extracellular matrix proteins or cellular membrane and/or transport across the cellular

membrane (Kurz and Fichtl 1983; Fenstermacher and Kaye 1988; Wang and Welty 1996; Kakee et al. 1997; Nicholson and Sykova 1998; Kalvass and Maurer 2002; Mano et al. 2002; Thorne and Nicholson 2006; Friden et al. 2007, 2009a; Longhi et al. 2011; Wolak and Thorne 2013; Wolak et al. 2015). The role of convection is more critical for the distribution of large molecules, however, the exact mechanisms are still heavily debated in the community (Cserr and Patlak 1992; Abbott 2004; Iliff et al. 2012; Abbott et al. 2018). Due to the immense dissimilarities between small molecular weight substances and biologicals, based on different size, charge and lipophilicity, there are differences in the predominant mechanisms of distribution, and hence, methods to study those processes are diverse.

The key parameters describing the extent of distribution of drugs within the brain are presented with focus on small molecular weight substances, and are presented in this section. In particular, two parameters $V_{u,brain}$ and $f_{u,brain}$ characterizing the relationship between the total concentration of a drug in brain tissue and the unbound concentration in brain ISF are important for the overall understanding of distribution.

2.2.1 Unbound Volume of Distribution in the Brain, $V_{u,brain}$

Underlying Principles

Wang and Welty (1996) were the first to introduce the parameter of the apparent volume of distribution of drug in the brain extracellular fluid denoted $V_{e,app}$ by using brain microdialysis derived data, describing the relationship between total and unbound drug concentrations in the brain (Eq. 6).

$$V_{e,app} = \frac{A_{brain,tot} - V_{blood} \cdot C_{blood,tot}}{C_{u,ISF}} \quad (6)$$

where $A_{brain,tot}$ and V_{blood} are the total amount of drug and the volume of residual blood per gram of brain, respectively. $C_{blood,tot}$ and $C_{u,ISF}$ are total and unbound concentrations of drug in blood and brain interstitial fluid, respectively. Wang and Welty explored the brain pharmacokinetics of gabapentin using microdialysis, making the comparison to less labour-intensive method of total brain to plasma concentration ratio, $K_{p,brain}$ (Wang and Welty 1996). They found that the understanding of intracellular distribution by means of $V_{e,app}$ was crucial for the characterization of gabapentin brain pharmacokinetics. In addition, they were the first ones to indicate over-prediction of BBB transport efficiency when utilizing only total brain to plasma concentrations. They also suggested to compare the obtained $V_{e,app}$ with the physiological volumes of the brain, and presented some ideas on how to interpret the values. For instance, gabapentin has a $V_{e,app}$ of $5.5 \text{ mL g brain}^{-1}$, which is significantly larger than the sum of intra- and extracellular water in the brain parenchyma, i.e. $1.0 \text{ mL g brain}^{-1}$, indicating intracellular accumulation. This approach is still very much used for interpretation of $V_{u,brain}$ values.

Consequently, $V_{e,app}$ was renamed the volume of distribution of unbound drug in the brain ($V_{u,brain}$) for high-throughput estimations (Friden et al. 2007) and this will be the terminology used hereafter. In short, $V_{u,brain}$ describes the relationship

between the total amount of drug in the brain per g brain and the unbound drug concentration in brain ISF and has the unit mL g brain^{-1} . The parameter is independent of BBB function and only describes the *intra-brain* distribution and binding. Brain microdialysis is the only *in vivo* reference method for estimating $V_{u, \text{brain}}$. Due to the limitations of microdialysis, alternative methods have been proposed for assessment of $V_{u, \text{brain}}$, among them is the brain slice assay, which is described below.

Current and Emerging Methodologies

As a result of developing an alternative method for assessing $V_{u, \text{brain}}$, the brain slice method was evaluated as a well-functioning approach (Friden et al. 2007, 2009a, b; Loryan et al. 2013, 2014). It has the benefits of being used in a regulated *in vitro* environment, while at the same time preserving much of the complex cellular integrity, including cellular barriers and circuitry, and as a result conserving the functionality of the *in vivo* brain. Therefore, the brain slice set-up permits investigation of specific and nonspecific drug brain tissue binding, pH gradient driven partitioning, passive diffusion as well as active transport across cellular membranes (Fig. 3).

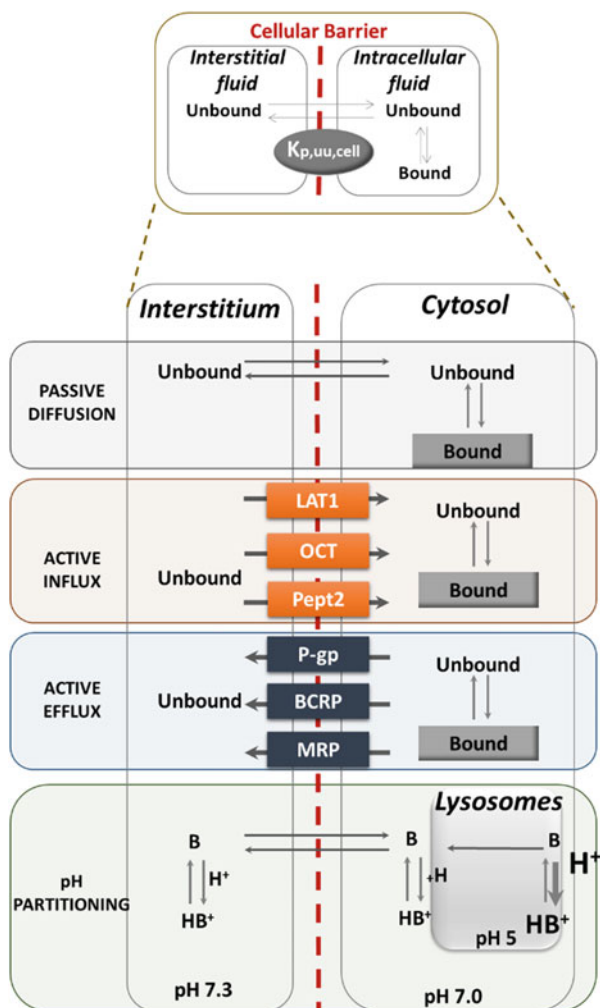
The first use of a brain slice method for evaluation of intracerebral distribution of substances aimed to estimate the uptake of nutrients such as glucose and amino acids into the brain (Newman et al. 1991). Later, the method was proposed for *in vitro* investigation of drug distribution (Van Peer et al. 1981; Kakee et al. 1996; Ooie et al. 1997). Fridén et al. proposed the brain slice method for assessment of $V_{u, \text{brain}}$ after successful validation of results against brain microdialysis using a set of 15 compounds (Friden et al. 2007). As the validation showed good alignment of data, the rat brain slice method was proposed as a high-throughput method, also extended to mouse brain (Friden et al. 2009a; Loryan et al. 2013). Briefly, six 300 μm brain slices obtained from drug naïve rats are incubated with the compounds of interest dissolved in artificial extracellular fluid until attainment of equilibrium or a maximum of 5 h. For screening purposes, the cassette dosing approach can be used; usually, a mixture of six compounds with summative concentration not exceeding 1 μM is investigated. The key assumption is that the concentration of the drug in the virtually protein free buffer at equilibrium is equal to the unbound concentration in brain interstitial fluid. Hence, $V_{u, \text{brain}}$ can be obtained as:

$$V_{u, \text{brain}} = \frac{A_{\text{brain}} - V_i \cdot C_{\text{buffer}}}{C_{\text{buffer}} \cdot (1 - V_i)} \quad (7)$$

where A_{brain} ($\text{nanomoles} \cdot \text{g brain}^{-1}$) is the total amount of drug in the brain slice and C_{buffer} ($\text{micromoles} \cdot \text{L}^{-1}$) the measured buffer concentration after reaching equilibrium. As a certain volume of the buffer remains on the surface of the brain slice even after removing the excess with filter paper (V_i , $\text{mL} \cdot \text{g slice}^{-1}$), this has to be accounted for. V_i was estimated in a separate experiment using [^{14}C] inulin and was found to be $0.094 \text{ mL} \cdot \text{g brain}^{-1}$ (Friden et al. 2009a). The method has been extended towards more mechanistic studies, e.g. aiming at evaluating the

Fig. 3 Diagram representing the key pharmacokinetic processes taking place in the brain parenchyma after a drug has entered across the BBB.

Some transporters are exemplified in the figure: LAT, the large amino acids transporter, OCT, the organic cation transporters, Pept2, the peptide transporter 2, P-gp, P-glycoprotein, BCRP, breast cancer resistant protein, MRP, multidrug resistance-associated protein. Modified from (Loryan et al. 2016) with permission from the publisher



contributive role of certain transporters (Friden et al. 2011; Chen et al. 2014; Puris et al. 2019), pH gradients (Friden et al. 2011; Loryan et al. 2017), as well as cellular heterogeneity between brain regions (Loryan et al. 2016).

Applications in Drug Development

The brain slice method is a robust technique for estimating the overall uptake of drugs into brain tissue, which is currently used and adopted by several pharmaceutical companies. The application of the brain slice assay in early drug development is presented in Fig. 2b for a set of 40 compounds. The method has been applied not only to drug candidates, but also to the process of selection of PET tracers, with the aim to identify low brain uptake and nonspecific binding tracers (Friden et al. 2014).

In addition, there is ongoing work on developing translational methods for assessment of unbound brain concentrations in the PET field, where brain slice data is used and compared with microdialysis (Gustafsson et al. 2017). So far, the brain slice assay is primarily used for investigation of small molecular weight drugs and peptides. Scaling up of the method to biologicals is still in progress and, in general, limited by bioanalytical methods and issues related to nonspecific adsorption to lab ware.

2.2.2 Fraction of Unbound Drug in the Brain, $f_{u, \text{brain}}$

Underlying Principles

The availability of equilibrium dialysis based methods to study drug plasma protein binding has facilitated the development of drug tissue binding theories and approaches (Kurz et al. 1977; Larsson and Tjalve 1979; Huang 1983; Kurz and Fichtl 1983; Fichtl et al. 1991; Ekblom et al. 1992b). Until now, there are still questions related to drug–brain tissue interaction, in particular to understand and evaluate driving forces, kinetics and relevance for drug effect. The most common approach to investigate this interaction is via equilibrium dialysis that provides assessment of the fraction of unbound drug in brain homogenate ($f_{u, \text{brain}}$). The method is often referred to as the homogenate method and provides information about the extent of drug brain tissue binding (Kalvass and Maurer 2002).

The semipermeable membrane between the buffer and the homogenate compartments in the equilibrium dialysis well acts as a molecular filter permitting diffusion of molecules smaller than the molecular weight cut-off of the membrane, normally up to 20 kDa. To be able to perform a brain binding study, the brain homogenate needs to be diluted with buffer, commonly with dilution factors of either three, five or ten. The brain homogenate (donor side) is usually spiked with drug (1–5 μM) and is dialysed against the buffer (receiver) side. As a general rule, the drug–tissue protein/lipid interaction is reversible and, in a majority of cases equilibrium occurs rapidly between the unbound and bound molecular species. At equilibrium, samples are taken from both receiver and donor sides and the unbound fraction is calculated as:

$$f_{u, \text{hD}} = \frac{C_{\text{receiver}}}{C_{\text{donor}}} \quad (8)$$

where $f_{u, \text{hD}}$ is the measured experimental fraction of unbound compound in diluted (D) brain homogenate, C_{receiver} and C_{donor} are the concentrations in the buffer and diluted brain tissue at equilibrium, respectively. The relative impact of dilution of the brain homogenate on the formation of drug–brain tissue protein complexes has been thoroughly investigated, followed by suggestion of how to account for the dilution in evaluation of f_u (Romer and Bickel 1979).

$$f_{u,\text{brain}} = \frac{\frac{1}{D}}{\left(\left(\frac{1}{f_{u,hD}}\right) - 1\right) + \frac{1}{D}} \quad (9)$$

The obtained $f_{u,\text{brain}}$ has a numerical value from 0 to 1, where 1 is no drug binding. It is important to mention that $f_{u,\text{brain}}$ has an inverse relationship to $V_{u,\text{brain}}$. Both methods give similar results if the nonspecific binding is the predominant mechanism driving the drug–brain tissue interaction.

Current and Emerging Methodologies

The necessity to assess the relationship between total and unbound drug in the brain tissue of thousands of compounds led to the development of high-throughput equilibrium dialysis method (Kalvass and Maurer 2002). The method rapidly became standard and it is widely used for a large number of chemically diverse compounds (Mano et al. 2002; Kalvass et al. 2007; Wan et al. 2007; Summerfield et al. 2008; Liu et al. 2009b; Friden et al. 2011).

When comparing the data obtained from brain homogenate with brain microdialysis, Fridén et al. observed over-prediction of in vivo $V_{u,\text{brain}}$ for compounds limited to intracerebral ISF distribution (e.g. morphine-3- and morphine-6-glucuronide) and under-prediction of the distribution of gabapentin, which has predominantly active cellular uptake (Friden et al. 2007). Comparison of $V_{u,\text{brain}}$ and $f_{u,\text{brain}}$ has shown that $f_{u,\text{brain}}$ is in general underestimating the brain distribution of basic drugs (Friden et al. 2011; Loryan et al. 2014). This has been attributed to the phenomenon of lysosomal tropism that is governed by pH gradients, which leads to the accumulation of protonated drug in the acidic cellular subcompartments, here collectively named lysosomes.

Along with equilibrium dialysis there are other methods that are used for brain tissue binding, although to a lesser extent. Among them are ultrafiltration, ultracentrifugation, gel filtration and absorption by brain lipid membrane vesicles stabilized on silica beads named TRANSIL (Kurz et al. 1977; Longhi et al. 2011).

Applications in Drug Development

The estimation of $f_{u,\text{brain}}$ is today a routine method to obtain unbound drug concentration estimates in preclinical pharmacokinetic studies. After the publication by Pfizer on lack of species differences in drug brain tissue binding assessed by ED (Di et al. 2011), many pharma companies are using one species whole brain tissue material in their screening programs, even further facilitating the process. Recently, brain regional drug tissue binding in rats and humans including healthy donors and Alzheimer's disease patients was investigated (Gustafsson et al. 2019). It was concluded that rat data in general overestimate regional $f_{u,\text{brain}}$ values in patient groups, bringing important questions to the pharma industry regarding the need to investigate binding in human brains with pathology. This is also a very important aspect for brain imaging, in particular PET, where there is successful example of the use of $f_{u,\text{brain}}$ values for estimation of unbound brain concentration in pigs based on measured total radioactivity (Gunn et al. 2012).

2.2.3 Unbound Intracellular to Extracellular Concentration Ratio, $K_{p,uu,cell}$

Underlying Principles

As direct assessment of unbound intracellular concentration is technically not feasible, intracellular pharmacokinetics has been neglected for decades. Fridén and co-authors were the first ones to introduce the concept of $K_{p,uu,cell}$. They proposed methodologies to assess the partitioning for average (not exactly defined mixture of cells) brain cells (Fridén et al. 2007). This is achieved by combining the two neuroPK parameters $V_{u,brain}$ and $f_{u,brain}$ determined using brain slice and brain homogenate, respectively.

$$K_{p,uu,cell} = \frac{C_{u,cell}}{C_{u,brainISF}} \approx \frac{A_{brain}}{C_{u,brainISF}} \cdot f_{u,brain} \approx V_{u,brain} \cdot f_{u,brain} \quad (10)$$

where $C_{u,cell}$ is the average intracellular unbound drug concentration in a brain slice, representing all subcellular compartments; A_{brain} is the sum of the amount of drug in all subcellular compartments. The tissue density is assumed to be 1 g mL^{-1} .

The inherent differences in the nature of the two parameters allow approximation of $K_{p,uu,cell}$. On the one hand, $f_{u,brain}$ is reflecting primarily intracellular binding of a drug to various subcellular constituents. As direct measurement of the extent of drug binding to various organelles is practically challenging, similar binding is assumed within all cell organelles and $f_{u,brain}$ is used. In this assumption, potential binding of the drug to the outer surface of cell membrane is neglected, despite the external surface area of a typical human cell membrane representing less than 0.5% of the total cell membrane surface area (Freitas 1999). The latter means that the extent of the drug binding measured by $f_{u,brain}$ is primarily governed by intracellular sites. On the other hand, $V_{u,brain}$ is determined by cell membrane permeability, as well as binding affinity for brain tissue components and pH partitioning into subcellular components. However, when the substance is predominantly localized in the extracellular space and significantly bound to the outer cell membrane and/or constituents of the interstitium, these assumptions may be violated. Large biologicals with specific protein interactions may have such a combination of properties.

The majority of small molecule substances pass across neural cell membranes by simple diffusion, i.e. the rate of transport is directly proportional to the concentration gradient across the membrane. At equilibrium the unbound and unionized intracellular concentration is equal to the concentration in virtually protein free ISF outside the cell. In this condition $K_{p,uu,cell} = 1$. Many neutral drugs, e.g. diazepam, lamotrigine, zidovudine, are good model substances for this condition.

However, asymmetry at the cellular barrier can be present similarly like across the BBB, as measured with $K_{p,uu,brain}$ (Fig. 2c). This is reflected in higher or lower than unity values of $K_{p,uu,cell}$. Intracellular accumulation of unbound drug could be a result of the involvement of various transporters (e.g., L-type amino acid transporter, organic cation transporter), or could be governed by physiological pH differences or electrochemical gradients (Kaufmann and Krise 2007; Fridén et al. 2011; Chen et al.

2014; Loryan et al. 2015, 2017; Puris et al. 2019). A majority of CNS active drugs are basic compounds, which is reflected in the occurrence of lysosomal trapping. This is a phenomenon, where the compound is accumulated in acidic intracellular compartments, mainly lysosomes. Lysosomal trapping is saturable and requires preserved cellular integrity. It is governed by the large pH gradient between the cytosol and acidic compartments. Weak bases in their unionized state permeate across cellular and lysosomal membranes and accumulate in the acidic lysosomes in the ionized form (Fig. 3). Opposite to bases, acidic substances undergo lysosomal depletion. In addition, several efflux transporters are expressed on the cellular membrane of neural cells representing a secondary barrier for drugs having intracellular targets (See examples in Fig. 3).

Current and Emerging Methodologies

After establishment of the $K_{p,uu,cell}$ concept (Friden et al. 2007), there have been several methodological developments, which lead to the extension beyond brain tissue applications (Mateus et al. 2017a; Trünkle et al. 2020). Currently, cell pharmacokinetics is gaining more popularity especially among groups working primarily with cell cultures (Zhou et al. 2011; Guo et al. 2018).

One of the critical progressions is associated with the development of a three-compartment pH partitioning model for prediction of $K_{p,uu,cell}$ based on pKa values of the compound (Friden et al. 2011). The model consists of interstitial, cytosolic and lysosomal compartments and includes information about their physiological volumes and pH values:

$$K_{p,uu,cell} = V_{ISF} + K_{p,uu,cyto} \cdot (V_{cyto} + V_{lyso} \cdot K_{p,uu,lyso}) \quad (11)$$

where V_{ISF} , V_{cyto} and V_{lyso} are the physiological volumes of ISF ($0.20 \text{ mL} \cdot \text{g brain}^{-1}$), cytosol ($0.79 \text{ mL} \cdot \text{g brain}^{-1}$) and lysosomes ($0.01 \text{ mL} \cdot \text{g brain}^{-1}$) in rodents, respectively (Weibel et al. 1969; Nicholson and Sykova 1998; Syková and Nicholson 2008; Friden et al. 2011). Estimation of the cytosolic-to-ISF unbound drug concentration ratio ($K_{p,uu,cyto}$) and the lysosomal-to-cytosolic unbound drug concentration ratio ($K_{p,uu,lyso}$) can be computed by introducing pKa values of the compound:

$$K_{p,uu,cyto} = \frac{C_{u,cyto}}{C_{u,brainISF}} = \frac{10^{pKa - pH_{cyto}} + 1}{10^{pKa - pH_{ISF}} + 1} \quad (12)$$

$$K_{p,uu,lyso} = \frac{C_{u,lyso}}{C_{u,brainISF}} = \frac{10^{pKa - pH_{lyso}} + 1}{10^{pKa - pH_{cyto}} + 1} \quad (13)$$

where $pH_{cyto} = 7.06$, and $pH_{lyso} = 5.18$, as determined by Fridén and co-workers (Friden et al. 2011). $C_{u,cyto}$ and $C_{u,lyso}$ describe the unbound drug concentrations in cytosol and lysosomes, respectively.

The main application of the pH partitioning model is related to $f_{u,brain}$, calculated from data determined with the brain homogenate method, and allows the

introduction of correcting pH partitioning processes for weak bases/acids. It can be applied for correction of $f_{u, \text{brain}}$ for the lysosomal trapping of basic drugs, where the brain slice assay is not available (Friden et al. 2011; Loryan et al. 2014). However, one should bear in mind that the pH-partitioning model is still incapable of identifying and/or correcting other processes, such as active uptake into or efflux from the cells. These processes are responsible for differences in the results obtained between the brain slice and homogenate methods. One of the emerging methodologies is related to understanding brain regional and cell type related differences in $K_{p, \text{uu}, \text{cell}}$ (Loryan et al. 2016).

The $K_{p, \text{uu}, \text{cell}}$ concept is a basic principle that can be translated and has been translated to other models. The “cell bioavailability” concept presented for in vitro cell culture studies provides insight into the intracellular exposure–target engagement relationship (Mateus et al. 2017a, b). However, there are remaining questions related to the presence of protein in the media, the role of transporters, as well as absence of flow in the system that may influence the parameter values.

Applications in Drug Development

Despite the fact that the $K_{p, \text{uu}, \text{cell}}$ concept was introduced more than a decade ago (Friden et al. 2007), it has not yet been fully utilized by most pharmaceutical companies. However, there are few progressive companies that have tested the concept in their programs. For instance, the assessment of $K_{p, \text{uu}, \text{cell}}$ for novel drug candidates in addition to measuring $K_{p, \text{uu}, \text{brain}}$ provides intricate details on the neuroPK that is beneficial and that can guide in decision making, e.g. for the risk of lysosomally related side effects (Fig. 3c, d). Although brain tissue is not as lysosome-rich as the lungs, liver and kidneys, lysosomal trapping could also influence neuroPK, given that they can transverse the BBB to a high enough extent. Many marketed and novel neurotherapeutics are cationic amphiphilic compounds. It is thus not surprising that they are lysosomotropic. In this regard, $K_{p, \text{uu}, \text{cell}}$ assessment can be crucial in understanding tissue-specific toxicity (Loryan et al. 2017). Hence, these methods allow special attention to be paid to lysosomotropism in CNS drug development programs.

3 Small vs Large Molecules: Pharmacokinetic Differences

A shift from small molecular drugs to larger biologics, e.g. peptides, antibodies and other proteins and protein fragments, was initiated for peripheral diseases already some 20 years ago. More recently this change of focus also involves drug candidates for central diseases. The size difference between small molecule drugs and protein drugs (Fig. 4) has a pronounced impact on the rate and extent of brain delivery. A full-sized IgG antibody is about 1,000-fold larger than a small molecule drug such as aspirin, and as a consequence neuroPK readouts and concepts need to be adapted. Antibodies display highly restrictive transport across the BBB. It is often reported that only about 0.1% of peripherally administered antibody reaches the brain (Poduslo et al. 1994; Bard et al. 2000), and it has even been questioned whether

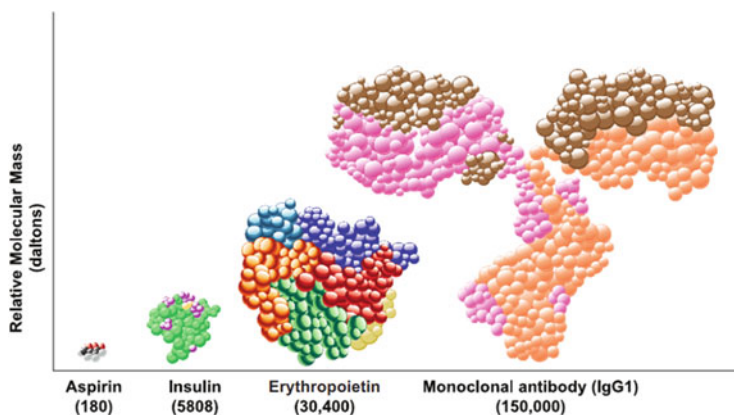


Fig. 4 Illustration of relative molecular mass (Da) of various drug classes. Figure obtained from (Mellstedt 2013) with permission from publisher

antibodies penetrate the brain parenchyma at all or if antibody concentrations measured in brain rather reflect transport from blood into the CSF, followed by potential but limited distribution into the ISF (Pardridge 2016). The time aspects are also generally different compared to small molecular drugs – antibodies showing systemic elimination half-life of weeks or even months, while central maximal concentration might be reached days or weeks after systemic administration.

The clearance of antibodies and other protein drugs from the brain is heavily debated, as it is unknown to what extent antibodies exit the brain via the BBB or if they are eliminated via the exchange between ISF and blood or CSF (sometimes referred to as the glymphatic system or the perivascular fluid system). It also appears that the unspecific and off-target binding of the often highly specific antibodies is very low. Thus, it might be difficult to relate the pharmacological effect of antibodies to the unbound concentration, as it could be that only the specifically bound antibody is producing down-stream pharmacological effects. Thus, the effect at a certain time point might be related to the amount of unbound antibodies present at a much earlier time point. Antibody delivery to the brain is often quantified as the per cent of the injected dose that reaches the brain. These values should be reported with information on the time of measurement even though changes in antibody concentrations may be slower compared to small molecular drugs due to the long half-life of antibodies.

As of today, there are only a handful of approved CNS active biologics, e.g. intravenous natalizumab (Polman et al. 2006), intrathecal nusinersen (Pane et al. 2018). Although there are systemically delivered antibodies and other biologics in clinical trials, it may be that modifications enabling facilitated transport across the BBB will be required for sufficient action at the intra-brain target site. In this respect, the TfR has been investigated as a potential brain delivery system of biologics by the fusion of a TfR binder to the primary drug, e.g. the therapeutic antibody (Li et al. 1999; Hultqvist et al. 2017; Johnsen et al. 2019). The specific characteristics of

receptor-mediated transcytosis (rate, capacity, expression, etc.) will then contribute to the pharmacokinetic and pharmacodynamics profile of the drug.

4 Optimization of Experimental Design for Neuropharmacokinetic Studies

In many CNS programs, only the plasma unbound drug concentration is used for monitoring drug exposure, relating it to the pharmacological effect. However, currently there is enough accumulated evidence supporting the fallacy of this kind of approach, particularly for drugs with transport-mediated passage across the BBB. In drug development programs, time for sophisticated experiments is often limited as quick results about CNS exposure are required. Hence, optimization of experimental design of neuroPK studies is essential, not to compromise the quality of experiments and relevance of obtained results for accurate assessment of CNS exposure. In addition, thorough evaluation of implicit and explicit assumptions should be performed prior to each experiment. Below, some examples are discussed.

Historically, AUCs of total brain and plasma concentrations after administration of a drug have been used for the assessment of $K_{p, \text{brain}}$ (Eq. 2). However, this implies that the experiments are terminal and only one concentration for the brain can be obtained per such experiment. This type of experiments require a large number of experimental animals to be used. There are emerging statistical methods for accurate evaluation of AUCs estimated from discrete samples, with assessment of variability (Wang et al. 2014).

In order to reduce the number of experimental animals, the concept of cassette or cocktail dosing, when administering several drugs simultaneously, has been proposed and even applied to microdialysis settings (Liu et al. 2012; Kitamura et al. 2016). With the aim to minimize the number of used animals, another more elaborate approach has been proposed with the focus on obtaining steady state plasma and brain concentrations (Hammarlund-Udenaes et al. 2008; Friden et al. 2009b). This requires prior knowledge on systemic pharmacokinetic parameters that will allow designing the intravenous infusion study aiming attainment of targeted steady state total drug concentration in plasma. There are several situations, when the $K_{p, \text{brain}}$ is measured using discrete samples, often 1 h after administration of a drug with the assumption, that $K_{p, \text{brain}}$ is similar to the one assessed by the approach in Eq. (2). Violation of this assumption, e.g. non-parallel blood and brain profiles, is very likely and may have significant impact on interpretation as the value may over- or under-predict the actual value.

Sampling of tissues is a critical aspect, and the time point of sampling requires optimization. Residual blood volume in brain tissue samples has to be accounted for. The assessment of total brain concentration could be affected by residual blood volume, something that is more pronounced for drugs with low BBB transport and high plasma protein binding (Friden et al. 2010). Consequently, the sampling techniques of brain tissue have to be adapted depending on the study purpose. With the aim to reduce the residual blood volume in the brain, some groups perform

cardiac perfusion before decapitation and/or optimize desanguination techniques. Others perform mathematical correction of measured total brain concentration accounting for ca 3–5% of residual cerebral blood volume. Each method should be evaluated before the application for a particular drug, as all have their pros and cons.

Another critical aspect is related to the species differences in BBB transport of drugs (Syvanen et al. 2009). Hence, $K_{p, \text{brain}}$ assessed in one species cannot be directly translated to other species. This requires specific experiments to be performed. The differences in BBB transporter expression are attributed to be the main driving force of species differences in the extent of drug BBB transport. However, there is accumulating evidence that it is not always easy to establish a causal link between the expression of transporters and functionality of the BBB (De Lange et al. 2018; Zhang et al. 2018). The latter requires more elaborate and optimized studies targeting this question.

5 Conclusions and Future Directions

Assessment of distribution of drugs into, within and out of the brain is essential for both preclinical and clinical phases of drug development, as it provides the pharmacokinetic foundation needed for establishment of exposure–response relationship. When evaluating drug brain disposition, it is critical to bear in mind the biological complexity of brain barriers, brain regional cellular and molecular diversity, flow of interstitial and cerebrospinal fluids in normal and pathological conditions along with the limitations of the methods used for assessment.

Expansion of the diversity of drug candidates from small molecules to proteins increases the level of complexity (Stanimirovic and Kemmerich 2015; Kumar et al. 2018) and requires thorough investigation of neuroPK of each novel class of molecules. Major advances are needed for the field of biologicals neuroPK, i.e. development of pharmacokinetic concepts, improvement of uptake across the BBB, understanding of brain elimination mechanisms and kinetics. All this cannot be achieved without sensitive and specific bioanalytical methods allowing detection of biologicals of interest in complex biological matrices. In this regard, PET brain imaging using radiolabelled antibodies opens up new perspectives in understanding the distribution of biologicals not only in the brain but also in the entire body (Sehlin et al. 2016, 2017; Syvanen et al. 2017).

In spite of the immense progress in the field of small molecular drugs' neuroPK we still face challenges in translating the readouts from in vitro to in vivo and from animals to healthy subjects and, eventually, to patients. This is primarily related to the fact that potentially translatable methods are not readily accessible (CSF sampling) and/or expensive (molecular brain imaging), limiting the speed of large-scale translational neuroPK studies. In addition, comprehensive characterization and understanding of the human BBB in health and disease is still lacking.

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Drug Delivery Strategies to Overcome the Blood–Brain Barrier (BBB)

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Abstract

The brain capillary endothelium serves both as an exchange site for gases and solutes between blood and brain and as a protective fence against neurotoxic compounds from the blood. While this “blood–brain barrier” (BBB) function protects the fragile environment in the brain, it also poses a tremendous challenge for the delivery of drug compounds to the brain parenchyma. Paracellular brain uptake of drug compounds is limited by the physical tightness of the endothelium, which is tightly sealed with junction complexes. Transcellular uptake of lipophilic drug compounds is limited by the activity of active efflux pumps in the luminal membrane. As a result, the majority of registered CNS drug compounds are small lipophilic compounds which are not efflux transporter substrates. Small molecule CNS drug development therefore focuses on identifying compounds with CNS target affinity and modifies these in order to optimize lipophilicity and decrease efflux pump interactions. Since efflux pump activity is limiting drug uptake, it has been investigated whether coadministration of drug compounds with efflux pump inhibitors could increase drug uptake. While the concept works to some extent, a lot of challenges have been encountered in terms of obtaining efficient inhibition while avoiding adverse effects.

Some CNS drug compounds enter the brain via nutrient transport proteins, an example is the levodopa, a prodrug of Dopamine, which crosses the BBB via the large neutral amino acid transporter LAT1. While carrier-mediated transport of drug compounds may seem attractive, the development of drugs targeting transporters is very challenging, since the compounds should have a good fit to the binding site, while still maintaining their CNS target affinity.

Receptor-mediated transport of drug compounds, especially biotherapeutics, conjugated to a receptor-binding ligand has shown some promise, although the amounts transported are rather low. This also holds true for drug-conjugation to cell-penetrating peptides. Due to the low uptake of biotherapeutics, barrier-breaching approaches such as mannitol injections and focused ultrasound have been employed with some success to patient groups with no other treatment options.

Keywords

Blood-brain barrier · Carrier mediated drug transport · Cell-penetrating peptides · CNS drug delivery · CNS drug design strategies · P-glycoprotein

1 Introduction

The barrier properties of the neurovascular unit, the “blood–brain barrier” is a major obstacle for pharmacological treatment of brain diseases. The present chapter will describe the drug delivery strategies commonly used to get drug compounds successfully delivered to the brain parenchyma. We will also describe experimental attempts to increase drug delivery by novel approaches, yet to be proven successful in the clinic. Finally, we will give a broader perspective on the CNS drug delivery field and point to future directions.

2 Drug Delivery and Blood–Brain Barrier Function

The brain capillary endothelium is the largest interface in terms of cross-sectional surface area, between blood and brain parenchyma. It serves as the main site for exchange of gases and water, uptake of nutrients such as glucose, amino acids, as well as excretion of metabolites. These exchange functions must be carried out, in order to maintain the microenvironment in the brain parenchyma and thereby sustain optimal neuronal signaling. Furthermore, the endothelium serves to prevent neurotoxic compounds in the blood from entering the brain parenchyma.

The brain endothelium is a very tight barrier for passive transcellular transport, in combination with solute transporters mediating uptake of nutrients and active efflux pumps, which keep xenobiotics out of the brain. The physical barrier is composed of intercellular tight junctions (TJs), which seal the spaces between the endothelial cells and allow only limited paracellular passage. The efflux pumps are active transporters of the ATP-binding cassette type, situated predominantly in the luminal membrane of the endothelial lining, as depicted in Fig. 1.

This barrier function (“blood–brain barrier,” BBB) is a major hindrance for the delivery of drug compounds from blood to brain. The physical barrier, i.e., the endothelial cell layer and the tight junctions simply prevent hydrophilic compounds above a certain size from entering the brain parenchyma, since only very small compounds may diffuse between the endothelial cells. This implies that all drug compounds passing from the blood to the brain parenchyma must enter via a transcellular pathway or, alternatively, cross the barrier in conditions where the junction tightness has been compromised.

Small lipophilic compounds may be able to permeate directly through endothelial cells, and most CNS drug compounds are molecules of this type. The strategy behind the design and test of such compounds is discussed further in the section “Delivery of ‘Small Molecules’ to the Brain.”

These small lipophilic molecules may be substrates of the efflux transporters in the luminal membrane of brain capillary endothelial cells. A number of studies have addressed whether co-dosing efflux pump inhibitors with small molecule drug compounds could be an effective strategy to increase brain exposure of the drug. This is treated in detail in the section “The efflux transporter inhibition concept.”

Brain drug delivery is shifting to larger drug compounds and constructs such as antibodies, liposomes, and other types of nano-particles. This is due to a number of reasons including emergence of novel technologies, low throughput with traditional approaches, and for the antibodies, a combination of very high specificity, low toxicity, and a large number of tools for modifying the antibody and the epitope-binding site. This is reflected by the fact that only few of the larger type molecules of biological origin are approved for treatment of CNS disorders (Salameh and Banks 2014; Freskgård and Urich 2017).

Drug delivery of larger molecules to the CNS is thus a formidable challenge, simply due to the size and the polarity of the compounds. An experimental approach to achieve this involves generating antibodies with dual affinity arms, one for a therapeutic target and the other for a protein, normally a receptor, expressed by brain

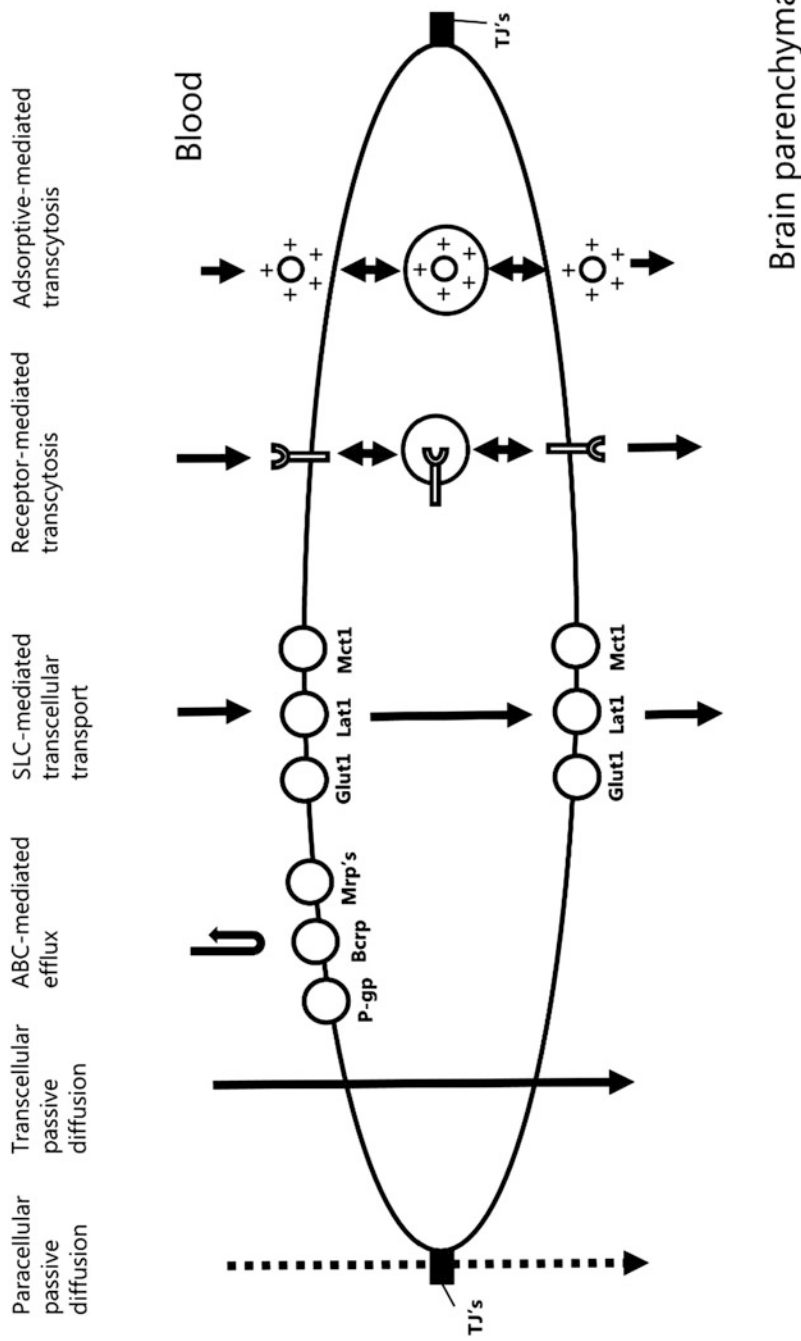


Fig. 1 A schematic overview of transendothelial transport pathways for drug compounds. *P-gp* P-glycoprotein, *ABC1*, *BCRP* breast cancer resistance protein, *ABCG2*, *MRP5* Multidrug resistance-associated proteins, *ABCC* family. *Glut1* Glucose transporter 1, *SLC2A1*. *Lat1* Large neutral amino acid transporter 1, *SLC7A5*. *Mct1* Monocarboxylate transporter 1, *SLC16A1*. *TJ's* Tight junctional complexes. Inspired by Abbott et al. (2010)

capillary endothelial cells. Examples of this approach are described in the section “Receptor-mediated uptake of therapeutic antibodies.” The section “Peptides as brain drug delivery vectors” deals with uptake of various types of targeting and non-targeting peptide sequences with therapeutic moieties. Another experimental approach has been to use viral capsids to cross the brain endothelium, which have been proposed for brain delivery of gene therapies, as described in the section “Viral Delivery to the Brain.” The above-mentioned approaches have shown promise, but a major breakthrough in terms of getting efflux transporter substrates and large, polar molecules across the barrier, is still lacking. Other experimental approaches have employed breakdown of tight junction integrity. The most established of these techniques are currently used for human treatment, albeit still at an experimental stage. These techniques, such as osmotic shock-induced by mannitol, or focused ultrasound techniques are described in the section “Invasive delivery; Osmosis and ultrasound.”

3 Delivery of “Small Molecules” to the Brain

The vast majority of CNS drugs can be categorized as “small molecules”; organic compounds with a molecular weight below 900 Da. Many small molecules are restricted from entering the CNS at therapeutic concentrations. Lipinski’s rule of five (Lipinski et al. 1997, 2012) has proven instrumental for designing small molecules for oral administration, and variations of this are similarly usable for designing small molecules with improved CNS access. The physico-chemical properties favoring CNS access have been extensively studied and have revealed a more confined space than for oral absorption of drug compounds (Pajouhesh and Lenz 2005; Ghose et al. 2012). This has given rise to a modified Lipinski’s rule termed the rule of CNS likeness. The exact boundaries for CNS likeness vary slightly from source to source, but numbers approximating those listed in the table below were reported by Goldberg (2011).

The increased confinement of the CNS drug space reflects not only limitations on the ability to reach therapeutic concentrations in the brain, but also certain structural confinements related to target affinity and potency (Rankovic 2017, 2019). This implies that BBB permeability confinement may not be as strict as previously assumed. Furthermore, existence of a strong interplay between different physico-chemical factors can make it difficult to predict BBB permeability of small molecules based on their physico-chemical parameters alone. The boundaries should therefore not be considered actual cut-off values (Banks and Greig 2019). Multiparameter optimization algorithms have been developed to integrate the different properties and weigh their contributions to classify compounds as CNS BBB permeating or BBB restricted (Wager et al. 2010). Nevertheless, whilst the rules of CNS entry should not be interpreted as inviolate, the fact remains that the majority of approved CNS drugs are small molecules with physico-chemical properties favoring passive permeability of cell membranes which are not substrates for efflux transporters (as discussed later) (Di et al. 2013). It follows naturally from this that

Table 1 Comparison of the selected physico-chemical properties of small molecules judged to favor their permeation across the gastrointestinal barrier (middle column) and across the blood–brain barrier (right column)

Property	Lipinski's "rule of five" ^a (Lipinski et al. 1997)	Modified CNS Lipinski's "rule of five" (Goldberg 2011)
Molecular weight (Daltons)	<500	<400
Calculated logP	<5	<5
Hydrogen bond acceptors	<5	<3
Hydrogen bond donors	<10	<7

^aThe favorable property-space for oral permeation was defined by Lipinski, hence the name Lipinski's "rule of five"

an effective strategy for getting small molecules from the blood to the brain is to design novel drug candidates so they are contained within the physico-chemical space determined for BBB-permeating drugs and preferably stay well below the boundaries. This approach has been considered in several recent studies designing new CNS active drugs (for example, see (Luo et al. 2018; Reis et al. 2018; Atkinson et al. 2019)).

Approximately 70% of all FDA approved CNS drugs obey the CNS-likeness guidelines (Table 1), thus supporting the relevance of confining new CNS drug candidates to this physico-chemical space (B. Fernandes et al. 2016). Conversely, staying inside the preferred space does not guarantee BBB passage. In other words, the BBB is not just a system of lipid membranes, interconnected by tight junctions. Several efflux and influx transport systems are present in the BBB (Fig. 1), which cause numerous deviations from the Lipinski's "rule." The Parkinson's disease prodrug, levodopa, is an illustrative example of this. Levodopa is a prodrug, which is converted to dopamine via decarboxylases. Levodopa's physico-chemical properties do not fit within the optimal space described above, and lipid membrane permeability of the compound is low. However, it is a substrate for an amino acid solute carrier, the large amino acid transporter-1 (LAT1), which is highly expressed in the brain capillary endothelial cells, at both the luminal and abluminal membranes (Gomes and Soares-da-Silva 1999; Kageyama et al. 2000). As a consequence, levodopa passes the BBB via LAT-1 mediated transport through the endothelial membranes. Solute carriers therefore present a strategy to improve BBB entry. A range of different solute carriers are expressed in the endothelial cells of the blood–brain barrier, most prominently the glucose transporter 1 (Uchida et al. 2011; Zhang et al. 2016). An overview of selected solute carriers and their RNA expression levels is shown in Fig. 2 (Zhang et al. 2016).

Glut-1 displays the highest expression level of the solute carriers and is essential for glucose supply to the brain (De Vivo et al. 1991). Expression levels of the individual transporters are highly relevant for their potential applicability to transport small molecules into the brain, since they follow Michaelis–Menten type kinetics

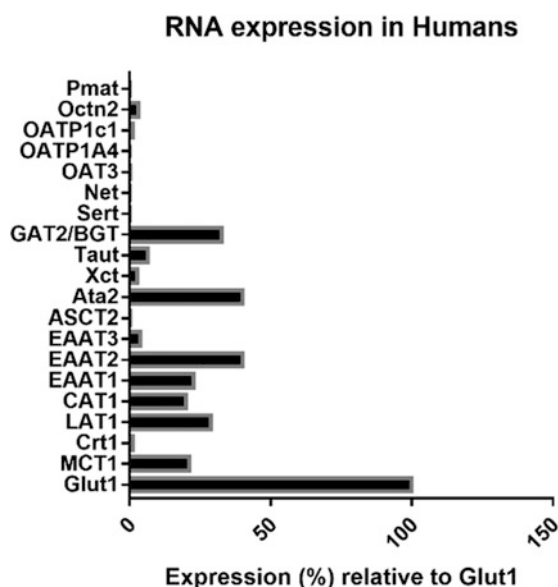


Fig. 2 mRNA expression levels of selected solute carriers in human brain endothelial cells. *Pmat* (*SLC29A4*) plasma membrane monoamine transporter, *Octn2* (*SLC22A5*) organic cation transporter 2, *OATP1c1* (*SLCO1C1*) solute carrier organic anion transporter family member 1C1, *OATP1A4* (*SLCO1A2*) organic anion-transporting polypeptide 1a4, *OAT3* (*SLC22A8*) organic anion transporter 3, *Net* (*SLC6A2*) norepinephrine transporter, *Sert* (*SLC6A4*) serotonin transporter, *GAT2/BGT* (*SLC6A13*) GABA transporter type 2, *Taut* (*SLC6A6*) taurine transporter, *Xct* (*SLC7A11*) cystine/glutamate antiporter, *Ata2* (*SLC38A2*) sodium-coupled neutral amino acid transporter 2, *ASCT2* (*SLC1A5*) alanine, serine, cysteine transporter 2, *EAAT1-3* (*SLC1A3*, *SLC1A2*, *SLC1A1*) excitatory amino acid transporter 1-3, *CAT1* (*SLC7A1*) cationic amino acid transporter, *LAT1* (*SLC7A5*) large neutral amino acid transporter, *CRT1* (*SLC6A8*) concentrative creatine transporter-1, *MCT1* (*SLC16A1*) monocarboxylate transporter 1, *Glut-1* (*SLC2A1*) glucose transporter 1. Data are from the Barres lab database (Zhang et al. 2014)

implying that the maximal transport capacity is dependent on the number of available transporters. Despite high glut-1 expression, this solute carrier does not play a prominent role in transporting small molecule drugs across the blood–brain barrier for several reasons, including a strict restriction on substrate size and competition from endogenous D-glucose (Mikitsh and Chacko 2014). At present there are no approved small molecule drugs known to utilize Glut1 to pass the BBB, although some studies have indicated that it may be possible to design pro-drugs with an attached glucose or galactose moiety to increase brain exposure (Bonina et al. 2003; Ruocco et al. 2008). This indicates that a solute carrier, used for ferrying drug compounds to the brain, should have a relatively broad substrate specificity in order to allow for some structural variations in drug design.

Another important factor is the localization of the solute carrier. Some highly expressed solute carriers such as the excitatory amino acid transporters (EAATs) and GABA transporter 2 (GAT2) are only expressed at the abluminal membrane facing

the brain side. This makes GAT2 and EAAT's less relevant for transport of small molecules from the blood to the brain (Takanaga et al. 2001; Helms et al. 2012), since carriers must be expressed at both the luminal and the abluminal membranes, in order to facilitate brain uptake.

The large neutral amino acid transporter (Lat1) is highly expressed, localized at both luminal and abluminal membranes and accepts a range of different branched amino acids and amino acid conjugates as substrates (Gynther et al. 2008). Besides the abovementioned levodopa, it has also been shown to facilitate blood-to-brain transport of the drugs gabapentin, melphalan, and baclofen, thus demonstrating its relevance for CNS drug delivery of small molecules (Scalise et al. 2018). However, these are the only examples of approved drug compounds utilizing LAT-1 to pass the BBB, and it is thus not a widely exploited transporter in approved CNS drug treatments, even though this is often stated. There are many reports of designing pro-drugs to transport non-LAT-1 substrates across the blood-brain barrier by coupling them to a LAT-1 substrate moiety, for instance to phenylalanine (Puris et al. 2019) or tyrosine (Gynther et al. 2008). Studies with these substrates have demonstrated a strong increase of LAT-1 mediated uptake both in cellular systems and in pharmacokinetic studies in mice. However, they also revealed some obstacles in utilizing this prodrug strategy. One is that the prodrug has to be bio-converted to the active drug compound in the CNS, which may be a slow process leading to low brain concentrations of the active compound (Puris et al. 2019). The other obstacle is that the bulky prodrugs often are transported at lower rates by LAT-1 than their parent amino acid substrates, even though the affinities for the transporter are retained (Puris et al. 2017, 2019).

The examples of Glut-1-, EAAT-, and LAT-1-mediated transport demonstrate that the absolute transporter expression levels, transporter localization and substrate affinity and capacity patterns are essential for a transporter to facilitate CNS access of drug compounds. A final major factor is the transport mechanism of the individual transporter. Some solute carriers, such as Glut-1, are uniporters that facilitate transport of a single solute, while others, such as LAT-1, are antiporters or exchangers, which simultaneously transport two substrates in opposite directions. A third form of solute carriers is symporters, for instance EAATs, which transport two or more substrates simultaneously in the same direction (For an overview of solute carrier transport mechanisms, see Nielsen et al. 2010). Transport through uniporters is governed by the concentration gradient of the compound moving from a high concentration on one side of the membrane to a lower concentration on the opposite side. The substrate transport mediated by exchangers is driven by the electrochemical gradient of both substrates, indicating that a substrate can be transported against a concentration gradient, driven by the "downhill" transport of the other substrate in the opposite direction. This is especially evident for symporters, where a high and constant concentration gradient of a co-transported substrate can lead to high accumulation of substrates against their concentration gradients and thus high brain uptake. A presumed, although not confirmed, example of this is the transport of the opioid drug, oxycodone, across the BBB. Oxycodone has been shown to reach equilibrium concentrations in the brain which are three

times higher than its corresponding blood concentrations, demonstrating an actual up-concentration across the BBB (Boström et al. 2006). The transporter responsible for oxycodone transport across the BBB has not been identified, but it is assumed to be a symporter utilizing another concentration gradient to facilitate oxycodone transport.

In conclusion, the vast majority of CNS drugs are small molecules with physico-chemical properties confined within the space of CNS-likeness. Novel drug candidates are therefore likely to have a better chance of reaching sufficient CNS concentrations if they are confined within this space. Solute carriers in the brain capillary endothelial cells can transport nutrients and thus also drug compounds resembling nutrient structures across the BBB and thus potentially provides an alternative pathway to the passive, “unassisted” diffusion into the CNS.

4 The Efflux Transporter Inhibition Concept

Drug efflux transporters constitute an important aspect of the BBB function by restricting the entry of xenobiotic compounds into the brain. Consequently, these efflux transporters are a major hindrance for drug delivery across the BBB. The currently known efflux transporters expressed in the brain capillary endothelial cells belong to the super family of ATP-binding cassette (ABC) transporters. They utilize hydrolysis of ATP to ADP to drive the transport of substrates against their concentration gradients. P-glycoprotein (P-gp, ABCB1), a 170 kDa transmembrane glycoprotein, has historically been the most studied efflux transporter of the BBB, since its function was described for the first time by Dano (1973) and subsequently identified by Juliano and Ling (1976). Breast cancer resistance protein (BCRP, ABCG2), which was discovered in 1998, may constitute an equally important efflux transporter at the BBB (Doyle et al. 1998; Eisenblätter and Galla 2002; Warren et al. 2009). While both P-gp and BCRP are ubiquitously expressed throughout the body, they seem to be expressed to a higher degree at the major barrier tissues including endothelial cells of the brain capillaries (Thiebaut et al. 1987; Maliepaard et al. 2001). Transport proteins belonging to the multidrug resistance-associated protein (MRP) family (ABCC) have also been shown to be expressed in endothelial cells of the small capillaries of the brain (Nies et al. 2004; Zhang et al. 2004; Roberts et al. 2008). The localization and physiological role of MRPs in BBB function have not yet been fully resolved.

The efflux transporters expressed at the BBB recognize and transport a wide range of structurally diverse substrates including, but not limited to, chemotherapeutic agents, calcium channel blockers, protease inhibitors, and steroid compounds, as listed in Table 2 (Sharom 2008). At present, little is known of the structural requirements for a compound to be recognized as a substrate for efflux transporters. P-gp substrates generally share some physico-chemical properties. P-gp substrates are often weakly amphipathic and hydrophobic compounds containing a functional group that is either electrically neutral or positively charged at physiological pH values and spatially separated from the hydrophobic moiety of the molecule (Sharom 2008). The substrate profiles for BCRP and MRPs are widely overlapping with that

Table 2 Examples of substrates and inhibitors of P-gp and MRP transporters expressed at the blood–brain barrier

Transporter	Compound class	Examples for substrates	Inhibitors
P-glycoprotein (ABCB1)	Cytostatic drugs	Vinblastine, vincristine, doxorubicin, daunorubicin, idarubicin, etoposide, teniposide, paclitaxel, methotrexate	Quinidine, quinine, amiodarone, surfactants (cremophor EL)
	Antihypertensive drugs		1st Generation, e.g., reserpine, quinidine, and yohimbine
	Immunosuppressants	Cyclosporin A, rapamycin, everolimus, tacrolimus	2nd Generation e.g., PSC-833 (valspodar), GF120918 (elacridar), VX-710 (biricodar), dexverapamil
	Corticoids	Dexamethasone, aldosterone, hydrocortisone, corticosterone, cortisol	3rd Generation, e.g., OC 144-093 (ONT-093), LY335979 (zosuquidar), XR9576 (tariquidar), R101933 (laniquidar), GF120918, epigallocatechin-3-gallate, baicalein, myricetin, wogonin, coumarins, Jatrophanes, lathyranes, tetracyclic diterpenes
	Opiates	Morphine	
	HIV protease inhibitors	Saquinavir, amprenavir, indinavir, ritonavir	
	Anthelmintic drugs	Ivermectin, abamectin	
	Cytokines	IL-2, IL-4, IFN- γ	
	Antidiarrheal drugs	Loperamide	
	Antiepileptic drugs	Phenytoin, carbamazepine, lamotrigine, phenobarbital, felbamate, gabapentin, topiramate	
	Anti-gout agents	Colchicines	
	Histamine H ₂ -receptor antagonists	Cimetidine	
	Calcium channel blockers	Verapamil	
	Antiemetics	Domperidone, ondansetron	

(continued)

Table 2 (continued)

Transporter	Compound class	Examples for substrates	Inhibitors
	Antipsychotics	Chlorpromazine, pimozide, fluphenazine, reserpine, triflupromazine	
	Antibiotics	Erythromycin, valinomycin, tetracyclines, fluoroquinolones	
	Cardiac glycosides	Digoxin	
	Diagnostic (fluorescent) dyes	Rhodamine-123	
	Antidepressants	Imipramine, amitriptyline, nortriptyline, doxepin, venlafaxine, paroxetine	
	Varia		Dexniguldipine, dofequidar fumarate (MS209)
MRP1 (ABCC1)	Anticancer drugs	Etoposide, teniposide, vincristine, doxorubicin, daunorubicin, melphalan, methotrexate	Sulfinpyrazone, probenecid, MK571, some Pgp inhibitors (e.g., cyclosporin A, verapamil, PSC 833)
	Glutathione, glucuronide, and sulfate conjugates		
	Immunosuppressants	Tacrolimus	
	HIV protease inhibitors:	Saquinavir, ritonavir,	
	Varia	Leukotrienes C4 (LTC4), D4, E4; 8-prenylnaringenin	
MRP2 (ABCC2)	Similar to ABCC1		Similar to ABCC1
MRP3 (ABCC3)	Considerable overlap in drug substrates with ABCC1 and ABCC2		Classical organic anion transport inhibitors such as sulfinpyrazone, indomethacin, and probenecid
MRP4 (ABCC4)	Anticancer drugs	6-mercaptopurine, methotrexate, thioguanine	
	Diagnostic (fluorescent) dyes	cAMP, fluo-cAMP	
MRP5 (ABCC5)		6-mercaptopurine, thioguanine	Probenecid and phosphodiesterase

(continued)

Table 2 (continued)

Transporter	Compound class	Examples for substrates	Inhibitors
			inhibitors such as trequensin or sildenafil
	Cyclic nucleotides	cGMP, cAMP	
	Diagnostic (fluorescent) dyes	Fluorescein	

Table information from (van Aubel et al. 2002; Sharom 2008; Liu 2019b)

of P-gp. In addition, BCRP and MRPs appear also to transport sulfate, glucuronide, and glutathione conjugated compounds (Liu 2019a). Co-expression in barrier tissues, the wide substrate profile and the extensive overlap between the substrate profiles of the different efflux transporters makes it difficult to identify substrates and inhibitors that are selective for one efflux transporter. When one transporter is inhibited, other efflux transporters will maintain the efflux function at the BBB to some degree. The expression and function of the different efflux transporters at the BBB therefore play a crucial role in maintaining the general homeostasis of the brain, but at the same time, also constitutes a major obstacle for drug delivery across the BBB.

Inhibition of efflux transporters, especially P-gp, as a tool for increased drug delivery to the CNS has gained considerable interest, and several compounds with an inhibitory effect on P-gp have been identified. P-gp inhibitors are divided into three generations of compounds, with different selectivities and potencies (Darby et al. 2011; Falasca and Linton 2012). The first generation of inhibitors include a variety of drug compounds developed with other clinical uses in mind, and later identified as P-gp inhibitors. Examples of first generation P-gp inhibitors include verapamil (a calcium channel antagonist), cyclosporin A (an immunosuppressant drug), tamoxifen (an estrogen receptor antagonist), and the antihypertensive drugs reserpine, quinidine, and yohimbine. The clinical use of the first generation inhibitors is limited by a low affinity for P-gp. This has the consequence that the plasma concentrations necessary for P-gp inhibition are often higher than those needed to achieve the primary pharmacological effects of these compounds, which may result in unacceptable side effects. Second generation P-gp inhibitors include analogs of first generation P-gp inhibitors such as dexverapamil (the R-isomer of verapamil) and valsopodar (PSC 833, an analog of Cyclosporin A). Other second generation P-gp inhibitors include dextiguldipine, biricodar (VX-710), and dofequidar fumarate (MS209). In contrast to first generation inhibitors, second generation inhibitors often lack the primary pharmacological effects seen in the first generation and inhibit P-gp at lower concentrations. However, second generation inhibitors are known to interact with other ABC transporters and inhibit cytochrome P450 enzymes, which results in complex drug–drug interactions and unpredictable pharmacokinetics for co-administered drugs. Third generation P-gp inhibitors, such as elacridar (GF120918), tariquidar, zosuquidar (LY335979), ONT-93 (OC144093), and laniquidar, show potent inhibitory activity on P-gp in vitro with no appreciable

effect on cytochrome P450 enzymes (Varma et al. 2003; Darby et al. 2011, 2017; Falasca and Linton 2012). Although early clinical trials with third generation inhibitors showed promising results of P-gp inhibition combined with acceptable toxicity profiles, later phase III clinical trials with tariquidar and zosuquidar were terminated prematurely. This was due to reports of adverse effects and a lack of response in the treatment groups (Leonard et al. 2003; Sandler et al. 2004; Kuppens et al. 2007; Kelly et al. 2011). Inhibitors of other ABC transporters such as Ko143 and fumitremorgin C against BCRP and MK571, agosterol-A and BAY-u9773 against MRPs have been identified, but they have also not yet been proven successful in clinical trials (Robey et al. 2011; Toyoda et al. 2019). Lessons related to toxicity and lack of efficacy learned from development of P-gp inhibitors have probably affected the clinical development of inhibitors targeting other ABC transporters.

Experience from clinical trials with P-gp inhibitors indicates that our current understanding of ABC transporters is insufficient, and we have a lot to learn before we can exploit inhibition of ABC transporters clinically to improve drug delivery to the CNS. Even though the existence and importance of P-gp and other ABC transporters for the BBB function has been known for more than two decades, the molecular mechanisms governing these efflux transporters, let alone the expression and interplay between the different transporters at the human BBB, are not yet fully understood. A more complete understanding of how ABC transporters mediate efflux of substrates at the molecular level and how they complement each other in different tissues would undoubtedly benefit the future development of ABC inhibitors. Insight into how the expression of ABC transporters differs between patients will be a prerequisite for proper experimental design and randomization in clinical trials. Ultimately, exploitation of ABC transporter inhibition to improve the drug delivery of CNS drugs will be patient-specific and require routine screening techniques for assessment of ABC transporter expressions at the BBB. PET imaging studies, using transporter tracers, may be valuable for this purpose (Tournier et al. 2018).

5 Receptor-Mediated Uptake of Therapeutic Antibodies

The introduction of humanized monoclonal antibodies as biomedical agents has added a new interesting class of approved drugs for a range of indications within oncology, inflammatory diseases, and autoimmune diseases (Walsh 2014). However, monoclonal antibody therapies for treatment of brain diseases have largely failed in the clinic. Whilst a few monoclonal antibodies have been approved for treatment of multiple sclerosis (Freskgård and Urich 2017; Voge and Alvarez 2019), their therapeutic effect is most likely driven by peripheral actions (Freskgård and Urich 2017).

Passive, unassisted diffusion of antibodies across the BBB is extremely limited because of their size (around 150 kDa for full IgG antibodies) and polarity. Their large size (and large hydrodynamic diameter) also restricts their ability to diffuse

through the extracellular spaces of the brain parenchyma (Wolak et al. 2015). As a consequence, significant efforts have been put into designing therapeutic antibodies that can utilize one of the receptor systems present in brain capillary endothelial cells. It has been speculated that antibodies may be bound to receptors at the luminal membrane, endocytosed and subsequently transcytosed to the parenchyma, allowing for global brain access (Freskgård and Urich 2017). In this section, we will briefly describe some of the most widely studied receptor systems and antibodies claimed to be transported across the BBB via these systems.

The insulin receptor was, along with the transferrin receptor, one of the early explored targets for receptor-mediated transcytosis of antibodies (Pardridge et al. 1985, 1995). Studies demonstrated brain accumulation of a high affinity monoclonal antibody against the human insulin receptor in rhesus monkeys (Pardridge et al. 1995). This antibody has since been applied in studies to deliver macromolecular therapeutics or imaging agents to the brain in pre-clinical studies in monkeys (Wu et al. 1997; Boado et al. 2008; Boado and Pardridge 2009). However, targeting the brain through the insulin receptor, and even the notion that insulin receptor transports insulin across the BBB, is controversial. A recent study from Genentech showed very low levels of brain uptake for an antibody targeting the insulin receptor (Zuchero et al. 2016). Furthermore, a study by Rhea et al. demonstrated that insulin transport to the brain was unchanged in an endothelial-specific insulin receptor knock-out mouse (Rhea et al. 2018). Hersom et al. demonstrated that the insulin receptor was functionally expressed, but did not transport insulin across the BBB in vitro (Hersom et al. 2018). As in all controversies, there are conflicting reports on the subject and other studies have demonstrated or indicated insulin receptor mediated transport across the BBB in vitro (for example, see (King and Johnson 1985; Gray et al. 2017)). Further research is therefore needed regarding the insulin receptor as a target for achieving BBB transcytosis. At the present time, it can be considered a somewhat uncertain, although not disproved concept.

The transferrin receptor 1 (TfR-1) is another receptor initially studied with biotherapeutic drug delivery in mind (Pardridge et al. 1991). TfR-1 is highly expressed in the brain capillary endothelium as compared to peripheral endothelium (Jefferies et al. 1984). The transferrin receptor mediates endocytosis of iron-bound holotransferrin from the blood. Once endocytosed, iron is released in the endosomes and transported to the brain parenchyma by a process, which is not fully elucidated. The receptor recycles back to the luminal membrane and the non-iron-coupled apotransferrin enters the blood (recently reviewed by Qian and Ke (2019)). Early experimental evidence also suggested that the receptor could be transcytosed, to release the transferrin at the abluminal membrane (Fishman et al. 1987; Friden et al. 1991). These studies were performed using radiolabeled ligands or antibodies and the brain stability of the compounds was not tested. The receptor-mediated transcytosis, defined as movement of receptor and transferrin (as well as bound antibodies) in an intact form across the barrier, has later been questioned and studies have indicated that transferrin is not transported intact across the BBB (reviewed by Lichota et al. (2010), McCarthy and Kosman (2015)).

The targeting of the transferrin receptor-1 with ligands or antibodies has been broadly explored and increases in brain exposure of antibody/drug conjugates have been reported. The number of studies has been large (excellently reviewed by Johnsen et al. (2019)) but has not yet resulted in drug candidates with success in clinical trials. Two of the most promising approaches are bispecific, affinity-modulated antibodies with a combination of TfR-1 and BACE-1 binding arms (Yu et al. 2011) and the so-called brain-shuttle where a trivalent construct with a conventional double-binding BACE-1 inhibitor part is combined with a monovalent TfR-1 binding arm (Niewoehner et al. 2014). The two types of constructs have shown reduced affinity to the TfR-1, and the authors have speculated that this may increase the exit step in the translocation pathway. Both have shown pharmacological effects in animal models (Yu et al. 2011; Ruderisch et al. 2017).

In general, however, the many studies employing the transferrin receptor system as a brain delivery pathway have shown low brain uptake of therapeutic doses, i.e., <1% of injected dose/gram, and none have shown success in clinical trials (Johnsen et al. 2019). A whole branch of studies investigating brain uptake of nanoparticles (dendrimers, PLGA particles, gold particles, liposomes) have been performed but also generally demonstrating low brain uptake and no approved medicinal products yet (Johnsen et al. 2019). It is evident that more research is needed to elucidate the endothelial cell endocytosis and endosomal sorting pathways of compounds targeting the TfR-1. There is solid consensus regarding the role of the TfR-1 in mediating endothelial cell uptake of receptor-targeting antibodies and ligands. However, the lack of mechanistic understanding of the cellular route of permeating TfR-1 targeting antibodies and ligands makes it a challenge to design therapeutics utilizing the receptor system as a transport pathway.

A large number of other approaches, utilizing receptor-mediated transcytosis, have been attempted. Pardridge and coworkers performed early studies with chimeric peptides, targeting various receptors (Bickel et al. 2001) and since then, a large number of studies have attempted to show biotherapeutic drug delivery, using a vector which targets a receptor system. There is an ongoing search for new targets, some of which may even be SLC membrane carrier proteins (Zuchero et al. 2016), and increased interest in the elucidation of identifying receptors which actually show movement from luminal to abluminal membrane (Siupka et al. 2017). Hopefully this will lead to increased understanding of the permeation mechanisms, and new possibilities for designing constructs that effectively increase brain exposure of biotherapeutics.

6 Peptides as Brain Drug Delivery Vectors

Peptides are attractive as vectors for drug delivery across the BBB due to their ease of production, versatility, and general low toxicity and immunogenicity. A disadvantage is their high susceptibility to enzymatic degradation *in vivo*, which in some cases calls for strategies to increase their stability in order to prolong plasma half-life (Bach et al. 2012; Wei et al. 2015).

6.1 Cell-Penetrating Peptides as Brain Drug Delivery Vectors

Cell-penetrating peptides comprise a family of peptides, which may be divided into groups based on their properties as cationic, hydrophobic, or amphipathic (Pooga and Langel 2015). Cell-penetrating peptides have demonstrated ability to deliver conjugated drugs into cells, reviewed by Ye et al. (2016) as well as across biological barriers, reviewed by Kristensen et al. (2016). In 1999, the 11-mer Tat peptide, derived from the HIV Transactivator of Transcription protein, was the first cell-penetrating peptide demonstrating an ability to deliver a conjugated cargo across the blood–brain barrier (Schwarze et al. 1999). Another study demonstrated that Tat conjugation to the therapeutic peptide moieties NR2B9c facilitated NR2B9c delivery into cortical rat neurons (Aarts et al. 2002). Moreover, intraperitoneal administration of Tat-NR2B9c to rats experimentally subjected to stroke diminished the infarct volume. Subsequently, the more potent *N*-dimer was developed (Bach et al. 2012) and intraperitoneal administration of this Tat-conjugated *N*-dimer to mice subjected to stroke demonstrated even greater reduction of the infarct volume when compared to Tat-NR2B9c. In addition, the ability of the Tat-*N*-dimer to translocate across the BBB in healthy live mice was visualized using two-photon microscopy (Kucharz et al. 2017). Tat-NR2B9c finished phase III clinical trial in early 2020 (Hankey 2020), whereas Tat-*N*-dimer will enter phase I trials in 2020.

Tat is the most widely applied cell-penetrating peptide as vector for brain drug delivery, but the brain delivery potential of other cell-penetrating peptides counting pVEC, SynB3, transportan 10, and transportan 10-2 has been demonstrated in healthy mice (Stalmans et al. 2015). In addition, the novel cell-penetrating peptide dNP2, which is of human origin, demonstrated BBB permeation potential following intravenous administration to mice (Lim et al. 2015). Furthermore, the cell-penetrating peptide penetratin was successfully used to deliver the copper chelating agent Trientine in surface modified liposomes into rat brain after intravenous administration (Tremmel et al. 2016).

The underlying mechanisms of barrier permeation by cell-penetrating peptides have not been fully elucidated. Endocytic uptake has been suggested as the cell uptake mechanism of Tat and dNP2 in splenocytes (Lim et al. 2015). Whether endocytic uptake is followed by transcytosis or segregation to lysosomes within the capillary endothelial cells is unknown. As for other systemically administered therapeutics intended for CNS action, a disadvantage of cell-penetrating peptides relates to their broad peripheral distribution (Sarko et al. 2010).

6.2 Blood–Brain Barrier Receptor Targeting Peptides for Brain Drug Delivery

In order to identify peptide-based drug delivery vectors targeting the BBB, phage display biopanning may be applied in the search for transferrin-, low-density lipoprotein-, and low-density lipoprotein related-1 receptor binding peptides. The brain delivery potential of peptides selected by phage display biopanning has been

demonstrated when displayed on AAVs (Xia et al. 2000), phages (Staquicini et al. 2011), or nanoparticles (Liu et al. 2013; Zhang et al. 2013). The HAI and THR peptides targeting the transferrin receptor did, however, effectively enter cells as single entities (Lee et al. 2001), thus potentially being relevant as brain drug delivery vectors.

Receptor-targeting peptides may translocate the BBB via receptor-mediated transcytosis. Whether the receptor itself is transcytosed is discussed as highlighted in section “Receptor-mediated uptake of therapeutic antibodies.” Nevertheless, receptor interaction seems a viable strategy for BBB recognition and binding prior to endocytic uptake. The transcytosis process – with or without the receptor – is less described mechanistically.

7 Viral Delivery to the Brain

Rare neurological disorders can be caused by genetic mutations resulting in loss of vital or gain of harmful functions. Viruses are interesting potential vectors for gene therapies as a remedy to correct these mutations, due to their inherent ability to deliver genetic cargo into cells. Adeno-associated viruses (AAVs) are probably the most studied viral vectors in gene therapy (for reviews, see (Daya and Berns 2008; Hastie and Samulski 2015; Naso et al. 2017)). Vectors based on herpes simplex virus (Neve 2012), lentivirus (Milone and O’Doherty 2018), adenovirus (Lee et al. 2017), and rabies virus (Kumar et al. 2007) have also been studied. Viral vectors may be injected directly into the local areas of the brain, which demands intensive surgery (Kaplitt et al. 2007). Studies demonstrated that viral vectors also can cross the BBB upon osmotic BBB disruption (Nilaver et al. 1995), focused ultrasound in combination with microbubbles (Wang et al. 2015). These procedures cause a transient breakdown of the barrier, and the permeation is therefore most likely due to bulk paracellular flow into the parenchyma. They have some limitations, as discussed later in the section “Invasive delivery; Osmosis and ultrasound.”

A number of studies have demonstrated evidence for permeation of viral vectors across an intact barrier. The AAV serotype 9 (AAV9) was the first AAV-based vector demonstrating broad transduction of astrocytes, and thus implying BBB translocation, after intravenous administration to mice (Foust et al. 2009). Subsequently, additional studies have demonstrated that a number of other AAV serotypes caused transfection of brain cells following intravenous administration (Zhang et al. 2011; Yang et al. 2014). The field has progressed considerably since the early studies three decades ago. There are, however, some considerations for exploiting virus capsids as drug delivery vectors, including: Low cargo loading efficiency, complexity of production, innate immunogenicity, inflammatory response, and toxicity (Lehrman 1999; Colella et al. 2018; Hordeaux et al. 2018).

7.1 Targeting Viral Vectors to the Brain

Lack of BBB tissue tropism remains a concern with respect to the safety of the AAVs as vectors for delivery of gene therapies to the brain. AAVs display broad distribution into peripheral tissues upon systemic administration (Zincarelli et al. 2008; Bevan et al. 2011; Gray et al. 2011), thereby potentially giving rise to multiple adverse effects. Therefore, strategies for AAV targeting to the BBB for the treatment of neurovascular or neurological diseases have been pursued. In one study, a BBB targeting AAV2 variant was generated by *in vivo* selection (Körbelin et al. 2016). An AAV2 capsid peptide library was intravenously administered to mice allowing selection of brain homing capsid peptides. The *in vivo* selection was repeated for multiple rounds resulting in identification of the BR1 peptide. AAV2 vectors displaying the BR1 peptide demonstrated impressive brain homing with limited transfection of peripheral tissues. AAV2-BR1 mainly transduced brain capillaries, but some neuronal transduction was also observed, thus implying that a limited fraction of AAV2-BR1 translocated across the BBB.

The BBB is altered during a number of diseases (reviewed by Sweeney et al. (2019)). Viral vectors preferentially binding to the healthy barrier may therefore not bind to the same extent to a diseased BBB. This was previously demonstrated in a study aiming to identify brain homing peptides by *in vivo* screening of a phage library (Chen et al. 2009). In this study, different peptide sequences were identified as a result of panning in wild-type mice as compared to mucopolysaccharidosis disease model mouse (MPS VII). When phage selected peptides were displayed on the AAV2 capsid, improved brain homing was observed in the model, in which the peptides were selected. The brain-targeting peptide sequences, identified in the wild-type mice, moreover differed from sequences earlier identified by the same methodology in wild-type rats (Work et al. 2006).

In addition to the limited BBB tropism, unmodified AAVs also suffer from low neuronal transduction rates upon intravenous administration to adult animals (Foust et al. 2009; Yang et al. 2014). A number of studies have therefore focused towards the development of AAVs with improved ability to deliver their genetic cargo into the target cells of the CNS. The AAV9-AS vector was generated by insertion of a polyalanine peptide at the N-terminus of the VP2 capsid protein and demonstrated improved transduction of the spinal cord and cerebrum upon intravenous administration to adult mice, when compared to control AAV9 (Choudhury et al. 2016). Moreover, AAV9-AS facilitated gene transfer to cat brain following systemic circulation. Another example is the AAV9-PHP.B vector (Deverman et al. 2016), which transduced neurons and astrocytes across the adult mouse brain, and the optimized version AAV9-PHP.eB24, with further improved neuronal transduction in the adult mouse brain. The AAV9-PHP.B and AAV9-PHP.eB vectors were selected *in vivo* using the CREATE methodology (Deverman et al. 2016). However, the CNS tissue tropism of the AAV9-PHP.B vector documented in C57BL/6J mice does not extend to all mouse strains (Hordeaux et al. 2018; Batista et al. 2020), whereas the CNS tropism of AAV9-PHP.eB appears to also apply for rats (Dayton

et al. 2018). Therefore, the applicability of the AAV9-PHP.B vector in human therapies may be questioned.

7.2 Mechanisms of Underlying Brain Delivery of Viral Vectors

The mechanisms underlying brain delivery of viral vectors upon intravenous administration are not fully understood. Some studies point towards involvement of initial binding to glycocalyx constituents presented on the capillary endothelial cells prior to transcytosis. Such studies are commonly carried out by uptake studies in mutant CHO cells being deficient in different stages of glycan chain synthesis. One study pinpointed N-linked galactose as important for cell interaction and subsequent uptake of AAV9 (Shen et al. 2011). Another study supported that conclusion, demonstrating that removal of terminal sialic acid from cell surface glycans improved AAV9 cell uptake (Bell et al. 2011), possibly due to better exposure of terminal galactose. However, simple cell uptake studies do not reveal whether endocytic uptake at the luminal membrane of the capillary endothelial cell is followed by exocytosis at the abluminal membrane. In order to study whether transcytosis actually takes place, representative barrier forming cell models must be included. An early mechanistic study showed transcytosis of AAVs across epithelia (Caco-2 and MDCK cell culture models and human primary airway cells) and endothelia (primary bovine brain microvascular cells) in a cell- and serotype-dependent manner (Di Pasquale and Chiorini 2006). Within recent years, more advanced BBB models of human origin have emerged. One study investigated BBB translocation of AAV9 and AAV2, using a model generated from brain microvascular endothelial cells isolated from human epileptic patients (Merkel et al. 2017). That study demonstrated that AAV9 permeated the barrier to a greater extent than AAV2, which supports earlier *in vivo* studies (Zincarelli et al. 2008; Zhang et al. 2011). Cell uptake of AAV2, however, resulted in transduction of the endothelial cells, whereas transduction of astrocytes at the abluminal side of the endothelial cells was observed with AAV9. AAV9 translocated across the BBB model in an energy-dependent manner without disruption of the barrier integrity. Moreover, tubular structures along the z-axis of the endothelial cells treated with AAV9 were observed. Such structures may correspond to the so-called sorting tubules, which have been suggested to regulate transcytosis across the BBB (Villaseñor et al. 2017). Recent studies have demonstrated that the expression level of the glycosylphosphatidylinositol-linked protein LY6A in brain capillary endothelial cells has an impact on the CNS transduction efficiency of AAV9-PHP-B (Hordeaux et al. 2019; Huang et al. 2019; Batista et al. 2020). It has not yet been established whether LY6A is transcytosed together with AAV9-PHP-B.

Some viral vectors may reach the brain parenchyma via the fenestrated capillaries of the circumventricular organs (Duvernoy and Risold 2007). Entry through that route was earlier suggested for some AAV serotypes, due to the observation of enhanced transfection around the circumventricular organs (Yang et al. 2014). The

extent and how these vectors can move beyond the ependymal barriers of the circumventricular organs have not yet been fully elucidated.

8 Invasive Delivery; Osmosis and Ultrasound

Among the different techniques to bypass the BBB, temporary BBB disruption using hyperosmolarity caused by intraarterial mannitol infusion has proven effective, although it is a relatively drastic approach. Neuwelt and colleagues developed the technique in animals, where they initially demonstrated increased brain concentration of methotrexate (454 Da) in dogs following BBB disruption, induced by a bolus injection of mannitol into the blood stream (Neuwelt et al. 1979). Shortly after, the procedure was tested in a clinical study demonstrating effective BBB disruption in patients with malignant brain cancer (Neuwelt et al. 1980). The extent of increased brain drug delivery is dependent on the size of the administered drug molecule. However the technique has proven efficient for increasing antibody delivery into the brain of both rats (Neuwelt et al. 1986) and humans (Neuwelt et al. 1987).

Mannitol/osmotic shock-induced transient BBB disruption is carried out in specialized hospital settings, primarily to treat primary and metastatic brain tumors. The principle is generally to fill parts of the CNS vasculature with a 25% mannitol solution, administered directly into the carotid artery. This hyperosmolar solution causes water to leave the endothelial cells in turn causing cell shrinkage and physical opening of the paracellular spaces (Rodriguez et al. 2015). Carotid artery infusion in itself is a complicated step, but nevertheless necessary to induce BBB disruption, since intravenous injection causes the mannitol to become too diluted in the circulation to induce sufficient hyperosmolarity (Chen et al. 2013). A study by Blanchette et al. showed that mannitol treatment results in an immediate increase in the BBB permeability, which remains for a period after the disruption process, varying from minutes to hours depending on the molecule administered (Blanchette et al. 2009). CNS levels of the contrast agents plateaued at levels approximately 2–3 times higher than in non-BBB disrupted animals. However, other studies have indicated that levels 10–100 times higher can be reached with BBB disruption and intraarterial chemotherapy as compared to intravenous chemotherapy alone (Kraemer et al. 2002).

BBB disruption treatment, in combination with intraarterial chemotherapy, has since been tested in numerous smaller clinical trials. In one trial, 41 patients with malignant astrocytoma were distributed in two groups. One group (28 patients) underwent the BBB disruption protocol in combination with intraarterial chemotherapy, while the other group (13 patients) received only the intraarterial chemotherapy (Kraemer et al. 2002). This study showed a significant longer survival for the patients receiving the barrier disruption procedure.

In 2009, an overview of 25 years of multicenter treatment of primary CNS lymphoma using BBB disruption together with intraarterial methotrexate showed promising results. The studies did not include a specific control group, and the fact that primary CNS lymphoma is a rare disease makes it difficult to recruit sufficient

patients for large multi-treatment modality studies. However, comparison with other study results indicated an improved median survival when comparing to trials with high doses of intravenous methotrexate alone (Angelov et al. 2009).

The BBB disruption procedure may cause adverse events such as focal seizures, increased intracranial pressure, temporary obtundation, and in the most severe case (only one reported) fatal brain herniation. Overall the procedure is considered relatively safe compared to its benefits, when performed by trained staff in a controlled setting (Doolittle et al. 2000). In addition, BBB disruption combined with chemotherapeutic infusion is not associated with a decline in neurocognitive assessments of patients following the treatment (McAllister et al. 2000). Thus, the main limitations for this technique seem to be the requirement for a highly specialized setting and extensive hospitalization. This is illustrated by the fact that the procedure is only being performed by members of the clinical “Blood–Brain Barrier Consortium”, consisting of eight north American institutions as well as an Israeli partner (*Blood–brain barrier consortium*, no date).

An alternative method to induce transient BBB disruption is through injected microbubbles in combination with focused ultrasound. This technique is based on the acoustic responsiveness of 1–10 μm diameter lipid or protein-encapsulated gas spheres upon entering an ultrasound field. Microbubbles have high compressibility and a tendency to cavitate under ultrasound, which transforms the energy from the acoustic wave to the microenvironment of the capillary through volumetric oscillations. This leads to mechanofluidic effects on the capillary endothelial cells causing transient barrier opening (Song et al. 2018). The procedure involves a range of parameters, physiological, acoustic and microbubble characteristics, which all need to be adjusted in relation to each other, in order to obtain a controlled and transient BBB disruption without long-term adverse effects (Song et al. 2018).

Animal proof of concept studies have demonstrated transient local BBB opening using contrast agents in mice (Choi et al. 2007), rabbits (Hynynen et al. 2001; McDannold et al. 2005; Marquet et al. 2011), and non-human primates (Marquet et al. 2011). The time-span and extent of permeability increases were further investigated in mice showing a 100-fold increase of permeability of gadodiamide (592 Da) relative to the contralateral side (Vlachos et al. 2010). Further studies have demonstrated increased local brain delivery for a range of differently sized compounds, from small molecules as the 543 Da compound doxorubicin (Treat et al. 2007) to the 40 kDa horse radish peroxidase (Sheikov et al. 2008) and the 148 kDa antibody, Herceptin (Kinoshita et al. 2014). However, there is a size cutoff for effective delivery into the brain, as demonstrated in a study showing delivery of 70 kDa dextran, but limited delivery of 2,000 kDa dextran (Choi et al. 2010). These and other studies have demonstrated the applicability of the method to improve treatment outcome in animal models for a range of CNS diseases, for instance, Alzheimer’s disease, glioma, Parkinson’s disease, and Huntington’s disease (Song et al. 2018).

Despite several promising studies in pre-clinical animal models, the translation to apply focused ultrasound and microbubbles to open the human BBB has been challenging. One important reason is the simple anatomical differences between

animals and humans. Rodents and other small animals have relatively thin skull bones, allowing for focusing in the brain with simple ultrasound transducers. The human skull bones are much thicker, absorbing up to 90% of the ultrasound energy, and thus the procedure requires much more advanced ultrasound equipment, takes several hours, and requires magnetic resonance imaging monitoring (Carpentier et al. 2016).

Over the recent years, several clinical studies have been initiated, investigating the safety and efficacy in treatments of glioblastoma, Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease with the ultrasound/microbubble-induced breakdown technique in combination with a therapeutic agent (Chen et al. 2019). Most trials are still ongoing or recruiting, but there are reports from two completed phase 1/2 trials. One is concerning the safety of opening the barrier without any additional pharmacotherapy in Alzheimer's disease (Lipsman et al. 2018) and the other concerning the safety and efficacy of combined BBB opening and carboplatin in the treatment of glioblastoma (Carpentier et al. 2016).

Both studies have low numbers of patients (5 in the Alzheimer's study and 15 in the glioblastoma study), which makes it difficult to draw firm conclusions so far, but both studies showed interesting perspectives. The Alzheimer's study demonstrated consistent increase in brain entry of the contrast agent, gadolinium, in the affected area, which returned to normal 24 h after treatment, with no apparent safety issues (Lipsman et al. 2018). The study on glioblastoma involved the insertion of an ultrasound transducer in the skull for repeated treatments during a three-month period. The study demonstrated the general safety of the procedure and a transient opening of the BBB for gadolinium in 68% of the treatments at a midrange ultrasound exposure. The actual carboplatin exposure in the brain was not measured, and neither were the exact effects on the disease outcome (Carpentier et al. 2016).

The long-term safety of repeated focused ultrasound and microbubble treatment has been questioned, since several studies have shown responses such as sterile inflammation (Kovacs et al. 2017) and neuromodulation (Chu et al. 2015). Behavioral responses in rats following repeated treatments at different acoustic exposure levels only showed effects when exposures beyond that needed for BBB opening were applied, indicating that the procedure is generally safe. It was highlighted however that several factors interact, and it is important to control microbubble dosage as well as acoustic exposure to ensure a safe procedure (Tsai et al. 2018). In conclusion, focused ultrasound in combination with microbubbles represents a promising, although still largely unexplored approach to bypass the BBB in a local and relatively safe manner to increase drug exposure to CNS sites. A recent interesting approach has been to modulate tight junction permeability with peptides and proteins interacting with proteins in the junctional zone (Bocsik et al. 2016; Dithmer et al. 2017; Neuhaus et al. 2018; Zeniya et al. 2018), thereby causing a transient opening for drug compounds. The techniques are, however, still at an early stage and will not be treated further in this chapter.

9 Conclusions and Perspectives

The field of CNS drug delivery progresses slowly at the moment. On the one hand, the development of small molecule drug compounds with passive permeation properties is fairly established. Many of these small molecule drug candidates eventually turn out to be P-gp substrates and are not taken further in the drug development process. The screening techniques for P-gp interactions (and interactions with other efflux transporters) are well established. The challenges associated with these small molecule CNS drug compounds are therefore related to the same challenges as those associated with drug compounds intended for oral delivery with peripheral targets. This includes identification of proper targets and binding pockets, generation of promising lead compounds with high affinity binding, modification of the physico-chemical properties of lead compounds towards optimal properties for lipid barrier permeability, modification of stability and investigations of the toxicity, ADME, and pharmacodynamics properties.

On the other hand, there is an intense focus on finding novel drug delivery principles and uptake routes for biotherapeutics to treat CNS diseases, and particularly brain delivery of antibodies. Antibodies are easy to produce and modify on a small scale and affinities/avidities to targets can be very high. Antibody constructs can be engineered to have two or three individual epitope-binding sites, plasma half-life can be long and the probability of generating toxic metabolic products is low. But the size and the polar nature of these molecular entities makes their passive BBB permeation negligible. They must therefore be targeted to the luminal membrane of the brain capillary endothelium and somehow undergo transcellular movement, for example via the postulated “receptor-mediated transcytosis” or via binding to nutrient transporters and subsequent uptake into endothelial cells, vesicular sorting, and abluminal release. These processes are poorly described and poorly understood at the subcellular level, and transport capacities have been found to be very low. Furthermore, antibody production on an industrial scale for treatment of common CNS diseases may not be feasible at the moment, due to the capacity of current production technologies (Freskgård and Urich 2017).

Other approaches for macromolecular delivery, e.g. nanoparticle (with or without targeting antibodies) and peptide delivery, also suffer from problems with limited transport capacities. As a result, no barrier-permeating peptides, antibodies, or nanoconstructs have been registered as drug compounds to date. Viral vectors are currently gaining intense attention for brain delivery of gene therapies. Especially variants of AAV9 have demonstrated broad transduction within the brain parenchyma in animals. However, several researchers report significant differences in brain transduction efficiency across species, and even strains, which constitute a major limitation for translation to the clinic.

Invasive methods such as osmotic opening of the barrier or focused ultrasound in combination with microbubbles may be promising treatments for some life-threatening diseases, but do not seem likely to become standard treatments for various mental health disorders or milder forms of neurodegenerative diseases, due to high cost, low compliance, and potential risk.

The main question is therefore where should researchers in academia and industry, decision makers, and funding bodies go from here? Small molecule drug delivery is feasible, but development of new drug compounds is expensive and with a high failure rate. Large molecule drug compounds like antibodies are easier to develop, but until now, none have reached the market. It should, however, be mentioned here that a number of antibodies intended to treat CNS diseases are being tested in clinical trials at the moment. Well aware of the inherent dangers in predicting future important steps, we still suggest the following avenues of research might be worthwhile:

Revisit small molecule drug delivery for treatment of CNS diseases. While large efforts in the industry and academia focus on antibody delivery and nanoparticle delivery, the development of novel design and drug delivery principles for small molecules have been somewhat underrated.

Increase focus on prediction of efflux transporter interactions of small molecules. Efflux transporter interactions and subsequent brain efflux contribute significantly to the high failure rate of small molecule drug compounds early in development. Better tools for prediction of efflux transporter interactions from molecular structure will, at least in theory, allow for faster and more efficient design of novel, BBB-permeating drug compounds.

Better understanding of “transcytosis.” “Receptor-mediated transcytosis,” “adsorptive-mediated transcytosis,” and transendothelial transport of compounds binding to BBB nutrient transporters are still not well understood at the cellular level. In order to fully utilize these pathways, a better understanding of the cellular machinery and the underlying processes is needed. An estimation of the kinetics of these systems will also allow us to estimate transport capacities better, and thus give us better tools to evaluate whether these systems have the necessary capacity for brain drug delivery.

As a final remark, it should be emphasized that a narrow focus on drug delivery will not be enough to accelerate successful CNS treatments. This should ideally go hand in hand with studies of disease etiologies and identification of novel targets. In other words, if we are not sure what to treat, and when to treat it, barrier permeation may be a minor issue in the greater picture. Academic drug delivery research groups may not necessarily work together with medicinal chemists, pharmacologists, basic researchers in disease etiology, or clinicians. This is often the case in the life-science industry. Company priorities may change on a relatively short time scale and accumulated knowledge may be lost. A way to accelerate generation of novel CNS drug compounds could be to increase national funding in basic brain and brain barrier biology, basic disease understanding and target identification, so as to generate novel concepts and find novel targets. Continuity and translational research could be supported by embedding national academic and company knowledge in transnational research networks, including both academia and industry.

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Part III

Experimental Methods to Study Drug Transport across the Blood-Brain Barrier In Vitro and In Vivo



Transport Studies Using Blood-Brain Barrier In Vitro Models: A Critical Review and Guidelines

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Abstract

Permeation is one of the most evaluated parameters using preclinical in vitro blood-brain barrier models, as it has long been considered to be one of the major factors influencing central nervous system drug delivery. Blood-brain barrier permeability can be defined as the speed at which a compound crosses the brain endothelial cell barrier and is employed to assess barrier tightness, which is a crucial feature of brain capillaries in vivo. In addition, it is used to assess brain drug penetration. We review traditionally used methods to assess blood-brain barrier permeability in vitro and summarize often neglected in vivo (e.g., plasma protein and brain tissue binding) or in vitro (e.g., culture insert materials or methodology) factors that influence this property. These factors are crucial to consider when performing BBB permeability assessments, and especially when comparing permeability data obtained from different models, since model diversification significantly complicates inter-study comparisons. Finally, measuring transendothelial electrical resistance can be used to describe blood-brain barrier tightness; however, several parameters should be considered while comparing these measurements to the blood-brain barrier permeability to paracellular markers.

Keywords

Brain capillary endothelial cells · Cell layer tightness · Drug transport · Paracellular transport · Penetration · TEER

1 Introduction

Until a decade ago, the focus of many central nervous system (CNS) drug programs was to increase the rate of blood-brain barrier (BBB) permeability, as it was considered to be the main factor determining CNS drug efficacy (Reichel 2010; Banks 2016). However, it became clear that not only rate but also the extent of CNS drug penetration and the intra-brain distribution should be evaluated, as BBB permeability results generally show poor correlation with pharmacodynamic and efficacy readouts (Hammarlund-Udenaes et al. 2008). Current focus is on simultaneous optimization of both rate and extent, and this is in line with the free drug hypothesis (Hammarlund-Udenaes et al. 2008; Banks 2016). Indeed, many efforts are still being made to improve drug permeation across the BBB, thereby focusing on specific transport systems (Neuhaus et al. 2006; Mahringer et al. 2012; He et al. 2018).

Brain penetration of molecules is controlled by the tight BBB endothelium, which results from the presence of functional tight junction proteins that seal adjacent brain endothelial cells to each other. It also depends greatly on the specific compound under study (Neuhaus et al. 2006; Bauer et al. 2014). The tightness of the brain endothelial cell (BEC) monolayer determines the restrictive paracellular permeability potential of the BBB and is therefore one of the most important BBB features, as it critically regulates BBB function (Günzel and Yu 2013). Any disruption of barrier tightness or function can lead to malfunctioning of the brain itself, contributing to different neurodegenerative disorders, including Parkinson's disease and Alzheimer's disease (Montagne et al. 2017; Sweeney et al. 2018).

The high complexity of in vivo models resulted in the development of a vast amount of in vitro approaches mimicking the BBB over the past decades. Therefore, the relationship between cell monolayer tightness and compound penetration is commonly evaluated preclinically using in vitro BBB models (Dehouck et al. 1990, 2011; Deli et al. 2005; Cecchelli et al. 2014; Avdeef et al. 2015; Aday et al. 2016; Helms et al. 2016).

These in vitro BBB models are highly valuable as they provide good tools to study barrier properties in both health and disease, as well as for studying mechanistic aspects related to drug transport (Neuhaus et al. 2006). They all aim to mimic the in vivo BBB; however, they generally reflect it only partially and in variable ways (Reichel et al. 2003; Helms et al. 2016). Model diversification and a further lack of standardization results in inter-study variability and complicates cross-comparison of data obtained from different models. BBB permeability is one of the BBB-related parameters that is often compared indistinctly across studies, which leads to inconsistent observations and misinterpretations (Bischoff et al. 2016). There are only few studies comparing different BBB models using the same permeability methods and set of markers or compounds (Hellinger et al. 2012; Veszeka et al. 2018) and even fewer studies comparing the in vitro BBB permeability results with in vivo brain penetration data (Dehouck et al. 1992; Garberg et al. 2005; Nakagawa et al. 2009). In order to enhance comparability and transferability of BBB permeability data, this chapter reviews the most frequently utilized methods to assess BBB permeability, provides guidelines, and draws the attention on multiple, often neglected, factors that should be considered when employing and comparing BBB permeability studies.

2 In Vitro Permeability Measurements

Permeability is a rate parameter that describes the speed at which a compound crosses the BBB. It can be assessed using in vitro BBB models with the purposes (1) to determine brain endothelial barrier tightness (i.e., evaluation of the functionality of complex tight junction proteins) by measuring BBB permeability of a well-known paracellular integrity marker and (2) to assess BBB penetration of compounds in, for example, CNS drug programs (Youdim et al. 2003; Wegener and Seebach 2014; Yusof et al. 2014).

BBB permeability of a compound is the result of both paracellular diffusion and transendothelial transport including passive transcellular diffusion of lipophilic molecules, active carrier-mediated transport via solute carriers, and [transcytosis](#) by receptor-mediated or non-specific transcytotic pathways (Deli et al. 2005; Abbott et al. 2010; Wegener and Seebach 2014). Paracellular space between neighboring brain endothelial cells is strictly regulated by the unique functionality of complex intercellular tight junctions including transmembrane tight junction and adherens junction proteins which restrict the passage of hydrophilic molecules (Wolburg and Lippoldt 2002; Neuhaus et al. 2006; Abbott et al. 2010; Wegener and Seebach 2014). Depending on their size and lipophilicity, most compounds are therefore transported through transcellular transport and their passive permeation across the lipid plasma membrane (Neuhaus et al. 2006). The parallel artificial membrane permeability assay (PAMPA) can be used to assess passive diffusion across the lipid membrane of the BBB (Ottaviani et al. 2006; Avdeef et al. 2015). However, artificial membranes lack BBB transporters, an issue that can significantly affect predictability of CNS penetration of a drug under study (Avdeef et al. 2015). By contrast, permeability assessment using an in vitro BBB model allows the more accurate reproduction of the complexity of the BBB in vivo, at least to some degree (Garberg et al. 2005; Helms et al. 2016), and may therefore facilitate compound selection in CNS drug discovery programs.

Multiple variants of in vitro BBB permeability experiments exist, but they all rely on the same principle. The transport rate of a molecule is assessed across a confluent cell monolayer, which is seeded on a cell culture insert with a highly permeable membrane. This insert is usually placed in a multiwell plate, thereby forming the interface between two fluid compartments, i.e., one compartment that mimics the blood and one compartment that mimics the brain side. Next, the test compound is added to the donor compartment, which can be one of the two compartments, and is then allowed to be transported across the cell monolayer to the receiver compartment in a defined amount of time, for example, 60 min (Dehouck et al. 2011). The permeability experiment ends with sample collection that is required for calculating BBB permeability. Ideally for translation experiments, the in vitro cell monolayer should be as tight as possible, thereby restricting the paracellular pathway to the highest possible extent, so that the contribution of transendothelial transport can be accurately investigated and estimated (Deli et al. 2005; Hammarlund-Udenaes et al. 2008; Helms et al. 2016). However, most in vitro models of the BBB only show a moderately tight brain endothelial monolayer and therefore reflect contributions of the transcellular pathway and the paracellular pathway. It is thereby often the case that the paracellular transport of especially hydrophilic or amphiphilic compounds represents a significant part of the total transport rate, which renders the study of the cellular and molecular mechanisms behind transendothelial transport extremely difficult.

BBB permeability is commonly calculated by two different types of methods with either consideration of a cell-free insert or not: the endothelial permeability coefficient (P_e) and the apparent permeability coefficient (P_{app}), respectively. Examples of

these methods are explained below together with important factors to consider when comparing permeability data from different studies.

2.1 Apparent Permeability Coefficient

The apparent permeability coefficient (P_{app} , in cm s^{-1}) is the most commonly used calculation method for the in vitro permeability. The parameter can be determined by the rate of appearance or flux (J , in s^{-1}) of the compound in the receiver, thereby normalizing for membrane surface area (S , in cm^2) and initial donor concentration at t_0 (C_0 , in mL^{-1}) (Eq. 1).

$$P_{app} (\text{cm s}^{-1}) = \frac{J (\text{amount.s}^{-1})}{S (\text{cm}^2) \cdot C_0 (\text{amount.mL}^{-1})} \quad (1)$$

The P_{app} does not calculate the true endothelial permeability coefficient (P_e) as it does not correct for the permeability across the cell-free insert. However, dependent on the transport route, the permeability of a compound across a cell-free insert is sometimes negligible, and therefore P_{app} is widely used. Nevertheless, the type of insert membrane can affect the permeability coefficient value (especially when comparing lipophilic with hydrophilic compound) which is frequently neglected in studies (Cecchelli et al. 1999; Wegener and Seebach 2014). To overcome this issue, endothelial permeability coefficient (P_e) could be calculated.

2.2 Endothelial Permeability Coefficient

The endothelial permeability coefficient (P_e , in, e.g., cm min^{-1}) is, like P_{app} , a calculation method for the permeability coefficient. However, it corrects for permeability across a cell-free culture insert and more closely represents the permeability across the endothelium. Among other factors, this calculation method takes into account that the thickness of the plastic support, on which the cells are cultivated, is significantly higher than the thickness of extracellular matrix (ECM) of the BBB in vivo. For example, plastic membranes of cell culture inserts are on average about 10 μm thick, whereas the ECM at the BBB is only about 20–60 nm (Thorne and Nicholson 2006). Therefore, the plastic membrane of the inserts represents an additional diffusion barrier for compounds as compared to the in vivo ECM. The P_e is calculated based on the clearance principle to obtain a concentration-independent transport parameter (Siflinger-Birnboim et al. 1987). The cleared volume (CL, in μL) is calculated by dividing the diffused amount of compound in the receiver compartment (A_r) with the concentration of compound in the donor compartment (C_d) (Eq. 2).

$$CL \text{ (}\mu\text{L)} = \frac{A_r}{C_d} \quad (2)$$

The average cumulative CL is subsequently plotted over time and the slope is estimated by linear regression analysis. This results in the permeability-surface area product (PS, in $\mu\text{L min}^{-1}$). To make a correction for permeability across cell-free inserts, the PS products are calculated for both cell-free inserts (i.e., PS_f, filter) and inserts with cells (i.e., PS_t, total = PS_f + PS_e).

The P_e can then be computed out of PS_f and PS_t (Eq. 3), normalized by the surface (S, in cm²) (Eq. 4) (Garberg et al. 2005; Dehouck et al. 2011; Cecchelli et al. 2014).

$$PS_e^{-1} = PS_t^{-1} - PS_f^{-1} \quad (3)$$

$$P_e \text{ (cm min}^{-1}\text{)} = \frac{PS_e}{S} \quad (4)$$

2.3 Efflux Ratio as a Measurement of Active Efflux

The P_{app} coefficient can give information on active efflux. In this type of bidirectional permeability assay, the transport of a compound is assessed in both directions (apical to basolateral (A-B) and basolateral to apical (B-A)) across the cell monolayer. This enables the calculation of the so-called efflux ratio, i.e., P_{app} (B-A)/P_{app} (A-B), which indicates whether or not active efflux is occurring for the compound under study. An efflux ratio greater than 2 indicates drug efflux (Hellinger et al. 2012). Reference compounds which are known efflux pump substrates, for example, talinolol, can be assessed in parallel. In addition, slow permeating compounds like atenolol or fast, transcellularly permeating drugs such as propranolol are frequently used in these assays as marker substances. Additionally, including reference compounds of active efflux, efflux pump inhibitors (e.g., verapamil for P-glycoprotein/ABCB1, MK571 for multidrug resistance proteins (MRPs)/ABCCs) can also be included in the study. If the test compound is an efflux pump substrate, the efflux should decrease in presence of an inhibitor of the efflux pump under study. This type of assay could also be used to assess active influx processes. In this case, an efflux ratio lower than 0.5 is a hint for an active influx transport.

3 Permeability Studies to Measure Barrier Tightness

When the goal is to evaluate the tightness of the brain endothelial monolayer, for example, to validate the permeability status or quality of an in vitro model, a typical integrity marker with low permeability is used. These markers are commonly characterized by their high hydrophilicity, high polarity, and absence of active transport. Therefore they are ideal probes to assess the contribution of the paracellular diffusion pathway, since the permeability will then only be determined

by the functionality of the cell junctions (Deli et al. 2005; Neuhaus et al. 2006; Wegener and Seebach 2014). The specific permeability value relies also on the properties of the tracer itself, including the type of molecule and molecular size. Several markers are currently on the market such as fluorescent markers including lucifer yellow (442 Da), sodium fluorescein (376 Da), and fluorescein isothiocyanate (FITC)-labeled dextrans (1–150 kDa) and radiolabeled markers including sucrose (342 Da), albumin (67 kDa), mannitol (182 Da), and inulin (5 kDa) (Neuhaus et al. 2006; Saunders et al. 2014; Helms et al. 2016).

Typically, one marker is used to assess the tightness of a BEC monolayer. The choice of the paracellular marker is often dependent on the experimental design and the barrier status of the cell layers. Smaller markers are preferred to study tighter cell layers, whereas bigger markers are used for cell layers with moderate to weak tightness. However, the usage of only one marker does not necessarily allow complete characterization of the paracellular permeability of both small and large molecules. Therefore, Neuhaus et al. developed a tool that characterizes paracellular transport or tightness of cell layers by employing a molecular weight ladder, the 8-aminopyrene-1,3,6-trisulfonate (APTS)-dextran ladder (Neuhaus et al. 2006).

Co-incubation of integrity markers with test compounds like neuropharmaceuticals allows the study of barrier toxicity of compounds. This method is therefore used to qualitatively assess barrier properties of a particular in vitro method. When comparing values obtained with this kind of method across studies, for data interpretation, it is essential to consider possible differences in type or molecular weight of the applied markers (Neuhaus et al. 2006).

3.1 Factors Influencing BBB Permeability Results

Modern model diversification hampers inter-study comparability of permeability results. However, generating a better understanding of the different factors that might influence BBB permeability can at least create awareness when comparing data. Inter-variability of in vitro models can originate from differences in isolation procedures of primary cells, cell culture conditions (e.g., assay format, medium), cell culture configuration (e.g., monoculture, co-culture, etc.) and origin (e.g., cell type, species), and experimental design (e.g., buffer, presence of plasma proteins, mechanical forces) (Reichel et al. 2003; Youdim et al. 2003). When the aim is to predict human in vivo findings, neglecting these factors might affect permeability and, so, the quality of the in vitro-in vivo correlation (Yusof et al. 2014; Avdeef et al. 2015). In general, considering these factors is helpful to produce relevant and comparable data that contributes to our knowledge of the in vivo BBB and that can be used in drug development programs for the production of reliable pharmacokinetic information of drugs and drug candidates.

3.2 Importance of Recovery Values

Assessing the mass balance or recovery (%) of the tracer or drug is essential. Poor solubility or binding to cell assay materials might result in loss of compound throughout the experiment and lead to an underestimation of the endothelial permeability. Poor recovery can result from adsorption of the tested molecule to materials (e.g., plastic), from metabolism by metabolic enzymes, from non-specific binding to cells, and from accumulation in the cell monolayer. Recovery is easily calculated out of the recovered amount of compound in both compartments at the end of the experiment as a percentage of the initial donor amount at t_0 . In addition, intracellular accumulation can be assessed when recovery values suggest poor recovery. The latter can subsequently be applied to determine whether or not the observed poor recovery is a consequence of compound binding to the cell monolayer.

Several groups adopted a quality threshold for permeability experiments, which can differ depending on the experiment and model. For example, Cecchelli et al. determined a mass balance threshold between 80% and 120% for the permeability values to be qualitative when using a human *in vitro* BBB model derived from hematopoietic stem cells (Cecchelli et al. 2014).

Poor recovery can be diminished by including serum albumin during the permeability assay as an absorptive driving force for lipophilic compounds and therefore to mimic the *in vivo* sink for lipophilic compounds (Youdim et al. 2003; Yusof et al. 2014). Addition of serum albumin in the experimental assay reduces non-specific binding of compound at the donor compartment but also influences the unbound concentration of compounds in the donor compartment, and both factors can influence its transport to the receiver compartment (Yusof et al. 2014).

3.3 Importance of the Materials Used in the Permeability Assay

Materials used during the permeability experiment can affect the permeability of compounds across the brain endothelial monolayer (Cecchelli et al. 1999; Wegener and Seebach 2014; Yusof et al. 2014). For example, permeability across a cell-free culture insert can provide essential information about how the membrane of the insert potentially influences permeability values. To ensure proper calculation of P_c , permeability across the cell-free insert should not be the rate-limiting step in a compound's diffusion process. The insert membrane should be highly permeable for the concerned compound, but at the same time, it should still support the cell layer mechanically (Cecchelli et al. 1999; Wegener and Seebach 2014). For this, pore size and membrane material are important parameters to consider as they can lead to divergent permeability values across studies using different inserts (Cecchelli et al. 1999; Yusof et al. 2014). Furthermore, assessing the MB in a cell-free experiment provides an efficient way to evaluate compound binding to materials. For example, transport tests with lipophilic ester compounds against trypanosome infections across cell-free PET membranes showed a significant loss of the substances within the plastic membranes (Cohrs 2008). Finally, this required an

adjustment of the plastic material and the cell seeding and culture procedure using hydrophilic PTFE (Teflon).

Another parameter to consider is the pH of the medium or buffer, as it is known to affect permeability when assessing the penetration of ionizable compounds like vinblastine, acetylsalicylic acid, and naloxone (Youdim et al. 2003; Yusof et al. 2014). In this regard, it should be mentioned that the medium composition could also influence the barrier properties of the cell layers; thus caution and control experiments for cell viability and the targeted transport process should be conducted to improve the interpretability of the results. For example, serum content not only affects the transport study results by different drug binding to serum proteins; it might also influence the barrier properties of the cell layer itself by, e.g., supplying different amounts of growth factors.

3.4 Influence of Mechanical Forces

Static in vitro models of the BBB do not reflect the physiological conditions in which the in vivo BBB operates, such as lack of cerebral blood flow and shear stress. In the last 8 years, new dynamic in vitro BBB models have emerged based on microfluidic devices to assess permeability of molecules. Similar to in vivo conditions, these flow models incorporate mechanical forces, i.e., shear stress, hydrostatic pressure, and cyclic stretch, that are reported to affect permeability by an overall reduction (Booth and Kim 2014; Wegener and Seebach 2014; Walter et al. 2016; Cochrane et al. 2019). This change in permeability mainly results from tightening of brain endothelial cell junctions, and its intensity depends on the cell type and experimental design (Wegener and Seebach 2014; Walter et al. 2016).

Many static in vitro models are currently being employed, and it is therefore necessary to consider other important factors that affect BBB permeability which follow from this static condition. Cell monolayers in static models show the presence of an aqueous boundary layer, adjacent to the cell layer, which is absent or minimal in in vivo conditions due to the existing blood circulation (Youdim et al. 2003). The absence of flow in vitro generates a concentration gradient between the compound in the upper part of the buffer or medium and the compound close to the membrane surface. The layer that results, the unstirred water layer, is recognized to affect P_{app} and P_e of compounds (Youdim et al. 2003). In particular, fast permeating, lipophilic compounds are hampered by this extra aqueous layer which might be the rate-limiting step for permeation of these compounds (Korjamo et al. 2008). This can lead to deviation from in vivo permeation values by underestimation of the permeability (Youdim et al. 2003; Yusof et al. 2014). Formation of this aqueous layer can be reduced by a stirring or shaking procedure during the permeability assay. However, complete reduction of this layer is difficult as a shaking velocity that is too high will compromise the endothelial barrier function by affecting tight junction integrity, and a shaking velocity that is too low will not efficiently reduce the aqueous-boundary effect, and hence a balance should be made (Yusof et al. 2014; Avdeef et al. 2015). Besides this, each compound is characterized by different

physio-chemical characteristics and may require different shaking speeds. Some correction procedures for permeability values have been reported, for example, the pCEL-X software correction method (Yusof et al. 2014).

3.5 Plasma Protein Binding and Brain Tissue Binding

In line with the free drug hypothesis, only the unbound compound is available for permeation across the brain capillary endothelial monolayer. However, compounds that are weakly bound to proteins such as albumin may still interact with membrane transporters. Thus the total biologically available compound is the sum of the unbound fraction and the weakly bound fraction (Banks 2016). This interaction depends on the compound's affinity for both the transporter and for proteins such as human serum albumin (Pajouhesh and Lenz 2005). Therefore, comparing in vitro BBB permeability of compounds from studies for which differences are present in the buffer or medium composition regarding the presence of plasma or serum proteins is cumbersome as BBB penetration has been demonstrated to be affected by protein binding (Pajouhesh and Lenz 2005). The latter should also be considered while comparing in vitro to in vivo data as plasma protein binding also influences other pharmacokinetic parameters of the drug, such as the half-life or volume of distribution, affecting BBB drug exposure (Banks 2016).

Similar discrepancies result when comparing studies that differentially mimicked potential brain tissue binding. Brain tissue binding is known to affect in particular the permeability of lipophilic compounds. This is not accounted for in the classical methods to assess permeability like P_{app} or P_e , leading to a poor in vitro-to-in vivo translation for those compounds characterized by high brain tissue binding (Summerfield et al. 2007; Heymans et al. 2018). In vitro, this discrepancy can be minimized by implementing glial cells, or very likely also other cells, in the receiver compartment that allow potential brain tissue binding. The latter requires the use of an adjusted permeability calculation resulting in a novel in vitro permeability coefficient, named P_{vitro} (Heymans et al. 2018).

3.6 Example of Normalization: Diazepam

The lipophilic compound diazepam is known to permeate rapidly into the brain using passive diffusion and is therefore extensively used as an internal standard for the assessment of transcellular, passive transport (Neuhaus et al. 2006; Culot et al. 2008). When comparing permeability using different in vitro models, diazepam is expected to result in a similar outcome, since it permeates very rapidly across a lipid bilayer through passive diffusion (Neuhaus et al. 2006). Therefore, very divergent permeability results for diazepam might indicate the significant influence of one or several factors on BBB permeability (described in Sects. 3.1–3.5) in one of the models under study. This was, for example, demonstrated by Zhang et al., who measured the permeability of diazepam in different, i.e., stirred vs. unstirred,

experimental conditions (Zhang et al. 2006). In this regard, some groups applied diazepam as marker compound within the same experiment in order to normalize for cell layer variabilities of single culture inserts (Appelt-Menzel et al. 2017). To assess the influence of the blank, cell-free insert membrane (PS_f) on the permeability coefficient of a compound, the so-called effect of correction could be used.

The effect of correction is calculated by the ratio of PS_e and PS_f . When this ratio equals one, the cell-free insert is considered to have no significant influence, and the brain endothelial monolayer is the major transport barrier. The compound in question must therefore most likely permeate this barrier paracellularly. In contrast to this, diazepam reveals effect of correction values significantly higher than 1 indicating a fast, transcellular transport with a significant influence of the plastic membrane to the total permeability coefficient (Neuhaus et al. 2006).

In addition to being a reference compound that reflects high permeability, it is useful to implement a control compound characterized by a low permeability in a way to relatively compare the permeability of different compounds, rather than compare them based on their absolute permeability value.

4 Transendothelial Electrical Resistance Measurements

The transendothelial electrical resistance (TEER) measurement is an additional method to evaluate the barrier tightness of in vitro BBB models (Deli et al. 2005; Wegener and Seebach 2014; Helms et al. 2016). TEER represents the paracellular permeability for small inorganic ions (i.e., Na^+ and Cl^-) and can be defined as a measure of electrical/ohmic resistance of ion flow across the cell monolayer (Neuhaus et al. 2006; Abbott et al. 2014). In accordance with Ohm's law, it is a direct current (DC) approach: voltage (U) is applied to two electrodes, from which one is above and the other one is under the cell layer enabling the ion current (I) measurement across the monolayer resulting in the recording of ohmic resistance ($R = U/I$) (Benson et al. 2013).

The two most common electrode types to measure TEER on BBB models grown on culture inserts are the chopstick (Fig. 1a) and chamber electrodes (Fig. 1b). One of the electrodes reaches the bottom compartment, assuring a stable position. The shorter electrode submerges into the medium in the top compartment but does not reach the cell layer. The handling of chopstick electrodes is fast and easy, but their positioning is key for reproducibility. The positioning angle, medium volume used, and the small interferences between measurement conditions, such as temperature and pH changes, can be a drawback of this method. Therefore, it is recommended to change the medium before each measurement and equilibrate the temperature by, e.g., incubating at room temperature for 30–45 min in the sterile working bench (Blume et al. 2010). This is generally considered as essential, since the composition (serum content, ion composition, viscosity (Gerhartl et al. 2020)), the age, and the temperature of the medium could influence the measured values significantly. In addition, it is also important to assess blank values without cells under the very same conditions (same age, same coating, same medium, etc.). Chamber electrodes have a

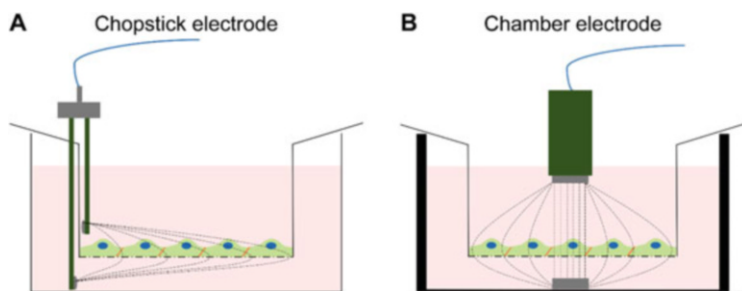


Fig. 1 Electrode types for transendothelial electrical resistance (TEER) measurements on BBB models using culture inserts. (a) Chopstick electrodes. (b) Chamber electrodes. The figures were adapted from the [EVOM2](#) instruction manual (World Precision Instruments, Sarasota, FL, USA)

more uniform current density across the insert membrane (Fig. 1b) resulting in low background TEER values across cell-free inserts. In general, the TEER values measured across cell-free inserts are ten times higher in the case of chopstick electrodes as compared to chamber electrodes. Modified versions of the chamber electrodes were developed (cellZscope, nanoAnalytics, Germany) to measure TEER in different multiwell format inserts (Benson et al. 2013).

Using DC for TEER measurement can result in the accumulation of ions at electrode and the cell layer; therefore the commonly used Epithelial Voltohmmeter (EVOM; World Precision Instruments) uses alternate current (AC) at a very low (12.5 Hz) frequency. When we measure TEER with AC, we register impedance. In this method the capacitance of the cell layer is also taken into account when calculating resistance. The total impedance reflects not only the TEER (paracellular ion pathway) of the cell layer but the resistance of the membrane (transcellular ion pathway), the medium itself, and the surface where the electrode and medium meet (Benson et al. 2013). It is important to note that the used frequency of the AC square wave current defines the information gained from the measurement. At low frequency (≤ 400 Hz), the paracellular ion current pathway is the dominant, and the impedance reflects cell-cell junction strength. At higher AC frequency (≥ 10 kHz), impedance measurement reflects the current pathway across cell membranes (Benson et al. 2013).

In experiments the TEER measurement is widely used as a quality control to select culture inserts with consistent and comparable cell monolayers representing similar integrity for further experiments (Reichel et al. 2003; Deli et al. 2005; Abbott et al. 2014; Helms et al. 2016). Thereby, studies generally adopt a TEER quality threshold to exclude cell culture inserts showing low integrity, but these threshold values can be very different depending on the BBB models (Gaillard and de Boer 2000; Abbott et al. 2014; Helms et al. 2016). Besides its use in quality control, the TEER method is employed in studies to identify agents that modulate barrier tightness (Bocsik et al. 2019; Santa-Maria et al. 2019). Moreover, supplements like lithium chloride (Veszeka et al. 2018), cAMP, or hydrocortisone influence TEER in different BBB models from different species (Deli et al. 2005). As shown in

Table 1 TEER values of in vitro models of the blood-brain barrier from different species

Species	Type of endothelial cells	Co-culture	TEER ($\Omega \times \text{cm}^2$)
Mouse	Primary BEC	Astrocyte	777 $\pm$ 15
Mouse	cEND cell line	–	300–800
Mouse	cerebEND cell line	–	500 $\pm$ 10
Rat	Primary BEC	Astrocyte	300–600
Rat	Primary BEC	Brain pericytes and astrocytes	350–723
Bovine	Primary BEC	Rat astrocyte	600–1,600
Bovine	Primary BEC (clone selection)	Rat astrocyte	600–800
Porcine	Primary BEC (isolation using enzymes)	–	250–790
Porcine	Primary BEC (isolation with density centrifugation)	–	400–1,500
Porcine	Primary BEC	Rat astrocyte/astrocyte cell line	800–1,800
Human	hCMEC/D3 cell line	–	40–200
Human	iPSCs	–	220 $\pm$ 51
Human	iPSCs	Astrocyte	700 $\pm$ 112
Human	iPSCs	Brain pericytes and primed NPC	2,500–5,350
Human	CD34 ⁺ -derived EC	–	60–90
Human	CD34 ⁺ -derived EC	Brain pericytes	150–180

Type of brain endothelial cells and co-culture with astrocytes and/or pericytes are indicated. Data are collected and adapted from Helms et al. (2016)

BEC Brain endothelial cell, *EC* endothelial cell, *cEND* immortalized cerebral endothelial cell, *cerebEND* immortalized cerebellum endothelial cells, *hCMEC/D3* immortalized human brain endothelial cells, *iPSCs* induced pluripotent stem cells, *CD34⁺* cord-blood CD34⁺ stem cell-derived endothelial cells, *NPC* neural progenitor cells

Table 1, co-culture and primary cell-based models present a higher TEER compared to monolayer or cell line models.

In general the TEER value measured depends on areas of the monolayer which possess the lowest electrical resistance, whereas BBB permeability depends on the total transport using all junctional or transport pathways (Deli et al. 2005). TEER is nonlinearly related to permeability coefficients (Fig. 2) (Gaillard and de Boer 2000; Neuhaus et al. 2006; Abbott et al. 2014; Thomsen et al. 2015).

More specifically, this relationship tends to be inverse. Permeability is related to conductance (g) that measures both ionic permeability across a cell layer and the total number of available ions, which is the reciprocal of resistance (Abbott et al. 2014). For example, leakiness of the brain endothelial monolayer may occur upon decreasing tight junction protein functionality, which therefore results in a higher P_e to an integrity marker and a generally lower TEER. However, brain capillary endothelial monolayers characterized by low TEER values fail to show this relationship as demonstrated by highly variable and inconsistent P_e or P_{app} values (Abbott et al. 2014; Bischoff et al. 2016). For low resistance cells or models therefore, it is

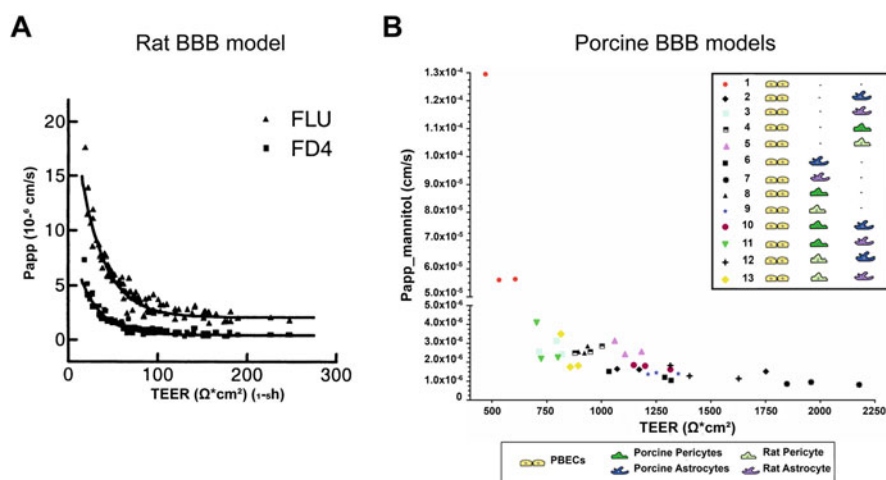


Fig. 2 Correlation of apparent permeability coefficients (P_{app}) and transendothelial electrical resistance (TEER) on in vitro blood-brain barrier (BBB) co-culture models. (a) Primary brain endothelial cells of bovine origin were co-cultured with primary rat astrocytes. In vitro permeability for sodium fluorescein (FLU) and FITC-labeled 4 kDa dextran (FD4) was assessed for 1–5 h (figure reprinted from Gaillard and de Boer (2000)). (b) Primary porcine brain endothelial cells (PBECs) in monoculture or co-cultured with primary porcine or rat pericytes and astrocytes (figure reprinted from Thomsen et al. (2015))

more convenient to employ permeability studies (Bischoff et al. 2016). It was suggested by some studies (Fig. 2) that as a general threshold, a TEER bigger than $150 \Omega \times \text{cm}^2$ should be adopted to ensure independence of P_{app} (Gaillard and De Boer 2000; Neuhaus et al. 2006). Hence, caution should be made when interpreting data obtained from leaky brain capillary endothelial monolayers (Bischoff et al. 2016). Besides this, time is another factor to be taken into account when relating TEER and permeability values, as the TEER method evaluates barrier tightness in real time compared to BBB permeability that evaluates barrier tightness for a given period of time (e.g., 30 or 60 min) (Wegener and Seebach 2014).

5 Conclusion

Over the past 20 years, many efforts were made to develop BBB in vitro models; however, much less attention has been paid to the comparison of data obtained by using these different models. As a consequence, comparison of data such as BBB permeability or transendothelial electrical resistance obtained with different models is cumbersome. Currently, low transferability and incompatibility of outcomes obtained by different in vitro models is the rule.

Tightness of the brain endothelial cell monolayer is one of the most critical BBB feature as it is the main contributor to proper BBB functioning. Methods, such as

BBB permeability tests, to assess barrier tightness in vitro are therefore broadly used. However, current model diversification results in inter-study variation which complicates the comparison of permeability values, as even subtle changes in, for example, cell culture conditions, experimental design, or methodology can result in significantly different outcomes. Because the list of in vitro models is still expanding, it is essential to always be aware of the different factors that potentially influence BBB permeability, especially when comparisons are made with data from other studies. From this aspect, a standardization of culture conditions and methodology would be highly beneficial and would very likely decrease the high prevalence of the current inter-laboratory variation. To compare permeability values, the implementation of a standardized ranking method by using control compounds that reflect a high- and low-permeability compound would be instrumental (Neuhaus et al. 2006). This would make it possible to compare relative permeability data rather than using absolute permeability values.

This chapter, describing the neglected factors relevant to in vitro BBB models, may help in a better understanding and prediction of the in vivo permeability of compounds.

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Human Blood-Brain-Barrier In Vitro Models: Overview and Applications

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Abstract

The human blood-brain-barrier (BBB) is a vital structure for brain health. Conversely it represents a challenge in drug development programmes that require breaching of the barrier in order to access the central nervous system. Very often

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brain disorders have early dysfunction of the BBB implicating an important role in pathogenesis and disease progression. The development of human in vitro models is a major advance to allow experimental studies and screening assays, although there remain outstanding questions for the field. In this chapter, the current state of the art will be reviewed, with the complementary innovative approaches to in vitro modelling described, from simple 2D-cultures to more complex multi-cell type micro-physiological systems.

Keywords

Blood-brain-barrier · Brain capillary endothelial cells · In vitro models · Induced pluripotent stem cells · iPSC · Microfluidics

1 Introduction

The blood vessels that supply the central nervous system (CNS) tightly regulate the movement of molecules and cells between the blood and brain and couple nutrient supply with neural activity (Parkes et al. 2018). A healthy blood-brain-barrier (BBB) and neurovascular coupling is essential for a healthy brain. Breakdown of the BBB is a feature of many neurological disorders ranging from epilepsy to dementia (Sweeney et al. 2019; Profaci et al. 2020). Even where the BBB is not directly involved, it remains an important consideration in drug development, since therapies for CNS disorders need to get to the site of brain pathology. Collectively CNS disorders impose the biggest burden upon health worldwide and cause widespread disability (Collaborators 2019). The development of CNS therapeutics is particularly challenging and for some disorders such as Alzheimer's disease, there remains no meaningful disease modifying therapy. In order to make progress, methods that enable experimentation upon the human BBB are essential.

Drug development requires the determination of blood-brain-barrier (BBB) permeability to predict whether a candidate therapy is likely to achieve appropriate CNS exposure to exert the desired effect (see Chapters “Considerations When Developing Blood-Brain Barrier Crossing Drug Delivery Technology; Brain Distribution of Drugs – Brain Morphology, Delivery Routes and Species Differences; Brain Distribution of Drugs – Pharmacokinetic Considerations; Drug Delivery Strategies to Overcome the Blood-Brain Barrier (BBB)”). Equally for systemic disorders not involving the CNS, drugs must be excluded from the brain to avoid cognitive side effects and neurotoxicity. Whilst in vivo non-human preclinical models can and are used for BBB transport studies, there are significant concerns around trans-species differences in BBB physiology. This is evident at the molecular level, with differing expression of tight junction proteins but also at the functional level, with different transport mechanisms for essential substrates in operation (O’Brown et al. 2018).

The availability of in vitro models permits mechanistic experiments as well as higher throughput screening assays. The advent of induced pluripotent stem cell (iPSC) technology (Takahashi et al. 2007) means that it is now possible to generate

relevant cell types from human subjects including patients, thereby capturing the range of human genetic variation that might affect BBB function and disease predisposition. The hope is that by using human cellular models, drug development has a higher chance of successful translation to an approved efficacious and safe therapy.

2 Key Features of BBB Endothelial Cells and Their Models

The selection of an in vitro model is a balance between physiological relevance (typically more complex models are required to mimic the native state), throughput and cost. Furthermore if the primary purpose is target discovery and validation then this may require a different type of model to one seeking to establish BBB permeability and transport. Nevertheless, an ideal BBB model should encapsulate properties that reflect the roles of the BBB as an effective barrier, mediating transport of substrates that need to reach the brain and as a signalling interface between blood and brain.

The fundamental cellular constituents of the BBB are the brain capillary endothelial cells. Other cell types of the neurovascular unit, such as pericytes and astrocytes, also serve critical roles but essentially the BBB is able to achieve its key functions through regulation of endothelial physiology in the following ways:

1. The suppression of pinocytic vesicles and fenestrae, which prevents transcellular pathways of diffusion (Ben-Zvi et al. 2014; Reese and Karnovsky 1967).
2. Expression of enzymes that metabolise bioactive substances before they can reach the brain (Banks 2016).
3. Polarised expression of membrane transporters ensuring efflux of a variety of small molecule substrates that would otherwise accumulate through passive diffusion. For example, P-gp, encoded by the multidrug resistance gene MDR1/ABCB1, is an energy-dependent efflux pump, predominantly expressed on the luminal membrane, that can engage a structurally diverse set of compounds including antibiotics, antihistamines, antineoplastics, calcium channel blockers and immunosuppressants (Cordon-Cardo et al. 1989; Juliano and Ling 1976).
4. Restricted paracellular flux and high electrical resistance achieved through cell–cell adherens and tight junctions such that the BBB is impermeable to polar molecules other than by a specific transcellular transport pathway. Tight junctions composed of occludin, the claudins and the junctional adhesion molecules (JAMs) are the main determinants of paracellular permeability (Bauer et al. 2014).
5. Mediate transport of substrates that need to reach the brain or to be taken out of the brain through passive diffusion, transport by solute carriers, receptor mediated transcytosis or adsorptive transcytosis. Specific BBB transporters exist for a large range of molecules, including electrolytes, glucose, amino acids, nucleotides and hormones (Hladky and Barrand 2016).

6. Enable bi-directional communication between the blood or brain through secreted signalling molecules, cell–cell contact such as astrocyte-pericyte-endothelial cell contact or matrix interactions (Osada et al. 2011). A critical task, for example, is ensuring appropriate neurovascular coupling between neural activity and blood flow.

Whether a particular in vitro model successfully incorporates these characteristics can be used as quality-control criteria to assess the physiological and disease relevance of the model. For example, permeability can be assessed through the use of reference compounds, or molecular analysis can evaluate the expression of relevant junctional proteins at mRNA and protein level. There also needs to be the appropriate subcellular localisation of junctional proteins and transporters for polarised transport and functional activity of key marker proteins such as the VEGF receptor. In addition to physiological relevance, the model should also be assessed for its reproducibility, scalability and cost. Finally, it must be kept in mind that these properties probably change in disease states and in particular transport mechanisms may be significantly up- or downregulated (see Chapters “The Blood-Brain Barrier in Alzheimer’s Disease; Blood-Brain Barrier Mechanisms in Stroke and Trauma; Brain Barriers and Multiple Sclerosis: Novel Treatment Approaches from a Brain Barriers Perspective; Epilepsy and Alterations of the Blood-Brain Barrier: Cause or Consequence of Epileptic Seizures or Both?; Novel Treatment Approaches for Brain Tumour from a BBB Perspective”).

3 Sourcing Brain Endothelial Cells

3.1 Primary and Immortalised Cultures of Cerebral Endothelial Cells

BBB models at their simplest will be a mono-culture of endothelial like cells. Very often, the aim is to reproduce the BBB at the capillary level, since this is where substance transfer occurs through strictly regulated processes. Brain capillary endothelial cells (BCECs) can be sourced from primary cultures or immortalised cell lines. Primary cultures are highly attractive for BBB models since they retain many of the physiological properties of the BBB including polarised expression of membrane proteins and formation of complex tight junctions (Cecchelli et al. 2007). Bovine and porcine tissues have been preferentially used over rodent because of the much higher yield of cells. However an important advantage of rodent models is the facilitation of in vitro/in vivo correlations, since particularly the rat has been widely used to generate in vivo data (Demeuse et al. 2002; Perriere et al. 2007). Immortalised brain endothelial cell lines such as rat RBE4 cell line or human cell lines such as hCMEC/D3 have been extensively used since they are easy to maintain and can be expanded to large numbers. However, collectively immortalised cell line cultures frequently do not exhibit functional barrier properties, with low TEER value ($<100 \Omega \text{ cm}^2$ (Helms et al. 2016)). Furthermore non-human primary cultures or

immortalised cell lines are likely to be very different to human BCEC and much of the failure of translation of drug discovery programmes has been attributed to the lack of access to human systems early on in the programme. Human primary tissue is available but typically much harder to acquire by research groups. Nevertheless, increasingly neurosurgery departments are recognising the value of access tissue obtained during neurosurgical procedures that can then be a source of human endothelial cells. Whilst these represent highly valuable resources, they cannot support high-throughput studies and there remains uncertainty on the ‘normality’ of tissues derived from epilepsy or tumour patients. Furthermore primary human BCECs de-differentiate in culture and expression of transporters and other physiological properties are unlikely to mimic the in situ state, including barrier tightness.

3.2 IPSC Derived Endothelial Cells

The ability to generate relevant human cell types, using induced pluripotent stem cell technology (iPSC), from healthy subjects or patients with particular conditions undoubtedly led to a revolution in biomedical research and drug discovery (Takahashi et al. 2007). Many different cell types can now be generated and in 2012, an important study identified a seemingly facile method to generate BCECs from iPSC (hereafter termed iBCECs) (Lippmann et al. 2012). The protocol involves a co-differentiation process that generates both neural and PECAM-1+ endothelial cells that appear spontaneously in undirected iPSC culture. Endothelial cells are then purified from the heterogeneous mixture by selective adhesion to collagen IV and fibronectin. The authors evaluated the identity of the differentiated cells by qRT-PCR and immunofluorescence for the expression of endothelial and tight junction markers. They confirmed barrier like properties with TEER measurements of $\sim 800 \Omega \times \text{cm}^2$ after co-culture with astrocytes as well as active efflux transporter expression and representative permeability to a panel of small molecules. Follow-up work to optimise BMEC differentiation methods used media supplemented with retinoic acid, optimised seeding density and defined media composition, and astrocyte and pericyte co-culture. These optimisations yielded cells with increased expression of VE-cadherin and occludin and TEER values to $\sim 6,000 \Omega \times \text{cm}^2$ and stable for up to 2 weeks (Lippmann et al. 2014; Hollmann et al. 2017). The reproducibility of the differentiation procedure has been subsequently confirmed in multiple other studies (Lim et al. 2017; Katt et al. 2019; Appelt-Menzel et al. 2017; Kokubu et al. 2017), although as discussed below there remains uncertainty as to whether the produced cells have a fully endothelial identity (see below).

Such iPSC derived cells can offer several advantages over primary or immortalised cell lines. Firstly, iPSCs are obtained from human subjects and therefore harbour the genetic material of the individual. This importantly means that the genetic component of disorders that the individual may suffer is also present in the iPSC and all derived cell types. Any environmentally induced epigenetic changes in the starting somatic cells is largely wiped clean (Rouhani et al. 2014). Hence iPSC models offer an extremely powerful platform to assess genetic causation without the

confounds of environmental influences. Nevertheless this does not preclude evaluation of specific gene–environment interactions and hypotheses, since cell can be exposed to a disease relevant perturbation and the resulting phenotypes assayed. In the case of monogenic disorders where a single deleterious gene mutation can cause disease, the causal role of the mutation in the observed phenotype can be directly addressed through genome engineering. Thus using CRISPR/Cas9, disease associated genetic mutations can be introduced or corrected to understand if the cellular phenotype is a direct consequence of the mutation. Secondly, iPSCs can be expanded to generate a potentially limitless amount of starting material that can then generate sufficient differentiated cells for high-throughput assays and screens. The cost can be significant but this is very dependent on the differentiation protocol method used and in particular the growth factors and specific media formulations required. Not surprisingly, given the growing availability of iPSC lines and methods, BBB in vitro models now routinely employ iPSC-BCECs (Appelt-Menzel et al. 2020).

4 The Importance of the BBB Niche

Whilst in its essence the BBB is a tightly sealed monolayer of BCECs, it nevertheless exists as a complex niche with several other cell types and the extracellular matrix (ECM) including the basement membrane that act in concert to produce a mature barrier. BBB in vitro models have therefore tried to recapitulate the niche by incorporating these additional cell types as well engineering extracellular matrix scaffolds in which BBB cells are seeded.

4.1 Astrocytes and Pericytes

Co-culture is frequently employed to improve in vitro cellular models since they can enhance maturity and physiological functions. Astrocytes, which through end-feet cover almost all of the cerebrovasculature (Mathiisen et al. 2010), can induce and maintain brain endothelial properties (Abbott et al. 2006). Pericytes share a basement membrane, cover a large part of the abluminal surface and make direct contact with BECs via peg and socket as well as gap junctions (Dore-Duffy and Cleary 2011). Pericytes may have a key role in reducing pinocytic vesicle formation (Daneman et al. 2010). In vitro, both astrocytes and pericytes can enhance barrier properties and expression of transporters (Daneman et al. 2010; Nakagawa et al. 2007; Dohgu et al. 2005), such as P-glycoprotein (P-gp) and GLUT1, as well as expression of metabolic enzymes. Since the BBB niche may be maintained through direct cell contact or secreted factors, in vitro models can attempt to reproduce the niche through the use of astrocyte-conditioned media or specific extrinsic factors such as cAMP stimulants or glucocorticoids. However since astrocytes and pericytes can be produced from iPSC lines using now well-established, validated and robust protocols, it may be preferable to directly co-culture with iBCECs. Indeed some

studies have generated isogenic models where different cells of the BBB are all produced from the same donor iPSC lines (Canfield et al. 2017). Equally the cell-specific pathogenic effects can be investigated by co-culturing different combinations of control and disease cells (Kelleher et al. 2019).

4.2 Extracellular Matrix

The extracellular matrix (ECM) or matrisome of cerebral blood vessels, secreted by cells of the BBB, is an often overlooked but key component of the neurovascular unit. It comprises highly cross-linked core extracellular matrix proteins such as fibronectin, laminin and collagen triple helix repeat as well as a variety of associated proteins, remodelling enzymes, cytokines and soluble factors (Novak and Kaye 2000). The composition of the human neurovascular matrisome has been recently characterised (Pokhilko et al. 2021). The basement membrane (BM) is a 50–100 nm thick, specialised form of ECM, consisting of laminin, fibronectin and collagen type IV (Wong et al. 2013). It covers the basal side of endothelial cells, includes the pericytes and is the interface with astrocytic end-feet.

The ECM is critical to the proper functioning of the BBB as it acts as a biomechanical scaffold, regulates BBB receptor activation involved in cell–cell signalling and regulates transport (Novak and Kaye 2000). Consideration of the ECM has been incorporated into models of the in vitro BBB through the culture of BCECs on self-polymerising scaffolds (Helms et al. 2016; Hartmann et al. 2007; Zobel et al. 2016). Furthermore gradients of trophic factors can be incorporated into the scaffolds in an attempt to reproduce some of the physiological features of the neurovascular unit niche. When endothelial cells are grown in these types of scaffolds they have been reported to display enhanced barrier function (Zobel et al. 2016; Campisi et al. 2018).

5 Types of In Vitro BBB Models

5.1 Transwell Models

The commonest type of model for barrier and permeability studies involves a two-chamber setup. BCECs are cultured in one chamber as an adherent monolayer upon a porous membrane in a Transwell insert, whilst other cell types can be cultured on the insert under-surface or in the lower chamber. The material, pore size and pore density of the membrane can be varied. This enables the relatively straightforward assaying of permeability, trans-endothelial electrical resistance (TEER), cell migration or measurement of secreted factors. Although easy to setup and even scalable for high-throughput assays, major limitations include the 2-D structure and absence of exposure to shear stress (Cucullo et al. 2011; Vigh et al. 2021). There can also be areas of the culture that may be more permeable, for example near edges (Hudecz

et al. 2014). Furthermore the porous membrane is a poor replica of the *in vivo* basement membrane and typically 100-fold thicker.

5.2 Dynamic Models

The development of dynamic models enables exposure of cultured BCECs to shear stress, which then seem to develop more physiological BBB properties than those observed with static platforms. However, flow-induced elongation seems absent in human BCECs in comparison with other EC, which instead retain their cobblestone morphology, perhaps through upregulation of ZO-1 expression. This may be important for BCECs to retain their barrier properties (Reinitz et al. 2015). Nevertheless early studies indicated other properties to be altered in response to fluid flow and shear stress including enhanced cell metabolism, higher TEER, cell polarisation and expression of specialised transporters and efflux systems (Colgan et al. 2007). However, the effects on human BCECs, grown *in vitro*, appear less prominent (DeStefano et al. 2017).

One approach to incorporate flow is through the use of artificial hollow fibre constructs. These attempt to recapitulate microvessels, engineered from thermoplastic polymers (Neuhaus et al. 2006). Cells are seeded and grow to mimic the spatial and topographical distribution of microvessels, whilst a pulsatile pump generates flow. Hollow fibre constructs have luminal and abluminal compartments that are accessible for transport studies and allow TEER measurements. However such models do not easily allow monitoring of cells using microscopy and the large diameter may not be representative of brain capillary beds as well as requiring a relatively large quantity of reagents and cells.

Smaller scale dynamic models have been engineered by moulding channels in elastomeric material by soft lithography (Duffy et al. 1998). Cells are cultured in the channels under flow through a pump or by gravity. The models can be visualised with microscopy, including with immuno-fluorescent staining, to enable assessment of cell morphology and cell–cell interactions. Systems can also be integrated with electrodes for TEER measurements (Booth and Kim 2012) and a variety of models have been produced using different microfluidic designs, incorporating different online biosensors for functional assays (Oddo et al. 2019). A limitation of this setup is that shear stress in microfluidic chips is typically lower than the physiological range of 5–25 dyne/cm² (Koutsiaris et al. 2007; Cucullo et al. 2013). An advantage of these types of models, which can be established as multi-well plates and integrated with high content imaging methods, is their potential for high-throughput drug screening efforts.

5.3 Organoid-Like Models

The concept of BBB organoids is that BBB constituents are cultured under low adherence which then leads to self-assembly into a multi-cellular structure, such that

cell–cell interactions help to recapitulate the *in vivo* state. The first example used a hanging-drop method whereby a mixed cell suspension of human primary brain endothelial cells, pericytes and astrocytes spontaneously form spheroid structures (Urich et al. 2013). Brain endothelial cells cover the outside of the spheroid as a shell by directly contacting pericytes and astrocytes positioned in the core region. Spheroids have been assembled in a staged process with up to six cell types including oligodendrocytes and microglia (Nzou et al. 2018). Capillary like networks with lumen and surrounded by basement membrane can also form in spheroid models prepared from rat or mouse cortical tissue although the structures were transient (Boutin et al. 2018). Similar capillary network formation using human brain endothelial cells has yet to be shown. Hence, in general, whilst these models are excellent for capturing cell–cell interactions, typically they do not incorporate fluid flow and developing BBB relevant transport assays can be challenging. Nevertheless, the utility of a spheroid model in small scale screening for BBB penetration of peptides has been demonstrated (Cho et al. 2017). Furthermore, recently a novel microfluidic spheroid array platform has been developed to study BBB transport with standardisation of spheroids (Eilenberger et al. 2021).

5.4 Extracellular Matrix-Based 3D BBB Models

An engineered three-dimensional ECM scaffold has the potential to reproduce the mechanical properties of the brain using either synthetic or natural materials. Synthetic ECMs can be reproducibly engineered for the desired density and stiffness and has been applied for *in vitro* cultures (Veiga et al. 2011; Barry et al. 2017). On the other hand, natural materials have the advantage of inherent bioactivity to support cell culture. Type I collagens are widely used due to good mechanical performance (Ferro et al. 2020). Matrigel, rich in laminin and Type IV collagen, is also commonly used but there can be substantial batch to batch variation in composition which can then impact on reproducibility (Hughes et al. 2010). Research groups have also attempted to combine different ECM components to better mimic the human vascular ECM, but it should also be noted that cultured cells will also secrete proteins and factors to contribute to the ECM and basement membrane.

With the space for 3D interactions between cells that are exposed to brain relevant ECM proteins, ECM-3D-BBB models are a plausible means of producing an *in vivo* like state. This has been evident from the ability of endothelial cells to form perfusable vascular networks when seeded onto or into ECMs that can be further stabilised with the addition of supporting cells such as pericytes (Campisi et al. 2018). Alternative to self-organised capillary networks, ECM models can be combined with microfluidics, with channels providing support for the ECM (Kim et al. 2013) and control over the geometry of the vascular network. A number of methods have been used to form the microfluidic channels and there are several examples of this approach being applied to multi-cell type cultures (Adriani et al. 2017; Partyka et al. 2017).

6 Applications of BBB Models

6.1 Disease Mechanism and Target Discovery

Recent genomic and human tissue molecular studies of brain disorders have identified numerous genes and proteins that are altered in the cerebrovasculature. Furthermore research is increasingly revealing complex mechanisms that link the cerebral vasculature to various CNS pathologies. The challenge is often distinguishing disease-related changes that are important for disease development and progression from changes that are inconsequential or indeed protective changes. Human cellular models derived from patient iPSC lines can now serve as a tool for target discovery, particularly the early genetic causal stages of disease without the encumbrance of non-genetic and confounding effects that are unavoidable in human post-mortem tissue.

The ability to understand genotype–phenotype relationships is a key strength of iPSC models as has been repeatedly shown across a wide range of disorders. An early example of the use of iPSC-BBB models was in the study of Allan-Herndon-Dudley syndrome (AHDS) – a rare disorder of psychomotor retardation, caused by inactivating mutations in the SLC16A2 gene (Dumitrescu et al. 2004; Friesema et al. 2004). SLC16A2 encodes the monocarboxylate transporter 8 (MCT8), a transmembrane transporter for thyroid hormones. AHDS iPSC derived BMECs exhibited thyroid hormone transport deficiency from the apical to basolateral direction (Vatine et al. 2017). The BBB model is also an exemplar of the power and utility of human iPSC since rodents express an additional thyroid transporter, *Oatp1c1*, and therefore a rodent based in vitro BBB model would not have been suitable to study pathogenic mechanisms of this disorder. A mutation in MCT8 would be expected to impact BBB transport but iPSC models can also be used to discover unexpected potential disease mechanisms. This was exemplified in iPSC derived BCECS from patients with Huntington's disease (HD); a disorder caused by pathogenic CAG repeat expansions in the huntingtin gene (Lim et al. 2017). In this study, the in vitro BBB model had defects in barrier formation with reduced TEER and increased transcytosis, which interestingly was correlated with the CAG repeat length. The detailed mechanistic linkage between CAG repeat length and disrupted barrier needs to be elucidated but it was proposed that it was due to endothelial cells becoming adopting an angiogenic state (Lim et al. 2017).

Two studies have investigated pathogenic mechanisms in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). CADASIL is one of the most common forms of hereditary cerebral small vessel diseases and is caused by mutations in NOTCH3 (Joutel et al. 1996). Mural cells differentiated from iPSC of three patients with CADASIL remarkably recapitulated pathogenic changes including increased PDGFR β , an abnormal filamentous actin network and N3ECD/LTBP-1/HtrA1-immunopositive deposits – a histopathological hallmark of this condition (Yamamoto et al. 2020). In separate second study iPSCs were generated from 2 CADASIL patients and 2 health controls and differentiated into either peripheral endothelial cells or brain mural cells

(Kelleher et al. 2019). The authors found that control but not CADASIL mural cells were able to stabilise an endothelial cell tubular network perhaps due to a deficiency in secretion of VEGF. CADASIL mural cell also demonstrated increased sensitivity to apoptotic challenge.

Genome engineering in iPSC models provides a powerful means to test and validate genetic hypotheses of disease mechanisms by creation of isogenic control or disease mutation carrying lines. As an example, a study employed lines with the PSEN1 $\Delta E9$ mutation that is a cause of familial Alzheimer's disease (FAD). Brain endothelial cells differentiated from three FAD iPSC lines carrying the PSEN1 mutation, two isogenic control iPSC lines where the PSEN1 mutation had been corrected, and one unrelated control iPSC line (Oikari et al. 2020). Overall the barrier properties of the BBB model were reduced as indicated by a lower TEER. However the molecular basis of this change remains unclear with expression of claudin-5 being increased in the disease mutation lines. This model was also used to highlight the potential of in vitro models to study methods to enhance CNS drug delivery, with the authors applying focused ultrasound and showing increased permeability of the barrier (Oikari et al. 2020).

Human in vitro BBB models have also been used to study host–pathogen interactions. *Neisseria meningitidis* is a bacteria that can cause meningococcal meningitis, which is a severe illness that has high mortality rates and frequent neurological long-term complications. The bacteria is internalised by endothelial cells through interaction with CD147 and beta2-adrenergic receptors but the effects on human brain endothelium remain to be fully characterised. In an iPSC-BBB model, exposure of BCECs to the bacteria led to increased expression of pro-inflammatory chemokines and disruption of tight junction proteins (Martins Gomes et al. 2019). This was associated with a significant reduction in TEER and increased sodium fluorescein permeability, potentially enabling paracellular transmigration of the pathogen (Martins Gomes et al. 2019).

6.2 Toxicology

Neurotoxicity of compounds in a drug discovery programme is a critical consideration and iPSC-BBB models provide a potentially means for early assessment in a human system. This could allow triaging of compounds to avoid later stage and more expensive failure of a drug candidate. The interaction of a compound with brain endothelial cells can be studied mechanistically to identify how it might cross the membrane, whether it is metabolised, if it is actively exported or if it interferes with endogenous transporter systems. As an example, the effects on BCEC stress responses upon repeated dosing of cyclosporine were recently examined using an iPSC-BBB model using a transcriptomic analysis (Wellens et al. 2021). Alterations in BBB permeability may itself be an important toxicity readout and in organ on a chip models, the penetration of a compound to particular cell types could also be evaluated. In order for iPSC-BBB models to be adopted, the assay protocols need to be validated and standardised for reproducibility and to satisfy medicines regulators.

6.3 BBB Barrier and Transport Function

To achieve a desired CNS effect, delivery of a minimum amount of drug into the brain is required since most therapies reversibly interfere with a specific aspect of cell function in a concentration-dependent manner. Hence the parameters that determine CNS exposure include a drug's permeability across the BBB, the pharmacodynamic properties of the drug and the pharmacokinetics of free drug concentrations in blood and brain (Rankovic 2015). For small molecule drugs, the simplest strategy is to improve passive diffusion through optimising lipophilic-hydrophilic balance and to reduce affinity to efflux membrane transporters. Biologicals such as peptides, antibodies and other large molecule are excluded and therefore require an active transport mechanism to enable entry and sufficient CNS exposure. The factors affecting CNS penetration are complex and iPSC based BBB models offer a platform to assess interactions between the therapy and endothelial cells including transcytosis, sequestration or non-specific interactions.

Disruption of the BBB *in vitro* can be measured by evaluating TEER and permeability of inert tracers across the monolayer (Srinivasan et al. 2015). TEER is mainly influenced by tight junction protein integrity and can be measured using a voltohmmeter or through impedance spectroscopy. *In vivo*, the BBB has been estimated to have very high TEER of up to $8,000 \Omega \text{ cm}^2$ (Wolff et al. 2015) although the TEER in the human BBB is unknown. Perhaps more critical than the absolute value of TEER is the relation between TEER and permeability since permeability of inert tracers appears to be largely minimised at TEER greater than $500\text{--}600 \Omega \text{ cm}^2$ (Mantle et al. 2016). Permeability measurements allow an assessment of the size of the molecule excluded by the BBB. Assays utilise inert tracers of varying molecular weights that are fluorescent or radiolabelled applied to the luminal chamber and then measuring the amount in abluminal side, conventionally expressed as the permeability coefficient (P_e). Whilst TEER depends mostly on the path with lowest resistance in the monolayer, permeability is the sum of the transport across all pathways (Erickson et al. 2020). An important strength of iPSC-BBB models is that the TEER and permeability coefficients achieved are close to that measured *in vivo*, although as noted this is presumed in the case of human BBB TEER. Practically the iPSC-BBB model with high TEER provides a more suitable assay starting point to evaluate drug transport properties.

The relatively straightforward means to assay barrier function allows iPSC-BBB models to address fundamental and disease-related barrier mechanisms. For example, iron transport into the brain is highly regulated as iron is essential for brain function but excess iron is toxic. Using an iPSC-BBB model, iron transport by brain endothelial cells was shown to be regulated by several mechanisms including ferroportin and DMT1 (Chiou et al. 2019). These mechanisms in turn may be potentially regulated by the brain parenchyma or endothelial cells depending upon the iron status of the brain. Another interesting study examined the involvement of the neonatal Fc receptor (FcRn) in antibody transcytosis in iBECs (Ruano-Salguero and Lee 2020). After showing that human IgG becomes endocytosed and targeted to the lysosome, the permeability was confirmed as very low, as was expected for a

macromolecule. Indeed the permeability of all tested IgGs was comparable and remained constant irrespective of concentration or competition with excess IgG, suggesting non-specific non-receptor mediated transcytosis.

As already described above, studies have started to examine how disease might affect barrier properties. A further study examined paracellular and transcellular transport across 8 iPSC lines from four neurodegenerative conditions (Alzheimer's, Parkinson's, Huntington's and Motor Neuron Disease) compared to controls using iBECs (Katt et al. 2019). This showed that four of eight disease lines had a decrease in TEER although since TEER remained over 500 Ω s, there were no large changes in measured small molecular paracellular permeability. Seven of eight disease lines showed evidence for reduced P-gp mediated efflux, which might indicate a common stress response. These types of studies are important for highlight that strategies for achieving optimal brain delivery need to take into account changes in transport processes due to disease. However given the number of lines per disease was small, it is difficult to confirm the changes are disease related rather than some other confounding factor.

7 Limitations and Challenges for Current Brain Capillary Endothelial Cell Differentiation Protocols

The protocol initially developed by Lippman and colleagues and subsequently modified to improve the resulting model properties has been widely adopted by many research groups, underpinning most of the studies described above. Other research groups have however found the protocol difficult to reproduce. However the successful implementation of the protocol likely requires each individual lab to test a range of cell seeding densities and evaluate specific media formulations (Kokubu et al. 2017; Rouhani et al. 2014). When the protocol is successful, the cells generated exhibit very high TEER measurements and low paracellular permeability – and can therefore serve well assays on transcellular transport.

A more persistent question by the field has been whether these protocols generate 'true' brain capillary endothelial cells or whether the resulting cells have a more epithelial molecular identity. The challenge at present however is that there is no well-defined benchmark for 'true' brain endothelial cells since isolation protocols are very likely to substantially change molecular and functional properties. Transcriptomic analysis of iPSC-BCECs using the Lippman protocol revealed relatively low mRNA expression of key endothelial genes but upregulation of claudins found in epithelial cells (Delsing et al. 2018). This finding may indicate that the cells have an epithelial cell trajectory, that they have a mixed identity or even that human BCEC express proteins traditionally considered epithelial as a mechanism to achieve strong barrier properties. In an attempt to address these issues, a recent study adopted an alternative approach to produce iBCECs through expression of three transcription factors (ETV2, ERG and FLI1) considered important for determining brain endothelial cell fate (Lu et al. 2021). Transcriptomic profiling confirmed an endothelial identity and a number of functional assays also validated an

endothelial phenotype – including response to TNF- α and VEGF and in vivo vascular tubule formation. Other transcriptional networks may need to be activated to achieve full brain capillary endothelial cell functionality as suggested by another study over-expressing transcription factors. In this case, over-expression of SOX18, TAL1, SOX7 and ETS1 increased tight junction expression, TEER and reduced permeability (Roudnicky et al. 2020). However it should be noted that in both cases, the TEER values are very low and therefore these models are not suitable for BBB transport studies.

8 Conclusions

Stem cell technology offers incredible opportunities to access human cells and understand physiological and pathophysiological processes. For tissues as important as the blood-brain-barrier, this application of iPSC has become exponentially growing area for research with studies examining developmental programmes, fundamental transporter mechanisms and disease biology. There remain further challenges and in particular refinement or completely new approaches may be required to establish brain endothelial cell models with both endothelial molecular signatures and brain capillary vascular functional responses. Strategies that are starting to be adopted include over-expression of transcription factors that might drive iPSC or progenitors to the correct cell type. At present it seems multiple transcription factor expression is necessary and indeed successive application of different transcription factors may be required. However efficient transcription factor delivery to iPSC can be challenging and may limit the ability to evaluate multiple disease iPSC lines. Hence the development of small molecule based differentiation methods remains important in order to enable conversion of different patient iPSC lines. As research progresses on signalling cascades involved in patterning towards human brain vasculature, the small molecule protocols to generate iPSC-BCEC will improve. This will then help support academic and industry research to identify novel transport mechanisms for CNS therapeutic delivery as well as reveal novel drug targets for neurological disorders.

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In Vivo Studies of Drug BBB Transport: Translational Challenges and the Role of Brain Imaging

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Abstract

Drug delivery to the brain is challenging to study due to the complexity of the barriers of the central nervous system (CNS). The present chapter describes and compares experimental methods such as microdialysis, two-photon laser scanning fluorescence microscopy and positron emission tomography (PET) that can be used for in vivo studies of drug transport across the blood–brain barrier (BBB). The selection of appropriate method is based on the research question, and the

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different methods will in most cases provide complementary information. Attention is also given to the fact that the BBB might undergo changes in integrity, protein expression and other morphological alterations as a result of disease. The use of animal models of human disease is therefore also discussed. Special emphasis is given to translational aspects of the different methods and readouts.

Keywords

Brain barriers · In vivo studies · Molecular imaging · Positron emission tomography (PET) · Microdialysis · Translation

1 Introduction to In Vivo Assessment of Drug Transport Across the BBB

Although highly advanced in vitro models of the blood–brain barrier (BBB) have been introduced during the last decade, the full complexity of the BBB is difficult to reproduce in vitro. Thus, as a complement to studies in cell systems, drug delivery across the BBB and into the brain parenchyma must often be investigated in vivo in preclinical studies to adequately predict drug delivery to the human brain. Further, the shift from small molecular drugs to larger biologics, not only for peripheral disease targets but also for central disease, requires increasingly refined experimental settings. This is because these drugs are characterised by lower and slower transport across the BBB compared with small molecular drugs. In addition, brain delivery of larger biologics is often dependent on various specific and unspecific modes of transport, e.g. receptor-mediated transcytosis (RMT) or adsorptive-mediated transcytosis (AMT), rather than passive transport across the BBB or paracellular transport. More attention should also be given to the fact that the BBB might undergo changes in integrity, protein expression, and other morphological alterations as a result of disease. Thus, preclinical in vitro and in vivo studies of drug delivery to the brain should be conducted in models taking into account these disease-related alterations. For this, the numerous genetically and chemically modified animal models have been vital.

Compared to other organs, the brain is more challenging to study, as biosamples are difficult to obtain. Hence, advanced invasive and non-invasive methods to study drug concentration in the brain have been developed and are increasingly utilised. Different modalities of brain imaging also enable translational studies, as these methods can often be conducted according to the same protocols in preclinical and in clinical settings.

The present chapter will focus on in vivo techniques for studying drug delivery to the brain and on the different types of translational in vivo models used preclinically.

2 Methods to Study BBB Drug Transport In Vivo

2.1 Brain Microdialysis

Brain microdialysis is the “gold standard” for brain drug delivery studies (Hammarlund-Udenaes 2000, 2017; Sawchuk and Elmquist 2000; Chaurasia et al. 2007). The main advantages of microdialysis include the possibility to obtain quantitative and time-resolved measures of drug transport to the brain in a limited number of animals. The technique has in a profound way contributed significantly to the understanding of BBB transport and role of efflux and influx transporters in vivo (Hammarlund-Udenaes 2000, 2016, 2017). The unique feature of microdialysis is that it directly measures the unbound tissue (and plasma) concentrations at the site of probe placement, thereby “purifying” the measurements and separating bound drug in plasma and tissue that otherwise disguise the information about transport properties. However, it also has methodological challenges limiting its use (de Lange et al. 2000; Chaurasia et al. 2007; Hammarlund-Udenaes 2017).

The microdialysis probe is placed in a specific brain region of interest. For studies of BBB transport of drugs, it is possible to place a probe also in vena jugularis for direct sampling of unbound plasma concentrations. Alternatively, regular plasma samples are taken followed by compensation for protein binding to obtain estimates of unbound plasma concentrations. This gives a direct relationship of concentrations across the BBB, resulting in mapping the $K_{p,uu,brain}$ relationship (see chapter “Brain Distribution of Drugs: Pharmacokinetic Considerations”). It is also possible to sample from CSF to determine the blood–CSF barrier (BCSFB) potential of drug efflux or influx (Yamamoto et al. 2017a).

An important issue to be considered in all microdialysis studies is the in vivo recovery across the probe, as the dialysate will contain less than 100% of the unbound tissue concentration surrounding the probe due to incomplete equilibrium across the probe membrane. The in vivo recovery will most likely differ from the in vitro recovery measured in a buffer, as the tissue transport properties profoundly influence the movement of drug molecules into the probe. The equilibration across the membrane is affected by the size of the membrane itself, the flow rate of the perfusate/dialysate, as well as tissue transport properties of the drug. The lower the flow rate, the higher the recovery, with common flow rates of 0.5–1 $\mu\text{L}/\text{min}$. Thus, there is a trade-off between the length of the sampling periods and the concentration obtained for analysis in the dialysate. Drugs that have more rapid movement in the tissue, like those that are actively effluxed at the BBB, will obtain higher recovery than those that are slowly diffusing. The microdialysis technique has been further developed with the MetaQuant probe to address these issues. It combines a very low flow through the tissue, thereby increasing recovery and certainty in estimation of tissue concentrations, with an added flow to improve sampling volume and speed between sampling time at the sampling site and at the sampling test tube on the other side of the tubing (Bunk et al. 2006; Cremers et al. 2009; Sood et al. 2009).

Push-pull probes with high cut-off membranes for peptides and proteins have also been developed, as the regular probes in general exclude molecules larger than 20 kDa. The first such probe was the Open Flow Microperfusion with a mesh instead

of a membrane (Birngruber et al. 2013, 2014; Birngruber and Sinner 2016). Other probes with high cut-off membranes have followed (Hillman et al. 2006; Chu et al. 2014; Hillered et al. 2014; Yamada 2018).

The main caveat associated with microdialysis is adsorption of drugs to tubings and probe membranes, thereby impeding the ability of the technique to correctly quantify tissue concentrations. This is especially an issue to consider for larger molecules that are prone to stick to plastics. It is therefore crucial to test adsorption to tubings and membranes *in vitro* before *in vivo* studies are initiated, if quantitative results are desired (Chaurasia et al. 2007). The choice of tubing material may be influential in this regard.

2.2 Molecular Brain Imaging

Molecular imaging methods include positron emission tomography (PET), single-photon emission computed tomography (SPECT), magnetic resonance (MR), optical imaging and molecular ultrasound. They differ from other diagnostic imaging modalities such as X-rays, computed tomography (CT) and ultrasound, which mainly provide anatomical information, while molecular imaging methods enable *in vivo* studies of chemical and biological processes.

In vivo brain imaging is an attractive approach due to the non-invasiveness of the methods that also allows them to be used in higher species including humans, and thus enabling translation. This section will focus on PET.

PET relies on the administration of radiolabelled drug-like molecules, i.e. radioligands. The radioligand is usually injected intravenously to the patient (or animal in preclinical PET studies) and will subsequently bind to its target. The target can be a receptor, enzyme, transporter, etc., and hence, the amount of detected radioactivity reflects the amount or function of the target. PET scanning is dynamic, which leads to time-resolved quantitative PET images that are based on the spatial distribution of radioactivity. In addition, due to its non-invasiveness, repeated PET scans can be obtained in the same subject allowing for drug intervention studies or longitudinal studies, for example with the aim to follow changes at the target site as a consequence of drug treatment or during progression of disease.

Many regions of interests can be defined in the PET images, allowing for the assessment of region specific pharmacokinetics. Application of appropriate pharmacokinetic models to the dynamic time-activity data allows for the estimation of semi-quantitative and quantitative biological parameters (Table 1). The reader is referred to the consensus paper by Innis et al. (2007) for further in-depth reading on PET pharmacokinetic concepts and the derivation of the different parameters (Innis et al. 2007).

The most frequently used PET based investigation involves the search and characterisation of tumours. For example, fluorine-18 radiolabelled glucose analogue [^{18}F]fluorodeoxyglucose, [^{18}F]FDG, a marker for glucose metabolism, is taken up by viable tumour cells to a larger extent than by healthy normal cells. Thus, even small metastases can be pinpointed by [^{18}F]FDG PET. The brain is

Table 1 Relationship between PET parameters and standard pharmacokinetic nomenclature

PET parameter	Description	Relation to standard field of pharmacology
SUV	Standardised uptake value: Tissue (radioactivity) concentration normalised for injected (radioactivity) dose per body weight	Not used
%ID/g _{tissue}	Tissue (radioactivity) concentration normalised for injected (radioactivity) dose	Same
V_T	Equilibrium partition coefficient; total brain-to-plasma concentration ratio	K_p
V_{ND}	Non-displaceable equilibrium partition coefficient; total brain-to-plasma ratio when no specific binding exists	K_p in a region devoid of specific binding
K_1 ($\text{mL min}^{-1} \text{cm}^{-3}$)	Rate constant for drug transfer from arterial blood to tissue	PS (permeability surface product), CL_{in} (net influx clearance), or K_{in} (rate constant)
k_2	Rate constant for drug transfer from tissue to plasma	CL_{out} (net outflux clearance); K_{out} (rate constant)
$\frac{f_{ND}}{f_p} \frac{C_{ND}}{C_p}$	Equilibrium partition coefficient: Ratio of unbound brain and unbound plasma concentration. The total brain concentration (C_{ND}) can be obtained from PET, while the total plasma concentration (C_p) is often obtained by blood sampling	Similar to $K_{p,uu}$, but also include non-specific binding that cannot be displaced
f_{ND}	Non-specifically bound radioligand fraction in brain tissue	Similar to $f_{u,brain}$, but also include non-specific binding that cannot be displaced
f_p	Unbound radioligand fraction in plasma	$f_{u,plasma}$

another organ with high glucose consumption and hence, [^{18}F]FDG can be used to study brain physiology and function in health and disease. Several neurodegenerative diseases are characterised by a decrease in glucose consumption and therefore also a decrease in [^{18}F]FDG accumulation. For example, patients suffering from Alzheimer's disease display decreased [^{18}F]FDG uptake especially in temporoparietal areas of the brain (Kadir et al. 2012).

In CNS drug development, PET has mainly been used to study target occupancy (Gunn and Rabiner 2017; Heurling et al. 2017). In this case, the radioligand and the drug candidates are usually separate entities binding to the same target, e.g. a receptor. A typical study includes a baseline scan with a radioligand at trace doses to quantitatively estimate the receptor availability. A subsequent scan, with the same radioligand, is then performed after administration of the candidate drug. The second scan is compared with the first scan to measure the fraction of receptors occupied by the candidate drug (Fig. 1). If several doses are used, a dose-occupancy relationship

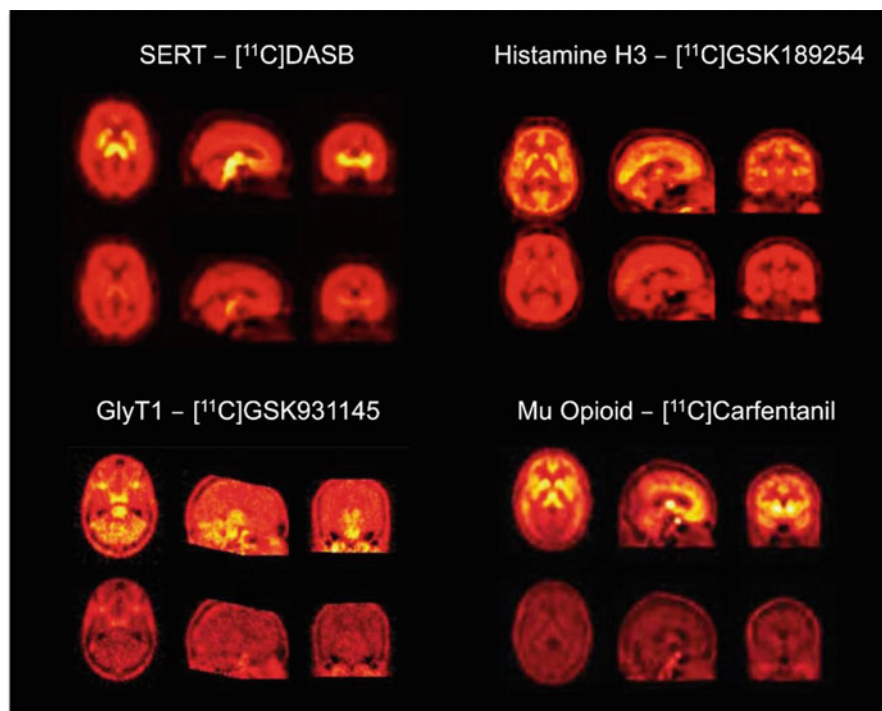


Fig. 1 PET target engagement studies of four different intra-brain targets (serotonin reuptake transporter (SERT), histamine H3 receptor, glycine transporter 1 (GlyT1) and Mu opioid receptor). Top rows illustrate baseline scans when all receptors/transporters are available for radioligand binding. Bottom rows illustrate the same subject imaged after the administration of a drug candidate that binds to the receptor/transporter of interest. Reduction in the overall signal and heterogeneity confirms that the candidate drug crosses the BBB and binds to the desired intra-brain target. Figure obtained from Gunn and Rabiner (2017) with permission from the publisher

can be obtained. In addition, several PET scans can be performed at different time points after a single dose of the candidate drug to measure the brain pharmacokinetics at the target (and off-target) site (Abanades et al. 2011; Heurling et al. 2017). In combination with measurement of drug concentrations in plasma, valuable information regarding the dose, plasma concentration and target site occupancy can be gained. These types of studies are often performed prior to larger clinical studies of CNS drug efficacy.

Studies of drug transport across the BBB using PET can be divided into two main categories. The first category utilises a labelled drug, i.e. the drug candidate under investigation, as the radioligand to understand its transport into the brain and elimination from the brain. The other category of studies use known transporter substrates that have been radiolabelled in combination with the candidate drug under investigation, e.g. a candidate drug whose ability to interact with the BBB transporter is examined. Although several CNS active drugs have been labelled with the

purpose to study their brain distribution and interaction with BBB transporters (Verbeek et al. 2012; Gustafsson et al. 2017), the second category is more common since these studies do not require radiolabelling of the studied drug and thus allows for a more high-throughput protocol that enables the investigation of several compounds in combination with the same transporter-specific radioligand.

The most studied BBB efflux transporter with PET is P-glycoprotein (P-gp). A frequently used radioligand for these types of studies is carbon-11 radiolabelled verapamil, i.e. [^{11}C]verapamil (Hendrikse et al. 1998). Under normal conditions [^{11}C]verapamil is distributed into the brain to a very low extent due to P-gp efflux. However, if P-gp function is inhibited, the brain concentration of [^{11}C]verapamil increases dramatically, up to ten-fold after administration of potent P-gp inhibitors cyclosporine A or tariquidar (Hendrikse et al. 1998; Syvanen et al. 2006). Several other radioligands have also been introduced for studies of P-gp, e.g. [^{11}C]loperamide (Kreisl et al. 2010), as well as radioligands for other efflux and influx transporters (Auvity et al. 2017).

Investigated drugs, suspected to interact with P-gp, can thus be studied in combination with the PET radioligand [^{11}C]verapamil (or other transporter radioligands). The studied drug can either be administered prior to the PET scan or during the PET scan. The later study protocol allows for the investigation of the complete inhibition process (Syvanen et al. 2006). In this setting, the radioligand can be administered as a constant infusion, or bolus followed by infusion, to enable direct estimation of the pharmacokinetic parameter of interest, K_p , from the steady state concentrations in brain and blood. K_p before and after the drug intervention can be compared. K_p can also be obtained by mathematical modelling of the PET data. If a single-tissue compartment model is used, K_p is the ratio of rate constants K_1 (transfer from blood to brain) and k_2 (transport from brain to blood), see also Table 1.

When using PET as a method to study in vivo drug transport across the BBB, it is important to keep in mind that the measurement is based on total radioactivity, and thus, if the radioactivity originates from a drug metabolite and not the parent compound, the method will not be able to separate the signal from the intact parent compound and the signal from the metabolite. Thus, the position of the radioactive label must be chosen carefully so that only hydrophilic and rapidly eliminated radiometabolites are produced. Hydrophilic metabolites are also less likely to enter the lipophilic environment of the brain and thereby contribute to the measured brain radioactivity and that must be corrected for. Another inherent feature of brain PET imaging is that radioactivity in the brain vasculature will contribute to the signal in the brain region. This signal may be separated from the “true” tissue signal by parallel blood sample and the assumption that 3–5% of the brain consists of blood (Todd et al. 1993; Hammarlund-Udenaes et al. 2008). In contrast to microdialysis, PET measures total brain concentrations, i.e. both unbound and bound radioligand. Since it is generally accepted that unbound concentrations drive the pharmacological effect, PET data should be corrected for brain tissue binding to estimate the unbound concentrations. This can be achieved by multiplication with in vitro estimated $f_{u,\text{brain}}$ values (Gunn et al. 2012; Gustafsson et al. 2017).

Single-photon emission computed tomography (SPECT) is analogous to PET, based on radioligands radiolabelled with radionuclides (George et al. 2015; Israel et al. 2019). However, SPECT compatible radionuclides do not decay by positron emission but instead with gamma emission. SPECT is in general regarded as a less quantitative method compared with PET.

MRI and CT are today frequently combined with PET, and hence, a PET/MRI or a PET/CT scan gives both functional and anatomical information. However, MRI can also be used to image function at the molecular level; functional MRI (fMRI) provides information of physiologic functions, e.g. blood flow, while magnetic resonance spectroscopy (MRS) can be used to gain spatial information on some specific metabolites, e.g. derived from neurotransmitters (Egerton 2019).

A general aspect of the above-mentioned techniques, when comparing to several high-resolution microscopy and fluorescence-based methods, is that the resolution of clinical scanners is in the range of 1 mm to 1 cm.

2.3 Two-Photon Laser Scanning Fluorescence Microscopy

In 1990, Denk et al. presented a new application of two-photon excitation of fluorescence to laser scanning microscopy (Denk et al. 1990). The technique is based on the phenomenon of simultaneous absorption of two infra-red photons by a fluorophore, for instance a fluorescent protein, and exogenous or endogenous autofluorescent substances. Up to 1 μm penetration depth combined with excellent spatial resolution enables optical in vivo studies of the brain at subcellular level. One of the greatest advantages of the method is the ability to perform longitudinal studies, for example by the implantation of a cranial window, with preserved natural neuronal environment. The use of two-photon microscopy for evaluation of BBB drug transport and BBB integrity has primarily a qualitative character, though there are several attempts to make it quantitative. For evaluation of BBB integrity in various physiological, pathological and/or induced conditions, fluorescently-labelled dextran molecules of various sizes can be administered intravenously and the degree of extravasation can subsequently be evaluated over time. For example, investigation of the passage of four different-sized molecules across a single cortical capillary confirmed that BBB transport properties are determined by three separate barriers: the glycocalyx, the endothelium and the extravascular compartment, all to be considered in CNS drug development (Kutuzov et al. 2018). Pathology impact on BBB transport, e.g. differences in BBB integrity, was discovered after transcranial focused ultrasound in wild type mice and transgenic mice (TgCRND8) with cerebral amyloid angiopathy (CAA); transgenic mice displaying slower “leakage” kinetics than the healthy wild type controls (Burgess et al. 2014). This method also provides unique opportunities to study nanodelivery (Baghirov et al. 2016).

2.4 CSF Sampling

Introduction of lumbar puncture (spinal tap) by Heinrich Quincke in 1902 enabled collection of cerebrospinal fluid (CSF) from humans. The close proximity of CSF and the brain has led to centuries of continuous investigations of anatomy of the ventricular system, physiology, pathophysiology as well as pharmacokinetics of drugs in CSF (Cushing 1914; Dixon and Halliburton 1916; Weed 1922; Sakka et al. 2011). CSF is often considered a relevant fluid not only for monitoring of the concentration of drugs and various biomarkers in the CNS, but also as a site for administration of therapeutics aimed to elicit a central pharmacological response. However, the question whether it is accurate to use CSF as a surrogate of brain ISF, in particular for investigation of drug concentrations, is debated (de Lange and Danhof 2002; Friden et al. 2009; de Lange 2013). Also, the use of CSF as a site for administration will require further evaluation.

CSF homeostasis is very well controlled and all stages of CSF secretion, circulation, and absorption involve several regulatory mechanisms. Briefly, CSF, primarily formed in the choroid plexuses, flows through the cerebral ventricles and the subarachnoid space to its ultimate sites of reabsorption into the blood via arachnoid villi of the dural sinuses and partially drains along cranial nerve sheaths, the cribriform plate into the lymphatic outflow system (Davson et al. 1970, 1973; Abbott 2004; Iliff et al. 2012; Louveau et al. 2015; Tarasoff-Conway et al. 2015; Abbott et al. 2018; Ahn et al. 2019). In addition, brain ISF also contributes to the CSF pool (Weed 1914; Cserr and Patlak 1992; Abbott 2004). The exchange between CSF and brain ISF is technically difficult to investigate, both in the context of CSF as a route for drug delivery to brain, or as a pathway for removal of waste products and drugs.

Despite technical limitations, advanced and quantitative clinical studies have been conducted aimed at understanding drug transfer between CSF and brain ISF. For instance, Shapiro et al. presented a pharmacokinetic study of methotrexate in patients with meningeal leukaemia and meningeal carcinomatosis after drug administration by intravenous infusion, intraventricular or lumbar puncture (Shapiro et al. 1975). They showed striking differences in methotrexate profile in intraventricular and intrathecal CSF spaces, reflecting differences in efflux of methotrexate at the barriers of the CNS when administered systemically. A model describing the differences in methotrexate concentrations in different brain and CSF compartments, and how they are correlated with each other, including the influence of transporters and disease confirmed the previous studies (Westerhout et al. 2014).

In addition, based on extensive compilation of CSF-to-ISF partitioning data, Shen et al. concluded that in the majority of cases, CSF concentration is higher than ISF unbound concentration obtained via microdialysis sampling (Shen et al. 2004). The difference was more pronounced for P-gp substrates, indicating that efflux transporters are likely to be differently expressed at the BBB and the BCSFB. This fact has later been confirmed by pharmacoproteomic approach (Gazzin et al. 2008; Braun et al. 2017; Zhang et al. 2018).

Increased interest in drug neuropharmacokinetics (neuroPK) and the acceptance of the free drug hypothesis has led to the use of the CSF to unbound plasma

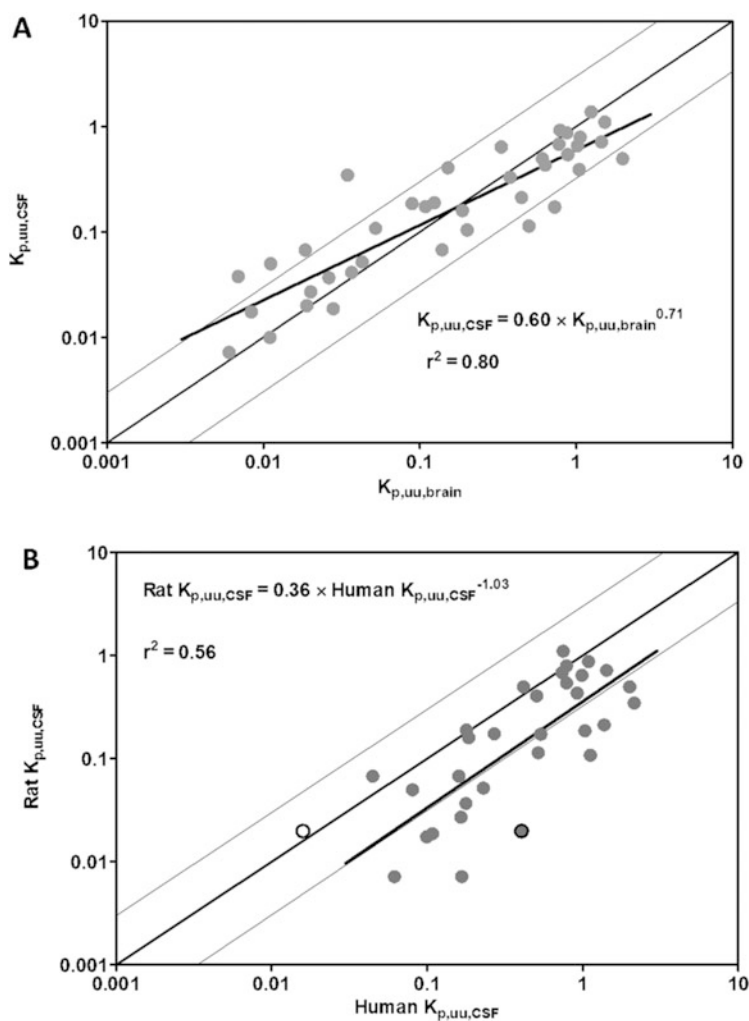


Fig. 2 Relationship between the extent of BBB transport and BCSFB transport assessed by $K_{p,uu,brain}$ and $K_{p,uu,CSF}$, respectively (a) and agreement between rat and human $K_{p,uu,CSF}$ (b). Fine lines represent identity and threefold difference. Solid lines are the result of linear regression analysis of log-transformed data. Figure obtained from Friden et al. (2009) with permission from the publisher

concentration ratio, also denoted as $K_{p,uu,CSF}$ as a measure of the extent of drug transport across the BCSFB (see also chapter “Brain Distribution of Drugs: Pharmacokinetic Considerations”, Sect. 2.1.2). In line with the debated assumption that CSF concentrations can be used as a proxy for ISF concentrations, $K_{p,uu,CSF}$ has been explored as an alternative for $K_{p,uu,brain}$. Comparison of $K_{p,uu,brain}$ and $K_{p,uu,CSF}$ values (Friden et al. 2009; Loryan et al. 2014) have indicated that the two parameters should not be used interchangeably without proper validation, in spite of a high

correlation ratio as indicated in Fig. 2a. Remarkably, even for a drug like acetaminophen without known transporter-mediated processes at the BBB, i.e. a model compound for passive transport, brain ISF concentrations measured in striatum via microdialysis are on average fourfold higher than CSF concentrations measured in the lateral ventricle and cisterna magna (Westerhout et al. 2012).

Evaluation of human vs rat $K_{p,uu,CSF}$ demonstrate reasonable correlation between species (Fig. 2b). However, for a given drug, human $K_{p,uu,CSF}$ is in general threefold higher than the corresponding rat $K_{p,uu,CSF}$ (Friden et al. 2009). This might be a reflection not only of the variances in the structure and function of the human and rat BBB and BCSFB, but also a consequence of species related dissimilarities in the hydrodynamics of CSF and dissimilarities in the sampling site. A study on protein expression of transporters in lysates of isolated canine brain capillaries and choroid plexuses revealed prominent differences (Braun et al. 2017). In particular, the transporters BCRP, P-gp, PEPT2 display very low expression levels in choroid plexuses, compared to brain capillaries, while OATP1A2, MRP4 and MCT1 are found abundantly at the BCSFB. Finally, the functionality of BBB and BCSFB is likely to change, and may change differently during disease. Thus, the presence of CNS pathology must be considered when evaluating the use of CSF as a surrogate for ISF.

Interplay between the CNS barriers is critical for maintenance of the CNS milieu. However, the contributive role of each barrier on achieving a certain level of CSF exposure for a given drug is challenging to investigate. Mathematical modelling, in particular PBPK models, that are driven by experimentally determined high quality data, carry extraordinary potential for facilitating the understanding of CSF translational pharmacokinetics. For example, de Lange and colleagues recently presented a generic physiologically based pharmacokinetic (PBPK) model for prediction of drug concentrations in physiologically relevant CNS compartments: plasma, brain extracellular fluid, cisterna magna, lateral ventricle, CSF lumbar site, and total brain tissue in rats (Yamamoto et al. 2017b) and humans (Yamamoto et al. 2018).

2.5 Combined In Vivo–In Vitro Assessment: The Combinatory Mapping Approach

In preclinical screening programs of novel CNS drug candidates, focused on evaluation of thousands of compounds annually, there is a high pressure to work within a short time-frame. Hence, elaborate and/or expensive methods such as microdialysis, brain imaging and even CSF sampling are not applicable for high-throughput screening purposes. Therefore, methodologies that will meet high-performance requirements without compromising the quality of the obtained data are required.

So far, there is no general consensus on the most appropriate screening cascade for CNS drug candidates. In addition, there is an absence of guidelines from both FDA and EMA on how to assess CNS exposure in preclinical settings. Overall, this is reflected by the big variety of methods that are used in different pharmaceutical companies to study the brain target-site concentration of a candidate compound in

relation to the systemic exposure and pharmacodynamic readouts. In this regard, the combinatory mapping approach (CMA, Fig. 3), that combines data from several in vitro and in vivo methods, has a potential to be used as a screening toolbox for the assessment of CNS exposure of new chemical entities. The method provides quantitative mapping of the extent of BBB transport by means of unbound brain to plasma concentrations ratio ($K_{p,uu,brain}$) and cellular barrier transport by means of unbound intracellular to extracellular concentrations ratio ($K_{p,uu,cell}$) along with the prediction of subcellular distribution (Loryan et al. 2014). The CMA approach is based on established pharmacokinetic concepts as well as novel methods, described in more detail in chapter “Brain Distribution of Drugs: Pharmacokinetic Considerations”. With these rather easily-performed methods, it is possible to map both the BBB and cellular barrier transport, providing important quantitative knowledge supporting the decision making in regard to selection of CNS drug candidates in the industrial settings.

3 In Vivo Models of Disease

3.1 Chemically Induced Models

Administration of toxins and other chemical substances to produce a certain phenotype of a disease has been used for decades. Injection of 6-hydroxydopamine (6-OHDA) or *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to dopaminergic parts of the basal ganglia in rodents results in a Parkinson’s disease-like phenotype (Bove and Perier 2012). Similar methods involving either systemic or central administration of neurotoxins have been used to generate models of epilepsy or various mental disorders (schizophrenia, anxiety, etc.) (Young et al. 2012; Levesque and Avoli 2013; Jefferys et al. 2016).

Neuroinflammation, associated with numerous neurological disorders, such as multiple sclerosis, amyotrophic lateral sclerosis, Parkinson’s disease, Alzheimer’s disease is often studied in preclinical models (de Vries et al. 1997; Tchessalova et al. 2018). Lipopolysaccharides (LPS), the major component of the outer membrane of Gram-negative bacteria, has been widely used to produce a resilient neuroimmune response. Numerous different protocols based on LPS have been published (Andersson et al. 1992; de Vries et al. 1997; Wyns et al. 2015). Variations include LPS from different strains, the use of different LPS doses, different routes of administration, induction of systemic inflammation vs primarily neuroinflammation. The different protocols often produce conflicting results; this is further discussed in a systemic review focused on BBB function in relation to systemic inflammation by Varatharaj and Galea (Varatharaj and Galea 2017).

It has been shown that LPS from *Salmonella typhimurium*, at a dose of 3 mg/kg administered intraperitoneally to mice, causes BBB disruption as measured by ^{14}C -sucrose and $^{99\text{m}}\text{Tc}$ -Alb (Banks et al. 2015). However, lower doses were not associated with any BBB disruption. LPS can also be used in higher species. Morphine concentrations were shown to be increased in an experimental pig

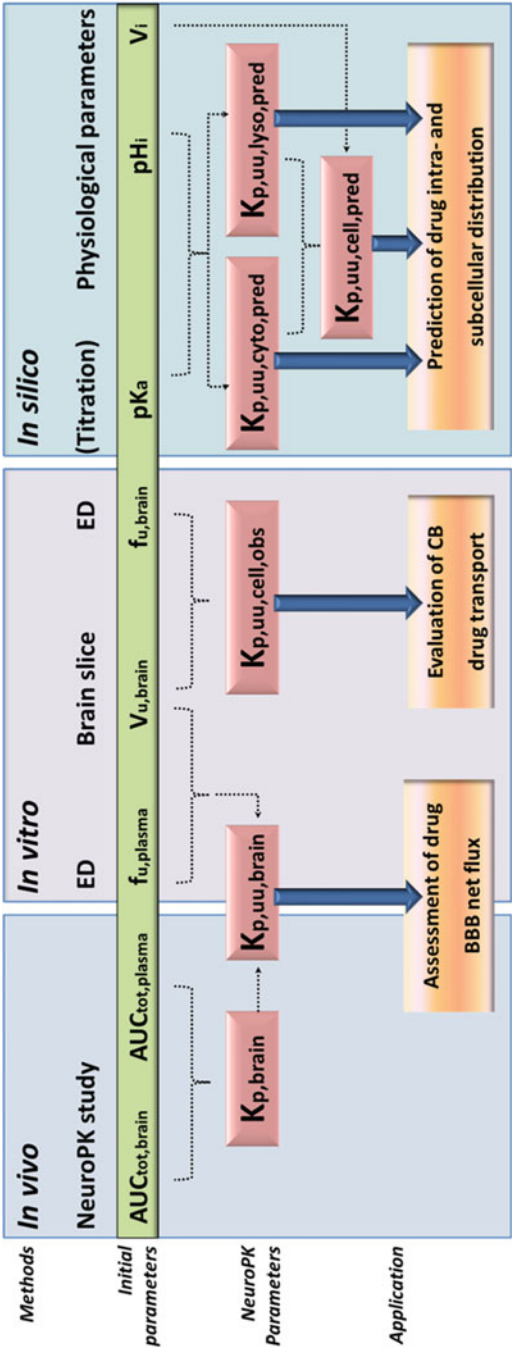


Fig. 3 Diagram summarising the combinatory mapping approach in the form of a screening toolbox for the evaluation of unbound drug CNS exposure required for selection of novel drug candidates. Figure adapted from Loryan et al. (2014). The platform comprising of in vivo, in vitro and in silico toolboxes. Total drug brain and plasma exposure (e.g., by means of area under the curve of concentration-time profiles, $AUC_{tot,brain}$ and $AUC_{tot,plasma}$) determined in an in vivo neuroPK study is essential for the assessment of the brain partitioning coefficient $K_{p,brain}$. In vitro measurements of drug plasma and brain tissue binding properties using equilibrium dialysis (ED) and brain slice techniques are required for estimation of $K_{p,uu,brain}$ and $K_{p,uu,cell}$ neuroPK parameters. Compound-specific pK_a values in combination with the physiological estimates of pH (pHi) of the relevant compartments (i = plasma, interstitial fluid, cytosol or lysosomes) are used for in silico calculation of drug subcellular distribution, i.e. $K_{p,uu,cyto,pred}$ and $K_{p,uu,lyso,pred}$. Assessed neuroPK parameters in conjunction with relevant pharmacodynamics readouts are recommended to be used for evaluation and selection of novel drug candidates

model of meningitis based on administration of 200 µg LPS from *E. coli* in *cisterna magna*, compared to healthy control pigs (Tunblad et al. 2004).

Interestingly, LPS itself has low transport across BBB even in cases when BBB disruption is observed (Banks and Robinson 2010). Thus, the LPS-induced neuroinflammation is likely an indirect effect. Yet, it has been shown that chronic neuroinflammation and progressive neurodegeneration can be observed even after a single injection of 5 mg/kg LPS to mice, and last months (Qin et al. 2007). Overall, chemically induced neuroinflammation models have potential in unravelling the role of brain barriers in multiple diseases.

Another widely used chemically induced model is experimental allergic (or autoimmune) encephalomyelitis (EAE) that resembles multiple sclerosis, which is a human inflammatory demyelinating CNS disease (Constantinescu et al. 2011). EAE can be induced in multiple species by immunisation with CNS tissue or myelin peptides, such as myelin basic protein and proteolipid protein. It has been shown that brain endothelial dysfunction precedes the development of symptoms and is primarily driven by disruption of tight junctions in particular zonula occludens 1 (Bennett et al. 2010). The EAE model performed in various species has been instrumental not only for the development of the drugs against multiple sclerosis but also for understanding the pathophysiology of EAE (Engelhardt and Ransohoff 2012).

Kainic acid and pilocarpine are neurotoxic substances that have been used to generate rat and mouse models that mimic some features of temporal lobe epilepsy. These models have been used in studies of BBB drug transport as well as in studies of anti-epileptic drug effects (Hellier et al. 1998; Klein et al. 2015; Welzel et al. 2020). Some studies report increased expression of BBB transporters that eventually may lead to drug resistance, while other studies report changes in transporter expression levels but not in functionality (Volk et al. 2004; Bankstahl et al. 2016; de Lange et al. 2018; Soldner et al. 2019).

3.2 Genetically Modified Models

Transgenic models, of which more than 90% are mouse models, are defined by the transfer of a foreign gene (or genes) into the host genome. Since the mid 1980s, these models have been vital for studies of human disease by the insertion of a disease causing human gene into, in most cases, the mouse genome. In most of the models, the gene has been randomly inserted and always “switched on”. Later models include more site-specific insertion and action, as well as means to switch on and off the inserted gene. More recent genetically modified models are not “transgenes”, as their own DNA has been altered into the human version of the gene (Saito et al. 2014). These “knock-in” models are considered to be more human like, since it has been shown that the random insertion of a foreign gene can result in effects simply because of the unnatural alteration of the host genome and the co-existence of the host and human gene.

Animal models of neurodegenerative diseases have been used extensively. For example, there are around 200, 20 and 50 genetically modified models listed as models for AD, PD and ALS, respectively (source: www.alzforum.org/research-models). They are mainly based on high accumulation of disease causing and/or associated proteins, e.g. amyloid-beta in AD models. Many of the models harbour several disease causing mutations.

Animal models characterised by lack of certain tight junction proteins have been widely used to study BBB function and integrity. For example, mice lacking tight junction protein claudin-5 display a compromised BBB and mice completely devoid of the protein die within hours after birth (Nitta et al. 2003). Claudin-5 has also been implicated to have a role in the development of schizophrenia, as well as other neurodegenerative disorders (Greene et al. 2018).

4 Translational Aspects of BBB Drug Transport

The overall aim of in vitro and preclinical in vivo studies of drug transport at the BBB is to predict human brain pharmacokinetics and effects, and to understand mechanisms of drug influx to and efflux from the brain parenchyma. Although in vivo studies in animals take into account the aspects of administration, distribution, metabolism and excretion of a drug, and therefore are likely to be closer to the clinical situation than studies in cell systems, several challenges persist for accurate translation. First of all, the BBB differs between species with regard to expression and function of transporters, receptors and other essential proteins. For example, the relative expression as well as the total amounts of efflux transporters appears to be different in mice and men (Shawahna et al. 2011; Uchida et al. 2011a, b). It has been shown that drugs that are P-gp substrates in mice are likely to be so also in humans, but the impact of P-gp on brain drug concentrations might be less pronounced in higher species compared to rodents (Syvanen et al. 2009). Disease might also induce or reduce the expression and/or functionality of BBB transporters, or in some cases it has even been speculated that changes in transporter function might lead to disease. For example, it has been shown that P-gp is downregulated in Alzheimer's disease (van Assema et al. 2012), which could have an impact on the accumulation of intra-brain amyloid-beta, while P-gp appears to at least locally be upregulated in epilepsy, potentially contributing to pharmacoresistance towards anti-epileptic drugs (Feldmann et al. 2013). Also the composition of the brain tissue itself (lipid content, etc.) might differ between species. This in turn can have an impact on the intra-brain distribution and the binding to brain tissue components. However, it has been shown, although with limited amount of samples, that $f_{u, \text{brain}}$ is relatively well preserved across species indicating that the free-to-bound drug ratio in the brain should be fairly similar across species (Summerfield et al. 2008; Di et al. 2011). These factors should be taken into account and be a basis for model selection when studying brain drug delivery in preclinical studies.

The development of animal models, especially transgenic mouse models, has been of great importance for studies of disease-related changes in BBB morphology

and function. However, it should be kept in mind that most models only reflect part (s) of the disease. The myriad of transgenic models for Alzheimer's disease are in fact only models of amyloid-beta pathology and in some cases also in parallel models for tau pathology or neuroinflammation. But the links between the different characteristics of the disease (amyloid-beta accumulation, tau aggregation, inflammation, atrophy, BBB breakdown) are to a large extent missing. Chemically induced models often produce a disease-like phenotype, but are not able to mimic the underlying biological changes that lead to disease. This should be considered when working with toxin induced models for epilepsy, Parkinson's disease and schizophrenia.

5 Conclusions and Future Directions

Even with the advanced techniques available today, drug delivery to the brain is challenging to study *in vivo*. The difficulties include the technical aspects of measuring drug concentrations in the CNS as well as the complex biology of the CNS and its barriers. Imaging methods enable target-site measurements of drugs but studies have to be well designed to account for potentially interference of factors such as radiolabeled metabolites (PET and SPECT), recovery (microdialysis) and invasive procedures potentially causing secondary reactions (microdialysis, cranial window). While it can be assumed that there exists a correlation between CSF and ISF concentrations, the correlation is likely to be dissimilar for different drugs and should therefore be confirmed before the use of CSF as a proxy for the drug target-site concentration.

Advancement of quantitative targeted proteomics has revealed heterogeneity in the expression profiles of transporters in BBB, BCSFB and blood–arachnoid barrier (BAB). Overall, the interaction of endogenous and exogenous molecules including drugs, with CNS barriers is very complex and specific, e.g. depends on the character of interaction with certain transporters in various interfaces. The complexity increases with the addition of disease associated pathology in CNS and at the barriers of the CNS.

With the increase in quality and quantity of data available, possibilities to develop mathematical models for brain drug delivery will increase. These models will likely contribute to greater understanding and improved prediction of processes involved in the rate and extent of drug transport into the brain.

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Part IV

The Blood-Brain Barrier and Diseases: Novel Treatment Approaches from a Blood-Brain Barrier Perspective



The Blood-Brain Barrier in Alzheimer's Disease

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Abstract

The accumulation of neurotoxic amyloid-beta (A β) in the brain is one of the characteristic hallmarks of Alzheimer's disease (AD). A β -peptide brain homeostasis is governed by its production and various clearance mechanisms. The blood-brain barrier provides a large surface area for influx and efflux mechanisms into and out of the brain. Different transporters and receptors have been implicated to play crucial roles in A β clearance from brain. Besides A β transport, the blood-brain barrier tightly regulates the brain's microenvironment; however, vascular alterations have been shown in patients with AD. Here, we summarize how the blood-brain barrier changes during aging and in disease and focus on

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recent findings of how the ABC transporter P-glycoprotein (ABCB1/P-gp) and the receptor low-density lipoprotein receptor-related protein 1 (LRP1) play a role in A β clearance from brain.

Keywords

Alzheimer's disease (AD) · Amyloid-beta (A β) · Blood-brain barrier · Clearance · LRP1 · Neurovascular unit · P-gp/ABCB1

Alzheimer's disease (AD), the most common form of dementia, is a devastating neurodegenerative disorder (Prince et al. 2013). In 1906, the German physician and neuropathologist, Alois Alzheimer, was the first to describe amyloid plaques and neurofibrillary tangles in the brain of his deceased patient who suffered from severe memory problems (Alzheimer 1906, 1907). To this date, plaques and tangles are considered the two characteristic hallmarks of the disease. Much effort has been devoted to study the underlying pathophysiological mechanisms of the disease. Despite tremendous effort, more than a century later, there is no treatment available, no cure in sight (Iadecola 2016).

On a molecular level, the presence of intracellular hyper-phosphorylated tau protein and extracellular aggregates of amyloid-beta (A β) protein in the brain of AD patients suggest that both proteins play a key role in disease pathogenesis. However, it is unclear whether these protein abnormalities are causative or rather incidental changes of the disease. Nevertheless, it is generally accepted that high levels of A β are toxic, not only to neurons, but also to other brain cells and lead to neurodegeneration, neuroinflammation, and neurovascular dysfunction resulting in behavioral changes (Iadecola 2004; Zlokovic 2005, 2011). Studies in animal AD models propose that A β acts upstream of tau, since amyloid models show tauopathies, whereas tau models do not display amyloid pathology (Bloom 2014). Recently, it was shown in humans that cerebrospinal fluid (CSF) levels of tau increase in response to A β deposition in the early stages of AD further suggesting that both pathogenic proteins are somehow linked (Mattsson-Carlsson et al. 2020). There is no generally accepted hypothesis on how AD develops or progresses. Over the years, researchers have developed multiple hypotheses. For example, in 1993, the "*Vascular Hypothesis of Alzheimer's Disease*" was formulated by de la Torre and his colleagues. This hypothesis is based on findings that two common medical conditions, chronic hypertension and carotid atherosclerosis, appear to be independently associated with AD (Iadecola 2016). The hypothesis postulates that vascular risk factors responsible for persistent cerebral hypoperfusion are key factors for converting normal aging into dementia. Ample epidemiological studies have shown that hypertension and cardiovascular risk factors are strongly associated with AD (Iadecola 2016). A recent multifactorial, data-driven analysis of PET and MR images of over 7,700 individuals showed that vascular alterations are one of the earliest events in AD pathology (Iturria-Medina et al. 2016). These changes are later followed by characteristic AD features such as A β brain accumulation, metabolic

dysfunction, functional impairment, and structural atrophy with significant volume loss of grey and white matter (Iturria-Medina et al. 2016). In 2004, Zhu et al. proposed the “*Two-Hit Hypothesis*” which states that although either oxidative stress or abnormalities in mitotic signaling can independently serve as initiators, both processes are necessary to propagate disease pathogenesis (Zhu et al. 2004). In 2011, this concept was modified and the “*Two-Hit Vascular Hypothesis*” was formulated to explain why vascular pathology often coincides with A β and tau pathology. This hypothesis states that vascular changes represent the first insult (*hit 1*), which is then followed by the second insult (*hit 2*) consisting of A β and tau accumulation in the brain (Zlokovic 2011). Several recent studies support the hypothesis, that vascular alterations precede neurodegeneration and cognitive decline (Iturria-Medina et al. 2016; Montagne et al. 2015; Nikolakopoulou et al. 2019).

Over the last decades, there is increasing evidence that the interfaces – blood-brain barrier, blood-CSF barrier, and meninges – that separate the central nervous system (CNS) from the periphery and tightly regulate brain homeostasis, play a major role in AD pathology. For example, the blood-brain barrier is involved in A β clearance from the brain, regulates infiltration of macrophages and other immune cells into the brain, and mediates inflammation (Zlokovic 2005). The reduction of neurotoxic A β brain levels is currently a major treatment strategy in AD either by targeting the generation or the removal of A β (Long and Holtzman 2019; Storck and Pietrzik 2017). It has been shown that in cases of sporadic AD and some form of familial AD, A β accumulation is the result of reduced brain clearance instead of overproduction (Bateman et al. 2006; Mawuenyega et al. 2010). The blood-brain barrier with its large surface area provides a big potential for therapeutic intervention (Storck and Pietrzik 2018). In this chapter, we focus on the role of blood-brain barrier function in A β clearance from the brain and how these processes could be used for therapeutic targeting.

1 Amyloid Dynamics at the Blood-Brain Barrier

The blood-brain barrier keeps neurotoxic blood-derived components, xenobiotics, cells, and pathogens out of the brain. At the same time, it supplies the brain with nutrients and oxygen and facilitates the clearance of waste products and neurotoxins out of the brain (Bell and Zlokovic 2009). The apicobasal polarized endothelial cells comprising the blood-brain barrier are equipped with specialized transport systems for nutrients, energy metabolites, signaling molecules, and waste products for transport in either direction. These include solute carrier-mediated transport of carbohydrates (e.g., glucose), amino acids, vitamins, fatty acids, hormones, nucleotides, and monocarboxylic acids (e.g., lactate); ion transporters, ATP-binding cassette transporters specialized for efflux of xenobiotics, and receptor-mediated transporters for peptides (Bell and Zlokovic 2009).

In AD patients, it has been shown that decreased A β clearance from the brain is a key event in AD pathogenesis (Bateman et al. 2006; Mawuenyega et al. 2010). In a

healthy individual, A β clearance through different clearance mechanisms in the brain exceeds its production (Bateman et al. 2006). In AD, however, this balance appears to be dysfunctional and the brain can no longer efficiently remove A β . To date, we have no understanding of how much each mechanism contributes to the overall clearance of A β from the brain. Several different mechanisms have been identified and impairment of any of these in mouse models of AD worsens A β pathogenesis. A β clearance mechanisms include extracellular enzymatic degradation, intracellular degradation by cerebral cells, transport into the periphery across the blood-brain barrier or the choroid plexus, glymphatic flow, lymphatic, or perivascular drainage (Da Mesquita et al. 2018; Storck and Pietrzik 2017; Tarasoff-Conway et al. 2015). Several independent studies suggest that the blood-brain barrier significantly contributes to A β clearance by translocating A β from the interstitial fluid (ISF) into blood (Cirrito et al. 2005; Shibata et al. 2000; Storck et al. 2016; Van Gool et al. 2019). Recently, the glymphatic system which is believed to act by water influx through astrocytic aquaporin-4 water channels has received attention. Glymphatic influx of CSF into the brain parenchyma along arteries is thought to create an ISF bulk flow between arteries and veins. This bulk flow drains nonspecific solutes including A β from the ISF into the CSF of para-venous spaces (Iliff et al. 2012; Kress et al. 2014). This hypothesis contradicts the traditional belief of ISF flow into the CSF relying mainly on diffusion along the perivascular route. Further studies need to be conducted to test whether distinct pathways exist, or whether the same pathway may give rise to different results depending on differing physiological or experimental conditions (Abbott et al. 2018; Iliff and Simon 2019; Smith and Verkman 2019; Tarasoff-Conway et al. 2015). Once in the CSF, A β seems to be eliminated by meningeal lymphatics (Da Mesquita et al. 2018) or can reenter the brain parenchyma by glymphatic-mediated CSF influx and access all the various clearance routes including blood-brain barrier-mediated clearance again.

Although it is believed that under homeostatic conditions proteins cannot passively cross the blood-brain barrier, a recent study in A β -overexpressing mice shows an involvement of a paracellular pathway under pathological conditions (Keaney et al. 2015). Keaney and colleagues showed that pathophysiological A β accumulation in the brain causes a transient downregulation of claudin-5 and occludin protein expression, allowing for its own auto-regulated paracellular passage across the blood-brain barrier. Interestingly, tight junction dysfunction has been described in patients with AD and/or with cerebral amyloid angiopathy (Biron et al. 2011; Carrano et al. 2011; Hartz et al. 2012). Under homeostatic conditions, A β is transported like other peptides via fast and efficient receptor- and/or transporter-mediated mechanisms across the CNS endothelium.

2 P-gp- and LRP1-Mediated A β Transport Across the Blood-Brain Barrier

Reducing A β transport across the blood-brain barrier in mouse models of AD results in accumulation of A β in the brain accompanied by cognitive deficits in learning and memory (Cirrito et al. 2005; Storck et al. 2016). The most studied clearance proteins are LRP1 (low-density lipoprotein receptor-related protein 1) and P-glycoprotein (P-gp), an ATP-binding cassette (ABC) transporter also known as ABCB1 or MDR1. While neither of them has been an obvious hit in genome-wide association (GWAS) studies, recent evidence has shown that Phosphatidylinositol binding clathrin assembly protein (PICALM), a highly-validated candidate found in these studies (Harold et al. 2009; Jun et al. 2010; Lee et al. 2011), regulates both LRP1 and P-gp trafficking in endothelial cells and therefore their function (Storck et al. 2018; Zhao et al. 2015). Another study recently found PICALM mRNA to be reduced in aged blood-brain barrier endothelial cells suggesting impaired A β transcytosis during aging (Yang et al. 2020).

LRP1 is a member of the low-density receptor (LDLR) family. Its expression decreases in aging and in AD, both in total brain tissue and brain capillaries (Kang et al. 2000; Osgood et al. 2017; Shibata et al. 2000; Silverberg et al. 2010a, b). Decreased blood flow in AD patients (Roher et al. 2012) could affect LRP1 as it has been shown that LRP1 protein expression is decreased during hypoxia (Bell et al. 2009; Kanekiyo et al. 2012). The membrane receptor can be shed from the cell surface and is then released as a soluble form (sLRP1) in the ISF or the bloodstream. Shedding of membrane-bound LRP1 in endothelial cells can influence brain clearance of A β (Shackleton et al. 2016; Zandl-Lang et al. 2018). Furthermore, sLRP1 in blood plasma is believed to drive A β clearance from the brain by binding A β in the blood and therefore acting as a peripheral sink (Sagare et al. 2007). Interestingly, AD patients show significantly higher amounts of oxidized sLRP1 with lower efficiency to bind A β (Sagare et al. 2007).

LRP1 is highly expressed in all cells that constitute the neurovascular unit (Kanekiyo et al. 2012; Liu et al. 2017; Storck et al. 2016) so that transcytosis from ISF through the cellular layer into the periphery is possible. Due to its fast endocytosis rate compared to other members of the LDLR family (Li et al. 2001), LRP1 rapidly removes soluble A β from the ISF. Immunohistochemical analyses in human brain capillaries suggest that LRP1 expression is restricted to the abluminal, brain-facing side of the endothelium (Zhao et al. 2015). For the longest time, it has been believed that LRP1 fully transcytoses A β through endothelial cells by binding the protein at the brain-facing side and releasing it into the blood at the luminal side. Recent studies, however, suggest that LRP1 needs luminal ABC transporters such as P-gp for transcytosis (Storck et al. 2018) for the actual release of A β into the vessel lumen. Although the exact transcytosis mechanism is not fully understood yet, studies suggest that in endothelial cells A β triggers LRP1 and P-gp internalization and localization to sorting endosomes, where LRP1-bound A β dissociates from the receptor and binds to P-gp (Storck et al. 2018; Zhao et al. 2015). It is thought that P-gp is then directed back to the luminal side and releases A β into the blood, whereas

LRP1 is redirected to the abluminal side of the endothelial cell. Both LRP1 and P-gp internalization and therefore their function seem to be regulated by the late-onset AD risk factor PICALM. This proposed mechanism could explain how luminal P-gp gains access to brain-derived A β in order to be able to efflux it. The exact mechanism of P-gp-mediated A β remains unclear.

In addition to LRP1 and P-gp, other members of the LDLR family (e.g., LRP2), other ABC transporters (ABCC1, ABCG2, and ABCG4) as well as the neonatal Fc receptor (FcRn) have been described to assist in A β clearance from the CNS (Bell et al. 2007; Deane et al. 2005; Do et al. 2012; Krohn et al. 2011; Lamartiniere et al. 2018). To what extent these transport systems contribute to homeostasis, whether these also act in concert with others, or if these transporters compensate for A β clearance when other systems fail is still unknown.

Recent findings further promote the role of LRP1 in AD pathogenesis. For decades, there had been no visible link between the pathogenic proteins that accumulate in AD patients' brains. Now, several recent reports provide insights into the pathways linking tau, A β , and neurodegeneration. A recent report now shows that LRP1 is also endocytosing the protein tau (Rauch et al. 2020). Another study in mice and humans shows that CSF levels of tau increased in response to A β deposition in the early stages of AD implicating that the clearance of LRP1 ligands is impaired when a certain threshold is reached (Mattsson-Carlsson et al. 2020). To our knowledge, there are no reports on LRP1-mediated transport of tau across the BBB. However, tau in blood is currently considered to be a new biomarker of disease progression (Barthelemy et al. 2020). All these novel findings help to understand that the trafficking of pathogenic proteins in and out of the brain is far more complex than initially thought and that future research is crucial to unravel the complex interactions in AD pathology.

3 RAGE-Mediated A β Transport Across the Blood-Brain Barrier

Studies suggest that receptor-mediated entry of A β into the brain is mediated by the receptor for advanced glycosylation end products (RAGE) that – in contrast to LRP1 – is mainly expressed at the luminal side of the capillary endothelium (Deane et al. 2003; Deane et al. 2012; Zhao et al. 2015). Therefore, RAGE mediates A β (re)-uptake from blood into brain. RAGE-mediated endocytosis is enriched within caveolae microdomains on the apical plasma membrane of endothelial cells and the receptor has been shown to colocalize with caveolin-1 (Stitt et al. 2000). Co-immunoprecipitation analysis revealed an interaction between caveolin-1 and RAGE, but not LRP1. Further, phosphorylation of caveolin-1 has been described to modulate the function of a number of membrane transporter proteins including RAGE and P-gp (Barakat et al. 2007; Zhu et al. 2018). Based on the recent finding that brain-to-blood transcytosis of A β via LRP1 requires ABC transporter function of P-gp (Storck et al. 2018), one could speculate that RAGE-mediated blood-to-brain transcytosis is dependent on/or restricted by similar mechanisms. Endothelial

transcytosis is believed to be mediated by a so-called common recycling endosome that connects basolateral and apical endocytosis events and directs cargo to different cellular compartments (Thuenauer et al. 2017). In this scenario, in health, when P-gp is high, the ABC transporter could efflux RAGE-mediated uptake of A β into the endothelium and thus limit RAGE-mediated A β transcytosis from blood into the brain. Dysregulation of this compensatory mechanism, either by downregulation of P-gp or upregulation of RAGE as seen in AD patients (Deane et al. 2003; Deo et al. 2014) would therefore promote brain uptake of peripheral A β by reducing the rate of efflux from intracellular vesicles. Evidence for an involvement of RAGE in AD pathophysiology in humans has been supported by its upregulation in the vasculature, astrocytes, and microglial cells in the hippocampus of AD patients compared to healthy individuals (Yan et al. 1996). Moreover, inhibition of RAGE-A β interaction, using either a soluble form of RAGE or an anti-RAGE antibody, results in reduced neuroinflammation, improved cerebral blood flow (CBF), reduced brain A β levels, and a lower rate of cognitive decline in Tg2576 mice (Deane et al. 2003, 2012). Based on these findings, a clinical Phase 2/3 trial with a RAGE blocker in patients with mild AD and impaired glucose tolerance (NCT03980730 June 2019–July 2023) has been recently initiated. In addition to RAGE, organic-anion-transporting polypeptide transporters (OATPs) such as Oatp1a4 have been reported to be involved in transporting A β from the periphery into the brain (Do et al. 2013). More studies are needed to establish the role of OATPs in A β clearance and AD pathology.

4 Transmission of A β Protein Pathology Via Peripheral Blood Sources

Data from several studies suggest that the blood-brain barrier might also play a role in the uptake of peripheral A β from blood into the brain (Bu et al. 2017; Eisele et al. 2010; Purro et al. 2018). Studies in mouse models have shown that small amounts of peripherally applied pathological oligomeric A β can act as a seed and accelerate A β pathology and its spreading within the brain (Eisele et al. 2009, 2014, 2010). Moreover, animal studies also suggest that medical and surgical procedures might pose a risk of transmission of AD. For example, Purro and colleagues showed that batches of human cadaveric pituitary-derived growth hormone (c-hGH) contaminated with A β and tau proteins can lead to A β plaques and cerebral amyloid angiopathy following intracerebral inoculation in mice (Purro et al. 2018). In a parabiosis experiment, Bu et al. recently demonstrated that surgical union of a human A β -overexpressing mouse with a wild-type mouse for several months resulted in the accumulation of human A β in the brains of wild-type animals. In this study, human A β was deposited in amyloid plaques and triggered tau phosphorylation, inflammation, and neurodegeneration in the wild-type mouse. These findings implicate that human A β produced in brains of AD mice was translocated from the brain into the periphery, entered the conjoined blood circulation, crossed the brain barriers in the wild-type animals, possibly by crossing the blood-brain barrier, and triggered aggregation and neuropathology in the brain of the wild-type

animals (Bu et al. 2017). The exact route of brain entry, its mediators, and relevance to humans is unclear and therefore more research is needed to understand cellular and molecular mechanisms underlying this process.

Overall, little is known about the relevance of transmission of AD pathology via peripheral blood sources in humans. Data from a retrospective study with 1,465,845 patients who received blood transfusions between 1968 and 2012 provided no evidence for the transmission of AD (Edgren et al. 2016). Therefore, further studies are needed to investigate the possibility of transmission of A β pathology via peripheral amyloidogenic proteins in humans.

5 Changes of Blood-Brain Barrier Function in Alzheimer's Disease

During aging and in disease state many of the described receptors, transporters, and carriers are dysregulated suggesting that A β clearance across the blood-brain barrier is altered. A recent study shows that there is an age-related shift in transport from ligand-specific receptor-mediated transport to nonspecific caveolar transcytosis in brain endothelium (Yang et al. 2020). The authors of this study found many receptors such as LDLR, leptin receptor, transferrin receptor (TfR), or insulin receptor downregulated with aging on RNA levels suggesting changes in specific trafficking of solutes into and out of the brain. No significant changes were detected for *Lrp1*. Interestingly, the authors found a significant downregulation of intracellular adaptor protein PICALM which is required for LRP1 and P-gp trafficking. *Picalm* reduction in mice reduces LRP1-mediated A β clearance without affecting LRP1 or P-gp protein levels (Zhao et al. 2015). Taken together, these studies nicely show that age-related changes in brain endothelium occur that affect A β clearance even when it seems that the RNA levels of transporters seem unaffected. Accumulation of A β in the brain triggers neurovascular stress (Deane et al. 2012), downregulates tight junction proteins (Keaney et al. 2015; Park et al. 2014), and allows the entry of blood-derived substances and immune cells penetrating the CNS which disturbs the tightly controlled homeostasis of the brain. In mouse models of AD, it has been shown that A β deposition in blood vessels, also known as cerebral amyloid angiopathy (CAA), causes blood vessels to degenerate which disturbs blood flow and angiogenesis throughout the brain (Meyer et al. 2008). Therefore, A β accumulation in the brain has a massive impact on cerebral blood vessel function and their distribution and consequently on brain health. Clinical studies have shown impaired P-gp transport function in AD patients (Deo et al. 2014). As P-gp acts as a gatekeeping efflux pump, P-gp impairment leads to reduced brain clearance of A β and potentially also to increased brain uptake of xenobiotics in AD.

Recent studies in mice show that meprin β , a protease upregulated in AD brain (Schlenzig et al. 2018), not only generates A β (Schonherr et al. 2016) but also affects blood-brain barrier integrity by cleaving tight junction proteins (Gindorf et al. 2020). In humans, it has been shown that the blood-brain barrier becomes leaky even before the onset of hippocampal atrophy typically seen in early AD (Montagne et al. 2015).

Cognitive impairment occurred regardless of A β or tau pathology (Montagne et al. 2020) – the hallmarks of AD. Moreover, blood-brain barrier leakage was enhanced in patients carrying the *APOE4* allele, the strongest genetic risk factor for AD. These findings again support the hypothesis that vascular abnormalities precede neuronal degeneration. Many other vascular alterations have been reported in the brain of AD patients. Microbleeds or microhemorrhages often coincide with AD leading to extravasation of blood-derived substances into the brain causing neuroinflammation and immune responses (Cullen et al. 2005). Blood-brain barrier transport of glucose, the most important energy source of the brain, has been demonstrated to be impaired in AD due to a lower endothelial expression of glucose transporters and reduced blood flow (Kalaria and Harik 1989). In line with an age-related shift to nonspecific caveolar transcytosis (Yang et al. 2020) along with an entry of serum proteins into the brain are other studies that report that an age-related dysfunction of the blood-brain barrier allows an influx albumin into the brain (Milikovsky et al. 2019; Senatorov Jr. et al. 2019). Albumin, then, activates Transforming growth factor-beta (TGF- β) receptors, overstimulates neuronal networks, and impairs cognition. The reports show that an opening of the blood-brain barrier correlated with localized slowing of cortical activity in epilepsy, AD patients, as well as in mouse models of AD (Milikovsky et al. 2019; Senatorov Jr. et al. 2019).

However, endothelial cells are not the only cell type of the neurovascular unit affected by changes occurring in AD. Pericytes, that are in direct contact with endothelial cells are critical regulators of blood-brain barrier function, are equally affected by AD pathology (Ben-Zvi et al. 2014; Daneman et al. 2010). It has been shown that pericyte numbers are decreased in brain samples from human subjects with AD (Sengillo et al. 2013) and that reduced pericyte expression correlates with barrier dysfunction in AD cases. The apolipoprotein E (APOE) is a major risk factor of AD. APOE 4 not only affects blood-brain barrier-mediated A β clearance (Bell et al. 2007; Deane et al. 2008), it also seems to have a direct effect on barrier integrity (Bell et al. 2012). In subjects with AD, it has been suggested that APOE 4 accelerates the loss of pericytes and causes microvascular reductions (Halliday et al. 2016; Sengillo et al. 2013). Studies in mice have shown that pericyte ablation leads to acute circulatory failure and rapid loss of neurons and behavioral deficits within days (Nikolakopoulou et al. 2019). The authors of the study postulate that circulatory stress, together with the loss of brain pericyte-derived neurotrophic support, is responsible for the observed neurodegenerative and behavioral phenotypes in these mice. Recently, it was shown that an age-related shift in receptor-mediated transcytosis to unspecific caveolar transcytosis occurs alongside a specific loss of pericyte coverage (Yang et al. 2020).

In the last decades, researchers have moved away from a neuro-centric view of AD and appreciated that other cell types of the brain contribute to AD pathology. As such, it has been recognized that neurons are not the only A β -producing cell type in the brain. To date, we know that also other cerebral cells such as astrocytes (Berkenbosch et al. 1990; Zhao et al. 2011), oligodendrocytes (Walter et al. 2019), vascular smooth muscle cells (Frackowiak et al. 2005), endothelial cells (Devraj et al. 2016; Kitazume et al. 2010), as well as peripheral cells (Citron et al. 1994;

Gowert et al. 2014; Jiang et al. 2003) contribute to A β production and might affect brain levels of A β . Therefore, the neurovascular unit could not only be an important mediator of A β clearance but also contribute to A β production.

However, not only production but also factors modulating A β clearance or A β deposition are increasingly recognized. As such, it has been suggested that pericytes play a significant role in the development of vascular deposition of A β , also known as CAA (Blanchard et al. 2020). Blanchard and colleagues recently devolved a humanized in vitro model that combined endothelial cells, astrocytes, and pericytes in a three-dimensional gel. The endothelial cells formed capillaries with tight junctions that had high electrical resistance mimicking the neurovascular unit in vivo. When pericytes in this model expressed the AD risk factor *APOE4*, they activated a transcriptional pathway leading to enhanced ApoE secretion, which, in turn, led to amyloid deposition on capillary walls. However, blocking this pathway prevented deposition.

6 Current Treatment Approaches

Currently, several cholinesterase inhibitors approved by the US Food and Drug Administration (FDA) are being used to treat AD, with some clinical improvement in cognition (Raina et al. 2008). However, these drugs can only improve cognitive symptoms of AD for a certain period and cannot modify the disease course. Therefore, we are in the need of new drugs that slow down disease progression or even prevent their onset.

Many of the current treatment strategies for AD are targeting elevated A β levels in the brain. Although the blood-brain barrier provides a potential for its therapeutic interaction, the physical barrier is at the same time an obstacle for drug delivery (Storck and Pietrzik 2017, 2018). One possible solution for this problem is a temporal opening of the blood-brain barrier by focused ultrasound which might allow the delivery of larger amounts of therapeutics, such as antibodies, to the brain (Meng et al. 2019). Interestingly, in mice focused ultrasound alone, without the need for any additional therapeutic agent such as anti-A β antibody, was efficient to reduce brain amyloid suggesting focused ultrasound to be a noninvasive method with therapeutic potential in AD (Leinenga and Gotz 2015). In humans, in a small study, focused ultrasound opened the barrier in the cortices of five patients with early AD with no obvious adverse long-term effects (Lipsman et al. 2018). Overall, focused ultrasound transiently affected neurologic function, but the functional organization was restored the next day and remained unchanged 3 months after. These first in human data have implications for the development of focused ultrasound as a drug delivery platform to the brain and its potential use. However, larger trials have to determine the method's efficacy and potential side effects. In mice, it has been shown that drug delivery works. A combination of an anti-tau antibody analog plus scanning ultrasound restored memory better than either the antibody or ultrasound alone (Nisbet et al. 2017).

We are just beginning to understand other possibilities that might enhance drug delivery to the brain. The expression of many blood-brain barrier genes is highly controlled by the circadian clock. For example, the expression of the drug efflux transporter P-gp (Zhang et al. 2018) and the tight junctional protein claudin-5 (Hudson et al. 2019) are highly dynamic and claudin-5 protein levels change up to 30–40% dependent on the time of day. Both proteins are tight controllers of blood-brain barrier passage and thus one could imagine that timing of drug administration in patients can alter the efficacy for brain delivery, even without physically altering the blood-brain barrier.

To date, targeting A β with anti-A β antibodies are a major treatment strategy. Just recently, it was announced that Biogen submitted a biologics license application to the FDA for aducanumab (Schneider 2020), a therapeutic antibody developed to clear amyloid plaques from the brain. While its efficacy in a robust improvement of cognitive decline is still debated due to inaccuracies in the conduction of the Phase 3 trial and therefore inconclusive findings, aducanumab's ability to remove brain amyloid is unquestioned and the medical need enormous. If successful, aducanumab would be the first anti-amyloid treatment – and the first new drug for AD in more than 16 years in the US, since Namenda was approved in 2003. Other pharmaceutical companies are also trying to develop anti-A β antibodies, which are currently tested in clinical trials. An improvement of brain uptake is facilitated by targeting receptors at the blood-brain barrier such as the TfR.

A recent study reports the development of Anti-BACE1 antibodies with an engineered fragment in the constant region of a human IgG1 (Kariolis et al. 2020). This engineered fragment binds to the apical domain of the human TfR at a site that is distinct from its binding sites for transferrin, a natural ligand for TfR and FcRn. Thus, these novel anti-BACE1 antibodies with engineered constant regions showed substantially enhanced brain uptake and decreased A β levels in mice and non-human primates.

Several therapeutic approaches target neuroinflammation in the AD brain. The abovementioned RAGE antagonists, currently tested in clinical trials, are not only believed to prevent the reuptake of circulating plasma A β into the brain but also regulate multiple other effects, including inflammatory processes, oxidation stress, and cerebral blood flow (Burstein et al. 2018). Other strategies target the triggering receptor expressed on myeloid cells 2 (TREM2) in microglia. By activation of this receptor, enhanced removal of amyloid plaques should be achieved. The TREM2-activating antibody AL002, developed by Alector and Abbvie, entered early clinical trials in 2019. In recent years, peroxisome-proliferator-activated receptor gamma (PPAR γ) has come to be a target of interest for AD drug development. PPAR γ activation has been shown to increase microglial A β phagocytosis and decrease cytokine release (Mandrekar-Colucci et al. 2012; Yamanaka et al. 2012). AD-4833 (Pioglitazone), a PPAR γ agonist, is currently tested in clinical trials.

Similar to A β , tau is targeted by either passive immunotherapy or by trying to prevent its aggregation with similar strategies. However, until today none of the clinical trials have led to a major breakthrough.

Looking at current targets for AD treatment, it seems that the blood-brain barrier with all its potential for therapeutic intervention is neglected. Up until recently, tools to modify specific gene expression at the blood-brain barrier had been lacking. However, using novel techniques, recently identified brain endothelial-specific targets or ways to manipulate the brain endothelium, which might be useful for the development of treatment strategies in the future. However, even today, we still do not know the causes of AD, which is crucial for the development of efficient treatment strategies. Therefore, it should be the major goal to improve our general understanding of pathophysiological pathways underlying disease progression. In this chapter, we have mainly focused on amyloid transport across the blood-brain barrier but also highlighted that AD is a multifaceted disease. The complexity and the various pathways that contribute to disease progression are beyond current understanding. It has been shown that in most AD cases, clearance pathways are affected. Moreover, in recent years, many novel AD risk factor genes have been discovered that affect A β clearance.

It remains uncertain whether targeting the blood-brain barrier will help to treat, prevent, or slow down AD pathology. However, the barriers of the CNS are crucial mediators for proper brain function. Therefore, neutralizing the alterations during aging and disease progression are likely to be beneficial for brain health.

7 Summary and Future Perspectives

The different cell types comprising the neurovascular unit act in concert as a gatekeeper and regulator of brain homeostasis ensuring proper brain function. In AD, however, this delicate system is disturbed and changes occurring at the blood-brain barrier, in astrocytes, pericytes, and neurons result in neurovascular dysfunction which has a massive impact on brain health. There is increasing evidence that neurovascular function is important for information processing, neuronal connectivity, and synaptic function suggesting a link between neurovascular dysfunction and memory decline seen in AD. To date, it is unknown whether barrier dysfunction is a cause or consequence of AD. As described in this chapter, studies support the hypothesis that vascular impairment precedes neurodegeneration suggesting that targeting the blood-brain barrier has the potential to slow the progression of the disease. In this regard, novel approaches are needed that specifically target mechanism(s) underlying blood-brain barrier dysfunction in AD.

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Blood–Brain Barrier Mechanisms in Stroke and Trauma

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Abstract

The brain microenvironment is tightly regulated. The blood–brain barrier (BBB), which is composed of cerebral endothelial cells, astrocytes, and pericytes, plays an important role in maintaining the brain homeostasis by regulating the transport of both beneficial and detrimental substances between circulating blood and brain parenchyma. After brain injury and disease, BBB tightness becomes dysregulated, thus leading to inflammation and secondary brain damage. In this chapter, we overview the fundamental mechanisms of BBB damage and repair after stroke and traumatic brain injury (TBI). Understanding these mechanisms may lead to therapeutic opportunities for brain injury.

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1 Pathophysiology of BBB Disruption After Brain Injury

Traumatic brain injury (TBI) and stroke comprise the two major forms of acute brain injury. TBI affects individuals of all ages, and results in complex neuropathology and functional damage. Stroke is a cerebrovascular disease that mainly occurs in elderly populations and is a major cause of neurological disability (Kearney et al. 2005; Lawes et al. 2008). Although these brain pathologies are initiated by different triggers and processes, many clinical and experimental studies suggest that blood–brain barrier (BBB) dysfunction is a common and key pathological feature in both TBI and stroke. Hence, it is important to develop an understanding of the cellular and molecular events that affect the BBB after traumatic and ischemic brain injury.

1.1 Time Course of BBB Dysregulation

The time course of BBB dysfunction is important because this significantly affects the progression of tissue damage as well as influences the therapeutic time window for potential interventions. Interestingly, in spite of the different initial causes of injury, a similar biphasic temporal pattern of early and late BBB impairment is seen after both TBI and stroke.

1.1.1 Time Course of BBB Dysregulation in TBI

Findings from clinical and experimental studies suggest that BBB damage after TBI proceeds in two stages (Chodobski et al. 2011; Price et al. 2016). In the acute phase, traumatic force causes stretching of microstructures, including axons and vessels, and leads to shearing injury, disruption of endothelial tight junctions, and enlarging of intercellular spaces. These injuries lead to further damage in brain structures, often resulting in contusions and/or hematomas, which further compress the microvasculature and decrease blood flow. These vascular changes are short-lived, lasting only several hours after injury, but they occur in vessels of various size and result in BBB dysregulation. Thereafter, secondary injuries are triggered several days after trauma, comprising complex events of cell death, transporter and junctional perturbations, cellular hyper-excitability, inflammation, and edema. These changes in the microvascular environment and disruption of BBB result in the dysregulation of normal neurovascular coupling (Shlosberg et al. 2010) and further brain dysfunction.

1.1.2 Time Course of BBB Dysregulation in Stroke

There are two types of stroke: ischemic and hemorrhagic, both of which are characterized by a sudden reduction in local cerebral blood flow. A large number of clinical and experimental studies have been performed for ischemic stroke, as it

comprises 80–90% of human stroke. Although stroke pathophysiology is highly heterogeneous and may differ between animal species, experimental models, detection methods, or ischemic severity, it is commonly thought that a similar biphasic time course of BBB dysregulation also exists in stroke (Kuroiwa et al. 1985); hyperpermeability in the early phase (4–6 h after ischemia) and delayed BBB opening in the following 2–3 days (Kuroiwa et al. 1985; Rosenberg et al. 1998). In the acute phase, cerebral blood flow is impeded by clots and/or microthrombi. Hypoxia and Adenosine triphosphate (ATP) loss induce hypoxia inducible factor-1 α (HIF-1 α), leading to an increase in activated Matrix Metalloproteinase 2 (MMP-2), causing BBB opening and vasogenic edema several hours after the onset of injury (Asahi et al. 2000, 2001; Belayev et al. 1996; Yang and Rosenberg 2011; Candelario-Jalil et al. 2009). This early BBB opening by MMP-2 is transient and reversible. Reperfusion-related metabolites, such as free radicals, also contribute to damage of endothelial cells and lead to primary BBB injury. Subsequently, inflammatory cytokines, such as Tumour Necrosis Factor α (TNF α) or Interleukin 1 β (IL1 β), mainly secreted from activated microglia in the ischemic core or the perinfarct area, induce activated MMP-9 and lead to secondary BBB damage (Asahi et al. 2000; Seo et al. 2012; Maki et al. 2013). This secondary impact after stroke is more severe than the initial changes and are irreversible; similar to the secondary damage after TBI.

1.2 Cell–Cell Interactions in BBB Dysregulation

The maintenance of BBB homeostasis is subserved by cell–cell interactions within the neurovascular unit, comprising multiple cell types from vascular and glial compartments. Endothelial cells, which form the basic element of the BBB, are strongly associated with pericytes and astrocytes. These interactions are important for maintaining BBB integrity, and in doing so, they play a crucial role in regulating the extracellular environment of neural cells (Blanchette and Daneman 2015; Guo et al. 2012). In the context of BBB leakage, disrupted crosstalk between endothelium, pericytes, and astrocytes is likely to play a key role.

1.2.1 Endothelial–Pericyte Interactions

Endothelial cells play a central part in BBB regulation after both TBI and stroke. In the ischemic penumbra after stroke, endothelial cells undergo proliferation and capillary sprouting, constituting the process of angiogenesis, starting days after ischemia and lasting weeks (Krupinski et al. 1994; Moisan et al. 2014). Pericytes are important cells that contribute to BBB function, and endothelial–pericyte interaction is necessary for BBB regulation after injury. Structurally, endothelial cells and pericytes are embedded within a common basal lamina interface, and connect with each other in a peg and socket type interaction that involves tight junctions, gap junctions (Cuevas et al. 1984; Tilton et al. 1979), and N-cadherin/ β -catenin adherens junction (Gerhardt et al. 2000). The interactions enable pericytes to support endothelial cells and to contribute to maintain BBB integrity (Bobbie et al. 2010; Cai et al. 2017).

The importance of pericytes in maintaining endothelial barrier function is highlighted by the fact that pericyte-deficient mice have an increase in permeability of the blood–spinal cord barrier (BSCB) even in the absence of injury (Winkler et al. 2012). In the clinical setting, arteriovenous malformations of the vasculature show a reduction of pericyte markers along with extravasation of blood markers (Winkler et al. 2018). These findings support the important contribution of pericytes to the function of the BBB.

1.2.2 Endothelial–Astrocyte Interactions

Endothelial–astrocyte interactions also play an important role in maintaining the homeostasis of the brain microenvironment (Goldstein 1988). The astrocytic end-feet are in close contact with the surface of microvessels and their function varies depending on their location. Water channel aquaporin-4 and ion/volume regulation Kir4.1 K⁺ channel co-localize with the astrocytic end-feet that are anchored in the basal lamina, and contribute to regulation of neurotransmitters metabolism and secretion of neuroactive molecules. In normal brain, the high density of astrocytic aquaporin-4 improves redistribution of water (Rash et al. 1998). Under ischemic condition, however, BBB damage triggers astrocytic aquaporin-4 upregulation, and this upregulation leads to astrocytic swelling, which further increases brain edema and adversely affects the injured brain (Verkman 2002; Tomas-Camardiel et al. 2005) (*Related to 1.3.4 Edema*).

In addition to their role in BBB regulation, astrocytes may affect other key aspects of endothelial function. For example, astrocytes regulate the pro- and anti-coagulation properties of microvessels by secreting transforming growth factor- β (TGF β), which downregulates capillary endothelial expression of the anticoagulant, thrombomodulin and the fibrinolytic enzyme, tissue plasminogen activator (Tran et al. 1999). In the acute phase of stroke and brain trauma, astrocytes may increase endothelial injury through the astrocytic bradykinin-receptor2-NF-kappaB pathway (Schwaninger et al. 1999; Vandekerckhove et al. 1991), in which bradykinin, a mediator of inflammation, leads to the release of Interleukin 6 (IL-6), which can exacerbate endothelial damage. On the other hand, astrocytes may also have beneficial effects on the vasculature. In the peri-infarct area after stroke, astrocytes upregulate pentraxin-3, which supports BBB integrity by regulating Vascular endothelial growth factor-related mechanisms in acute phase of ischemic stroke (Shindo et al. 2016). Thus, under pathological condition, astrocytes may interact with endothelial cells via paracrine signaling in various beneficial and detrimental pathways (Schwaninger et al. 1999).

1.3 Common Events in BBB Dysregulation After TBI and Stroke

Although the initial events causing the brain injury in TBI and stroke are different, previous studies indicate that there is overlap in the downstream events of BBB pathology after TBI and stroke. Therefore, it is important to understand the common events in BBB injury in order to develop an effective therapeutic target.

1.3.1 Gene Expression

The complex and heterogeneous pathophysiology in stroke and TBI is associated with a tremendous amount of gene expression changes. Molecular profiling investigations of BBB breakdown after TBI and stroke (Lindgren 2014; Asano et al. 2016; Francis et al. 2007; Bennett et al. 2016) demonstrate that these disease entities widely affect BBB-related gene expression, as well as brain endothelial protein expression, also known as the “vasculome” (Guo et al. 2012, 2016). Recent research also suggests that the brain vasculome provides an important source of trophic signals to the parenchyma. Hence studies that map the brain vasculome could yield information on mechanisms of BBB dysfunction after stroke and trauma (Regenhardt et al. 2018; Vanlandewijck et al. 2018).

1.3.2 Immune Response

The immune system is also activated in the acute phase after TBI and stroke. Neutrophils have been generally recognized as having a detrimental effect on the brain during the acute response to TBI and stroke. Recent studies provide evidence that neutrophils have the ability to switch between a more pro-inflammatory and a less pro-inflammatory phenotype. Similar to the switch between M1 and M2 phenotypes in macrophage and microglia, the new concept of N1 and N2 phenotypes for neutrophils suggests that this switch may be a crucial event for resolution of inflammation (Cuartero et al. 2013).

Crosstalk between the immune system and the BBB is exemplified by the observation that activation of regulatory T cells can prevent protect BBB integrity after stroke (Mao et al. 2017). Regulatory T cells works to suppress excessive immune responses, which would ameliorate BBB damage after stroke. The number of circulating regulatory T cells in stroke patients was dramatically and temporarily reduced after stroke onset; however, stroke patients with t-PA administration showed a sustained suppression of regulatory T cells in the blood. In a mouse model of stroke, delayed tPA treatment caused hemorrhagic transformation, but when regulatory T cells were intravenously co-administered with tPA, hemorrhagic transformation was decreased. Therefore further understanding of the regulation of the immune system may lead to potent therapeutic targets for BBB protection after stroke and TBI (Choi 2017). The knowledge gained will help to address current obstacles in the application of immune targets to future therapies, including the transport of immune cells across the BBB (Enzmann et al. 2013), and differences between the rodent immune systems studied in the laboratory and the human immune system which will be important for translational therapies (Shay et al. 2013).

1.3.3 Inflammation

Normal BBB function involves continuous immune surveillance of pathogen invasion and when needed, recruitment of peripheral blood cells across the BBB via transendothelial leukocyte migration (Keaney and Campbell 2015). In response to ischemic stroke and trauma, brain endothelial cells become activated and release pro-inflammatory molecules and upregulate cell adhesion molecules such as

Vascular cell adhesion protein 1, Intercellular Adhesion Molecule 1, and E-selectin (Greenwood et al. 1995). These adhesion molecules interact with neutrophil integrins such as $\alpha 4\beta 1$, $\alpha M\beta 2$, and $\alpha L\beta 2$, and enhance neutrophil infiltration into the central nervous system (CNS) (Choi et al. 2009). Recent studies of human clinical samples and rodent models with TBI and stroke have demonstrated that increased influx of neutrophils and monocytes is associated with increased edema and brain tissue damage (Semple et al. 2010; Perez-de-Puig et al. 2015). A series of biological processes facilitate neutrophil-induced BBB damage, including the release of proteases, proteinases, and reactive oxygen species (ROS), causing endothelial dysfunction and disorganization of tight junction proteins (Li et al. 2018a).

Among these proteases, MMP-9 is considered to have a major role in ischemic brain injury because increased MMP-9 contributes to ischemic brain injury and perihematomal edema (Rosell et al. 2006). Neutrophils are a major source of MMP-9 and thereby contribute to further BBB injury (Borregaard et al. 2007). In a rat ischemic stroke model, neutrophil infiltration significantly increased MMP-9 in the ischemic brain by releasing the MMP-9 pro-form, thus enhancing the inflammatory reaction surrounding the injured areas (Justicia et al. 2003). In human brain samples from patients who have suffered ischemic and hemorrhagic stroke, higher levels of MMP-9 are found in the infarct core, mainly around blood vessels and associated with neutrophil infiltration and activated microglia (Rosell et al. 2006). In an in vitro study using cultured dorsal root ganglion cells that were treated with neutrophil-conditioned media, the addition of MMP inhibitors, ROS scavengers, or TNF α neutralizing antibodies improved cell survival (Nguyen et al. 2007). Whether these MMP and ROS mechanisms also operate in CNS neurons requires further study. In preclinical studies involving rodent models of TBI, treatment with monoclonal antibodies to CD11d/CD18 and CD11b/CD18 or to ICAM1 after injury has shown reduction of infiltrating neutrophils, a decreased loss of neural tissue, and an improvement of recovery (Clark et al. 1996; Weaver et al. 2000; Knoblich and Faden 2002; Utagawa et al. 2008). In ischemic stroke, the increases of ICAM-1, VCAM-1, and CD11B/CD18 were significantly inhibited by D-allose during Middle cerebral artery occlusion (MCAO)/reperfusion injury, which prevented the reduction of Zonula occludens-1 (ZO-1), occludin and claudin-5, and decreased BBB permeability (Huang et al. 2016).

There is no doubt that neutrophil-mediated inflammation, as a secondary injury response following TBI and stroke, is an important factor in neuronal injury. However, while many experimental studies indicated that therapies against excessive inflammation resulted in good outcome, clinical studies did not demonstrate the same degree of effectiveness (Ghajar and Hesdorffer 2004; Jones et al. 2005; Sandercock and Soane 2011; Llovera et al. 2015). These results suggest that the inflammatory response can have both beneficial and detrimental effects, and successful translational strategies must take both of these aspects into account. In part, these biphasic aspects of inflammation and BBB response may be related to microglia activation (discussed in Sect. 3.2 below).

1.3.4 Edema

Among the events following TBI and stroke, the development of cerebral edema is considered the most clinically important secondary consequence and a significant predictor of outcome. Although the pathophysiology regarding edema is complex, it is generally recognized that there are 2 types of edema: cytotoxic and vasogenic. Cytotoxic (intracellular) edema is most commonly seen in the acute phase of ischemic stroke, in which extracellular water goes into the cytoplasm and results in cellular swelling caused by dysregulation of ATP-dependent Na^+/K^+ membrane pumps and upregulation of astrocytic aquaporin-4 (*please see previous discussions on endothelial and astrocyte interactions*) (Simard et al. 2007). Vasogenic (extracellular) edema is mainly seen in as a secondary change after cytotoxic edema or in the ischemic area or in the area around a traumatic and hemorrhage lesion, in which the BBB is disrupted (Marmarou et al. 2006).

Cerebral edema following TBI or stroke affects the morbidity and mortality of the patients. Most importantly, post-traumatic and post-ischemic cerebral edema can cause increased intracranial pressure (ICP), since the combination of endothelial cell damage, tight junction disruption, and abnormal transcellular transport (Nag et al. 2007; Campbell et al. 2012) exacerbate brain swelling. Reducing ICP and maintaining cerebral perfusion pressure (CPP) are recommended in the Brain Trauma Foundation guidelines in 2017 (Carney et al. 2017) and American Heart Association (AHA) guideline for the early management of patients with acute ischemic stroke in 2018 (Powers et al. 2018; Wijdsicks et al. 2014). However, current treatments for increased ICP are limited, consisting mainly of mannitol (Sakellaridis et al. 2011), hypertonic saline, Cerebrospinal fluid (CSF) drainage by ventriculostomy, and hypothermia. Corticosteroid therapy has been tried, but there is no sufficient evidence of its therapeutic benefits in the setting of TBI and stroke (Roberts et al. 1998; Hutchison et al. 2008). The use of short interfering RNA against claudin-5 was proven effective in suppressing early BBB damage through increasing beneficial soluble factors in TBI model mice (Campbell et al. 2012). The study also demonstrated that the mice with claudin-5 siRNA treatment showed improved cognitive outcome after TBI, suggesting that early therapeutic protection of the BBB is important for prevention and treatment of edema, and for easing neurological deficits after TBI. Future therapeutic options would ideally include early interventions that prevent BBB deterioration (Jha et al. 2019).

1.3.5 Cell Death

In cerebral ischemia, oxygen and nutrient deprivation leads to pathological processes, such as BBB disruption, neuroinflammation, and apoptotic cell death (Moskowitz and Lo 2003; Lo et al. 2003, 2005). The detrimental effects of oxygen depletion involve the inhibition of ATP production, which leads to the failure of ATP-dependent ion transport. These deficiencies result in an increase in intracellular calcium concentrations, resulting in cellular over-excitation, cytotoxic edema (*Related to 1.3.4 Edema*), and apoptotic cell death. ATP-binding cassette (ABC) transporters have also been investigated in pathological conditions following TBI/stroke. ABCC8, which is known as sulfonylurea receptor 1 (SUR1), does not

have transport functions, but regulates the activity of the Ca^{2+} -activated, ATP-sensitive nonselective cation (NCCa-ATP) channel (Chen et al. 2003). This SUR1-regulated NCCa-ATP channel is activated by ATP depletion, and causes endothelial swelling/death in the injured CNS. It is linked with the transient receptor potential melastatin 4 (Trmp4), a nonselective cation channel, which induces astrocyte swelling after TBI (Chen et al. 2003). These studies suggest that optimal strategies for stroke or TBI would involve combination therapies that target the different cell types that form the BBB and the neurovascular unit (Lo et al. 2003).

2 Molecular Mechanisms of BBB Dysfunction in Stroke and TBI

In rodent models, both stroke and TBI are involved with dysfunction of BBB following the initial insult (Keaney and Campbell 2015). Common cascades of events have been described, including hypoxia and reoxygenation, glutamate release, and ionic disturbances, that all lead to BBB breakdown (Prakash and Carmichael 2015; Lochhead et al. 2017). It has been demonstrated that under pathological conditions, alteration of junctional proteins in cerebral vascular endothelial cells is associated with an increase in BBB permeability (Wolburg and Lippoldt 2002). Another important feature related with BBB disruption is that inherently low transcytosis rates of CNS endothelial cells (ECs) are increased after stroke and trauma, contributing to enhanced vascular permeability (Siegenthaler et al. 2013).

2.1 Paracellular Mechanisms

Junctional proteins, including tight junctions (TJs) and adherens junctions (AJs), form a circumferential zipper-like seal between adjacent endothelial cells, maintaining distinct tissue spaces through separation of the luminal side from the abluminal side of the plasma membrane (Sandoval and Witt 2008; Lu et al. 2018). Hypoxia/reoxygenation and systemic inflammation induce disruptions in cerebrovascular junctional proteins after stroke or trauma (Petty and Lo 2002; Witt et al. 2008). As a major reason underlying increased paracellular permeability of the BBB after stroke or trauma, dysregulation of junctional proteins warrants attention.

2.1.1 Down-Regulated Expression of Junctional Proteins

After both stroke or trauma, a temporary or permanent decrease of blood supply to specific brain region induces the reduction of glucose and oxygen supply to brain, which results in a rapid decline in ATP and Ca^{2+} movement (Sims 1995; Dohmen et al. 2008). Ionic imbalances including changes in Zn^{2+} then accumulate in cerebral microvascular compartments, which activates matrix metalloproteases (MMPs), a family of endopeptidases that can proteolytically degrade various components of extracellular matrix (ECM) including collagen, fibronectin, laminin, and proteoglycans (Klein and Bischoff 2011). Proper control of MMPs helps to maintain

the dynamic integrity of BBB by remodulation of ECM (Seo et al. 2012), and once MMPs are over-activated under the conditions of stroke and TBI, cerebral microvascular basement membrane proteins are degraded, leading to the disruption of brain endothelium stability followed by increases of BBB permeability (Yang et al. 2007). In the early stages of ischemic stroke, upregulation of MMP-2 and MMP-9 exacerbate the loss of TJs on ischemic microvessels, including Occludin and Claudin-5 (Rosenberg et al. 1992; Abdul-Muneer et al. 2016). Similarly, after blast-induced mild TBI, MMP-3 and MMP-9 are activated within 24 h, which degrade perivascular units and reduce the expression of TJs-ZO-1, Occludin and Claudin-5, leading to BBB leakiness that causes immune cell infiltration to activate inflammatory responses in the neurovascular unit (Abdul-Muneer et al. 2013a). In transgenic mice overexpressing the tissue inhibitor of metalloproteinase (TIMP-1), after controlled cortical impact (CCI), 24 h-MMP-9 levels were significantly reduced (Tejima et al. 2009). At 7 days post-trauma, brain lesion volumes were also significantly decreased by TIMP-1 overexpression (Tejima et al. 2009). Similar results were also observed in a model of transient 2 h focal cerebral ischemia (Tejima et al. 2009). For in vitro studies, primary human brain microvascular endothelial cell cultures were subjected to hypoxia and IL-1 β , and the expressions of junctional proteins-VE-cadherin, ZO-1, Occludin and Claudin-5 were all significantly decreased (Li et al. 2018b).

2.1.2 Translocation of Junctional Proteins

Junctional proteins translocated from ECs membrane to cytoplasm are detected during the early phase of stroke and TBI, and subsequently aggravate brain injury. In brain microvascular endothelial cells (BMECs), after oxygen-glucose deprivation (OGD) for 90 min and reperfusion with glucose for 30 min, glucose causes significant decrease in TJs expression and particularly ZO-1 redistribution from membrane to cytoplasm of BMECs (Lv et al. 2018). As stated in the last paragraph, hypoxia and IL-1 β induced the translocation of VE-Cadherin and ZO-1 in cultured human brain microendothelial cells, resulting in the compromised transendothelial integrity (Li et al. 2018b). Consistent with these in vitro findings, using fluorescence tracer extravasation, increased interstitial MMP-2, Occludin protein reduction and Claudin-5 redistribution are also detected in ischemic cerebral microvessels. This leads to BBB disruption at early stroke stages, which is relevant to acute thrombolysis because the early disruption of BBB leakage after reperfusion is due to structural changes of endothelium–pericyte–astrocyte interactions (Liu et al. 2012). Under physiological conditions, BMECs are fused by intercellular junctional proteins that are anchored to the actin cytoskeleton, which is important for maintaining junctional proteins on the cell membrane. Therefore, dynamic interactions between the cytoskeleton and junctional proteins are important for junctional proteins translocation (Shi et al. 2016). The very early disruption of the BBB in experimental stroke may indicate early structural alterations in BMECs, including actin polymerization, which results in junctional proteins redistribution, damaging the BBB before the onset of junctional and ECM degradation by MMP-9 (Shi et al. 2016). Experiments involving primary cultures of murine brain endothelial cells have shown that

chemokine ligand 2(CCL2) which is rapidly increased by the Rho signaling cascade after stroke and trauma increases the paracellular permeability of endothelial monolayers by inducing the formation of actin stress fibers and causing the redistribution of ZO-1, ZO-2, Occludin and Claudin-5 (Stamatovic et al. 2003). Moreover, several studies have demonstrated the importance of protein kinase C (PKC) pathway in regulating junctional proteins-cytoskeleton connection and cellular localization of junctional proteins (Andreeva et al. 2001; Rao et al. 2002). nPKC- θ and aPKC- ζ are activated in a global hypoxia rat model, and nPKC- θ and aPKC- ζ activation modulate distributions of Claudin-5, Occludin and ZO-1 at endothelial cell margins during hypoxia and posthypoxic reoxygenation (Willis et al. 2010). These molecules and pathways induce translocation of junctional proteins from extracellular cell-cell contacts to the cytoplasm, loosening the paracellular pathway (Jiang et al. 2018).

2.2 Transcellular Mechanisms

In addition to specialized junctional proteins between BMECs that strictly limit movement of ions and molecules within the paracellular spaces, the BBB also contains low rates of transcellular pathways (Siegenthaler et al. 2013). The transcellular transport systems in the BBB restrict the CNS entry and exit of hydrophilic molecules (Keaney and Campbell 2015). Carrier-mediated transport is the major route to transport essential nutrients, such as glucose, amino acids, and fatty acids into the CNS (Abbott et al. 2010). Recent studies are revealing the importance of maintaining low rates of transcytosis at the healthy BBB (Ayloo and Gu 2019). Under pathological condition, BBB-enriched components not only transport essential molecules, but are also important regulators of BBB permeability (Keaney and Campbell 2015).

2.2.1 BBB Transporters

The BBB possesses several functional transporters that play critical roles in maintaining brain homeostasis. After stroke or TBI, transporters may become dysregulated, thus exacerbating the of brain microenvironment homeostasis (Villasenor et al. 2019). Here, we will review the roles of two types of BBB transporters – nutrient transporters and ABC transporters.

Nutrient Transporters

Nutrient transporters play a critical role in brain uptake of amino acid brain uptake. They are governed by either an electrochemical gradient utilizing an inorganic or organic solute as a driving force or the transmembrane concentration gradient of the substance actually being transported (Ronaldson and Davis 2015). Mfsd2a (major facilitator superfamily domain-containing protein-2a), which was considered as an orphan transporter, has recently gained attention for its regulatory role in the maintenance of BBB formation and integrity (Nguyen et al. 2014; Ben-Zvi et al. 2014). Genetic ablation of Mfsd2a results in a leaky BBB from embryonic stages through adulthood, but the normal patterning of vascular networks is maintained

(Ben-Zvi et al. 2014). Further, patients with *Mfsd2a* mutations exhibited a lethal microcephaly syndrome linked to inadequate uptake of lysophosphatidylcholine (Guemez-Gamboa et al. 2015). In zebrafish model, the mutation in *Mfsd2a* caused the disruption of BBB integrity (Guemez-Gamboa et al. 2015), further supporting the role of *Mfsd2a* in BBB tightness. In an intracerebral hemorrhage (ICH) model, *Mfsd2a* protein expression in perihematoma brain tissues was significantly decreased after ICH. Knockdown and knockout of *Mfsd2a* in mice markedly increased BBB permeability, neurological deficits and brain water content after ICH, and these were rescued by overexpressing *Mfsd2a* in perihematomas (Yang et al. 2017a). Moreover, the regulatory effect of *Mfsd2a* on BBB permeability after ICH correlated with changes in vesicle number (Yang et al. 2017a). There are no differences in expression of junctional proteins in *Mfsd2a* knockdown, *Mfsd2a* knockout, and *Mfsd2a* overexpression mice (Yang et al. 2017a), supporting the idea that *Mfsd2a* regulates the BBB function through vesicular transcytosis across the cerebral endothelium. A recent *in vitro* study reveals that endothelial *Mfsd2a* expression can be inhibited by miR-155, and reduced *Mfsd2a* may lead to the increased permeability in cultured endothelial cells (Awad et al. 2018).

ABC Transporters

ABC transporters contain 48 members grouped into 7 sub-families according to structural homology. For BBB function, the most significant are P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs), and breast cancer resistance protein (BCRP). Among them, the roles of P-gp and MRPs in BBB function/dysfunction have been studied in stroke and TBI, and this section will focus on these two sub-families of ABC transporters.

P-gps transport a diverse range of lipid-soluble compounds out of the brain capillary endothelium and CNS, thus limiting the ability of these substances to accumulate in brain parenchyma (Tome et al. 2016; Abdullahi et al. 2017). In experimental rat models, P-gp, predominantly expressed in brain microvascular at baseline, is reduced 2 months after CCI (Pop et al. 2013). Using mice MCAO model, P-gp is upregulated on capillary endothelium after focal cerebral ischemia (Spudich et al. 2006). In another rat permanent MCAO model, elevated expression of P-gp at the BBB and decreased concentration of P-gp substrates in the ischemic brain tissues were observed within 4 h after MCAO. However, at 6 h after MCAO, the concentration of P-gp substrates in the ischemic hemisphere began to rise even though the expression of P-gp was still increased. Moreover, the expression of claudin-5 was decreased; contrarily, the expression of MMP-2, MMP-9, TNF- α as well as nitric oxide synthase (NOS) gradually increased within 6 h after MCAO (Cen et al. 2013). Taken together, these studies suggest P-gp plays a crucial role in limiting the entrance of agents into the brain after brain injury and the specific regulation of P-gp may provide an important approach for the improvement of pharmacotherapy (Spudich et al. 2006; Cen et al. 2013).

MRPs contain 13 members including ion channel, surface receptors, and truncated protein. The increased expression of MRPs at the BBB could actively efflux the endogenous antioxidant glutathione (GSH), resulting in reduced

endothelial cell concentrations of GSH, an alteration in cellular redox status, and increased potential for cerebral endothelial cells injury and death (Ronaldson and Davis 2015). A previous study has shown that MRP1 levels are mildly reduced in response to focal cerebral ischemia. In isolated brain microvessel fractions, western blot revealed that MRP1 expression decreased at 3 h and partly recovered within 24 to 72 h after stroke (Kilic et al. 2008). However, in human neocortex after TBI, the expression of MRP1 in brain microvessels is increased (Willyerd et al. 2016). Therefore, changes in MRPs activity at the BBB in response to brain injury and diseases warrant further investigation.

2.2.2 Transcytosis

Caveolin proteins are major regulators of a variety of intracellular signaling pathways. In caveolae, caveolin-1 is the major transcytosis molecule that is important for the uptake of a number of macromolecules into the brain (Simionescu et al. 2009). Previous study has reported that caveolin-1 was down-regulated and the production of nitric oxide (NO) induced the loss of caveolin-1 in focal cerebral ischemia and reperfusion injury (Shen et al. 2006). Moreover, caveolin-1 plays important roles in regulating MMP activities and BBB permeability in focal cerebral ischemia and reperfusion injury (Gu et al. 2012). However, other studies also showed that after MCAO, the expression of caveolin-1 was increased in new blood vessels in the lesion and in reactive astrocytes in the peri-lesion areas (Blochet et al. 2018). In caveolin-1 knock out (KO) mice, the lesion volume was larger and the behavioral outcome was worse than in wild type mice (Blochet et al. 2018).

In a rat TBI model, caveolin-1 expression profiles in blood vessels after TBI parallel the resolution of the BBB disruption using Claudin-5 and P-gp expressions as markers (Badaut et al. 2015). Using a CCI model of TBI, a study also showed that caveolin-1 and caveolin-3 KO mice exhibited enhanced lesion volume and cytokine/chemokine production after CCI (Niesman et al. 2014). In TBI patients, the level of caveolin-1 in the brain of all patients was higher than that of the control group at 8 h, 24 h, 48 h, 72 h and 1 week after the onset (Wang et al. 2017). Moreover, the expression of caveolin-1 in brain was related to the severity of craniocerebral injury (Wang et al. 2017).

Another important mechanism of endothelial transcytosis is clathrin-mediated endocytosis. This is a primary endocytic mechanism with central importance for nutrient uptake, cell signaling, cell adhesion, migration and morphogenesis, as well as for neurotransmission, among many other processes (Haucke and Kozlov 2018; Kaksonen and Roux 2018). In TBI, activated capillaries show predominantly an enhanced abluminal orientated vesicular transport by means of small, medium, and large uncoated and clathrin-coated vesicles (Castejon 2013). With in vitro experiments, lipopolysaccharide (LPS), a major pathogenic factor involved in neuroinflammation, induces human vascular endothelial cells hyperpermeability (Zhang et al. 2014). Clathrin-mediated endocytosis was dominant early after LPS treatment, and caveolae-mediated endocytosis was dominant hours after LPS treatment (Zhang et al. 2014). Moreover, the authors also demonstrated that structural changes in the actin cytoskeleton, specifically from polymerization to

depolymerization, were important reasons for the switching of the VE-cadherin endocytosis pathway from clathrin-mediated to caveolae-mediated (Zhang et al. 2014).

3 Therapeutic Interventions for BBB Dysfunction After TBI and Stroke

The clinical relevance of many molecular mechanisms of BBB dysfunction in stroke and TBI remains to be fully translated (Obermeier et al. 2013). However, increasing evidence suggests that oxidative stress and neuroinflammation are major contributors to dysfunction of paracellular and transcellular pathways in stroke and TBI. Hence, various antioxidant and anti-inflammatory therapies are currently under investigation (Chodobski et al. 2011; Madden 2012).

3.1 Antioxidant BBB Therapies for TBI and Stroke

Antioxidant therapies have been reported to protect the BBB, reduce brain edema, and improve neurological outcome in rodent models of TBI or stroke, as well as in clinical and TBI or stroke patients in clinical trials.

In the setting of brain trauma, ROS and reactive nitrogen species (RNS) are generated and contribute to oxidative stress in the brain (Abdul-Muneer et al. 2015). Superoxide ($O_2^{\cdot-}$), a free radical produced mostly in the mitochondria by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) (Zhang et al. 2012), is another mediator of oxidative injury (Sastre et al. 2003). Nox is a target of many antioxidant therapies under investigation, with the goal of inhibiting downstream production of $O_2^{\cdot-}$. This is an active area of research, utilizing in vitro models of BBB (Rochfort et al. 2014) as well as in different kinds of in vivo models of TBI (Wang et al. 2016; Choi et al. 2012; Yuan et al. 2016a; Kim et al. 2013; Abdul-Muneer et al. 2013b) and stroke (Kahles et al. 2007; Kleinschnitz et al. 2010).

An additional strategy to reduce oxidative stress after TBI (Gao et al. 2017) involves inducing mitochondrial autophagy, thereby reducing cellular apoptosis and the oxidative stress associated with apoptosis. During oxidative injury, hydroxyl radicals interact with membrane lipids in cells that form the BBB, leading to disruption of the BBB and increased permeability (Hall et al. 2010; Mertsch et al. 2001; Tolia et al. 2004). Administration of the lipid peroxidation inhibitor tirilazad mesylate was shown to attenuate post-injury damage of the BBB in a rat CCI model (Smith et al. 1994) as well as in a mouse stroke model (Hoda et al. 2009; Logsdon et al. 2018; Yuan et al. 2016b). Post-injury administration of a NOS inhibitor, N(G)-nitro-L-arginine methyl ester (L-NAME), also decreased BBB disruption in rodent models of TBI (Logsdon et al. 2018) and stroke (Mohammadi et al. 2011, 2012).

Another method under investigation for protecting the BBB involves the use of hyperoxia, either under normobaric conditions (Liu et al. 2011) or hyperbaric conditions (Rockswold et al. 2010). In several clinical trials of TBI (Tolia et al.

2004) and stroke (Mazdeh et al. 2015), normobaric hyperoxia therapy reduced intracranial pressure and improved the outcome of the patients. One of the mechanisms is believed to be BBB protection by inhibition of NADPH oxidase, which has been demonstrated in a mouse model of ischemic stroke (Liu et al. 2011, 2008). Hyperbaric oxygen has been shown to stabilize brain mitochondrial function in rats after CCI (Palzur et al. 2008), reduce brain edema, and improve BBB integrity. It also reduced intracranial pressure in a rabbit model of CCI rabbits (Wei et al. 2014) and rodent models of stroke (Veltkamp et al. 2005; Michalski et al. 2011). Hyperbaric oxygen therapy has been reported to have beneficial effects in several clinical trials for treatment of stroke and TBI (Deng et al. 2018). However, oxygen therapy, in both normobaric and hyperbaric conditions, has potential side effects such as acute cerebral toxicity and increased production of ROS. Therefore, it is important to optimize the duration of hyperbaric oxygen therapy to minimize the detrimental effects (Eve et al. 2016).

In addition to pre-clinical studies of anti-oxidant therapies, there are clinical studies, which explore the anti-oxidant actions of several substances that are in clinical use for repurposing. These include dexamethasone, vitamins C and E, progesterone, N-acetylcysteine, and enoximone, which have been used in clinical trials for TBI (Shen et al. 2016). Vitamins C and E appeared to decrease brain edema in TBI (Razmkon et al. 2011). Edaravone, a free radical scavenger, was also reported to suppress brain edema and to improve functional outcome in patients with acute cerebral ischemia (Houkin et al. 1998; Edaravone Acute Infarction Study Group 2003). In an acute ischemic stroke clinical trial, sodium tanshinone IIA sulfonate (STS), an inhibitor of store operated calcium entry (SOCE), was found to inhibit oxidative stress (Han et al. 2018), ameliorate BBB damage, and improve outcome in acute ischemic stroke patients (Ji et al. 2017). Mild hypothermia is another therapeutic approach which is thought to have anti-oxidant effects by reducing nitric oxide expression, and is currently used in the treatment of patients with TBI (Lewis et al. 2017) and stroke (Hong et al. 2014; Piironen et al. 2014; Ma et al. 2017). It has been shown to reduce edema after TBI (Eroğlu et al. 2017) and to increase the rate of successful recanalization after strokes (Hong et al. 2014).

In conclusion, antioxidant therapies show promise in the treatment of brain injury after stroke and brain trauma. Their actions support mitochondrial function and inhibit the formation or actions of ROS or RNS, and may protect the BBB and reduce edema after TBI and stroke.

3.2 Anti-inflammatory BBB Therapies for TBI and Stroke

Inflammation is an important contributor to the pathophysiology of brain injury after trauma and stroke and is in large part mediated by microglia. Microglia may become activated within a few minutes after injury and can remain activated for weeks to months after injury (Kabba et al. 2018). Microglia activation results in the expression of many inflammatory mediators including cytokines (IL-1, IL-6, TNF- α), chemokines (CCL2, CX3CL1, MIP-1), proteases (MMPs), and free radicals

(Thurgur and Pinteaux 2019). Microglia that are activated to express these pro-inflammatory proteins are referred to as having an M1 phenotype. In addition to the M1 phenotype, microglia may also assume an M2 phenotype, which expresses anti-inflammatory cytokines, including IL-10, TGF- β , and arginase 1 (Arg1) (Thurgur and Pinteaux 2019). Promoting microglia polarization toward the M2 phenotype significantly reduced BBB degradation in rats after ischemic stroke rats (Yang et al. 2015) and demonstrated strong neuro-protective function by reducing brain edema in mice after TBI (Lv et al. 2018; Mazdeh et al. 2015; Braun et al. 2018). The polarization of microglia into different phenotypes may be affected by up-stream transcription factors – signal transducer and activator of transcription 1 (STAT1) and interferon-regulatory factor 5 (IRF5) promote M1 polarization, while the peroxisome proliferator-activated receptor- γ (PPAR- γ), STAT6, and IRF4 promote the M2 polarization (Lawrence and Natoli 2011). Further research into the regulation of microglia phenotypic changes may be a useful tool in the modulation of the inflammatory response after brain injury.

Among cytokines released by microglia, IL-1 is a major regulator of neuroinflammation in TBI. Both IL-1 α and IL-1 β levels are increased at 3 h following TBI (Shojo et al. 2010) and 2 h after cerebral ischemia (Zhai et al. 1997). IL-1 binds to the type 1 IL-1 receptor (IL-1R1) which is expressed in brain endothelial cells (Mazdeh et al. 2015), and induces a downregulation of ZO-1 and occludin (Konsman et al. 2004; Kangwantas et al. 2016). The deleterious effects of IL-1 could be reversed by treatment with laminin (LM-10) or with an IL-1 receptor antagonist (IL-1ra) (Kahles et al. 2007; Newell et al. 2018; Zhang et al. 2017), both of which led to a reduction of brain edema and of neuroinflammation following TBI and stroke. TNF- α is another cytokine which is released at early time points after TBI and stroke, at 3 h following TBI (Lang et al. 2015) and 2 h after stroke (Zhai et al. 1997; Qiu et al. 2008). It binds to the TNF receptor type 1 (p75) on endothelial cells and is associated with increased BBB permeability during inflammatory conditions (Nishioku et al. 2010). Treatment with a neutralizing antibody to TNF- α , as well as therapies that inhibit overexpression of TNF- α were found to reduce brain edema in rats after CCI (Mashhadizadeh et al. 2017) and MCAO (Khaksar and Bigdeli 2017). These results suggest that an effective approach may be the use of agents that inhibit the overexpression of both TNF- α and IL-1 (Hall et al. 2010; Hashimoto et al. 2018; Li et al. 2018c; Gao et al. 2018). In addition to cytokines, some chemokines are increased after brain injury, including CXCL1, CXCL2, CCL2, CCL4, and CCL5 (Yang et al. 2017b), which are expressed by neutrophils. CCL2 expression is increased in the following models of injury: an in vitro stretch injury model, an in vivo TBI model (Yang et al. 2017b; Salvador et al. 2015), and a mouse MCAO (Strecker et al. 2013) model, and decrease of CCL2 results in a reduced BBB-leakage and reduced infarct sizes after cerebral ischemia (Strecker et al. 2013).

The actions of MMPs constitute another avenue by which inflammation causes BBB injury. MMP-2 and MMP-9 are activated as early as 3 h after TBI and 2 h after MCAO in mice (Amantea et al. 2007), and disrupt the BBB by degrading the extracellular matrix of the BBB (Liu et al. 2008; Palzur et al. 2008). MMP-9 knockout (Amantea et al. 2007; Wang et al. 2000), knockdown (Muradashvili

et al. 2015) or intravenous injection with MMP-9 Inhibitor SB-3CT (Liu et al. 2012; Lee et al. 2015) significantly reduced BBB degradation and edema, and improved the neurological function of mice after TBI and MCAO.

Astrocytes also participate in inflammatory pathways that may be associated with both neuroprotection and neurodegeneration following TBI (Karve et al. 2016) and stroke (Pekny et al. 2014). Reactive astrocytes can produce pro-inflammatory cytokines, chemokines, and MMPs, all of which can disrupt the BBB by degrading the extracellular matrix, causing neuroinflammation (Carpentier et al. 2005). Reactive astrocytes have also been shown to prevent the infiltration of inflammatory cells into brain areas with intact cortical neurons (Karve et al. 2016). The severity of CCI may influence the functions of reactive astrocytes after TBI – ablation of reactive astrocytes after moderate CCI in transgenic mice led to inflammation and neuronal degeneration after moderate CCI, but not in severe CCI (Myer et al. 2006). Another indication that astrocyte play a role in BBB damage after stroke is that knockout of astrocytic Na^+/H^+ exchanger isoform 1 significantly reduced astrogliosis, inhibited BBB damage, and improved neurological function after ischemic stroke in mice (Begum and Song 2018).

Pericytes constitute another group of cells with actions that may be beneficial or detrimental to BBB integrity. Pericytes affect the BBB in different ways depending on the particular physiological condition. For example, pericytes help to maintain endothelial tight junction integrity under in vitro hypoxic conditions (Jackson et al. 2017). When endothelial cells alone were exposed to hypoxic conditions, their permeability to Evans blue-albumin and sodium fluorescein increased significantly; but when endothelial cells were co-cultured with pericytes, their permeability after 6 h of hypoxic conditions was unchanged from baseline (Hayashi et al. 2004). On the other hand, pericyte actions can be detrimental to BBB function. After exposure to IL-1 β , human pericytes may increase BBB injury by expressing CCL2, adhesion molecules (ICAM-1), and cytokines (IL-8, and IL-1 β), exacerbating inflammation (Rustenhoven et al. 2015; Hurtado-Alvarado et al. 2014). Similarly, upon TNF α stimulation, rats pericytes secrete MMP-9, which may decrease BBB integrity (Machida et al. 2017; Takata et al. 2011). In an acute ischemia stroke model, pericytes increase their expression of MMP-9 (Underly et al. 2017) or VEGF (Bai et al. 2015), both of which may injure the BBB.

There is also evidence that pericytes may provide guidance for peri-infarct angiogenesis (Dalkara et al. 2019), thereby contributing positively to post-stroke outcome (Ergul et al. 2012). In a rat TBI model, approximately 40% pericytes were found to have migrated from their microvascular location within the first hour injury, which may represent a pro-survival strategy, as nonmigrating pericytes displayed cytoplasmic alterations and rapid degenerative changes (Dore-Duffy et al. 2000). In support of this theory, inhibition of pericyte death with IV injection of CO-releasing molecule (CORM)-3 1-h post-CCI promoted neurogenesis and improved neurological function of mice after TBI (Choi 2016), suggesting that pericyte survival is important in the preservation of overall brain function. Pericyte loss may affect BBB permeability, as biomarkers of pericytes including PDGFR- β , NG2 and CD13 were significantly decreased 12–48 h after mild and moderate fluid percussion injury (FPI)

in mice, with a subsequent reduction in the expression of adherens junction proteins and tight junction proteins, and an increase in BBB permeability (Bhowmick et al. 2019).

To develop therapeutic strategies for stroke and brain trauma, it is important to understand the roles of different inflammatory pathways that affect the BBB after stroke and trauma. Minocycline is an example of an anti-inflammatory compound, which has been used in clinical trials of treatment of brain ischemia. Minocycline is known to decrease pro-inflammatory cytokine output and inhibit apoptosis by attenuation of TNF- α actions. Minocycline inhibits microglia activation by blocking NF- κ B nuclear translocation (Pang et al. 2012) and interferes with the interactions between microglia and T cells, thus impairing microglia-induced cytokine production in T cells (Giuliani et al. 2005). In another clinical study, treatment with minocycline was found to reduce plasma MMP-9 levels (Switzer et al. 2011).

In several clinical trials of patients with acute ischemic stroke, patients in the minocycline groups had improved neurological function compared with patients receiving placebo (Lampl et al. 2007; Sheng et al. 2018). These studies illustrate the importance of understanding the role of different pathways in inflammatory injuries of the BBB after stroke and brain trauma.

4 Conclusions

Stroke and TBI comprise the major forms of acute brain injury. Although there are many differences in the initial triggers of primary injury and subsequent steps in secondary injury, both stroke and TBI elicit profound responses in the BBB that significantly influence clinical outcomes. In this chapter, we have attempted to survey and summarize the many advances that have been made in the molecular dissection of signals and pathways that mediate BBB dysfunction in stroke and TBI. A deeper understanding of these complex and multi-cellular mechanisms in vascular, glial, and neuronal cells should lead to new therapeutic opportunities for not only rescuing BBB function, but more broadly restoring homeostasis in the damaged neurovascular unit.

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Brain Barriers and Multiple Sclerosis: Novel Treatment Approaches from a Brain Barriers Perspective

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Abstract

Multiple sclerosis (MS) is considered a prototypic organ specific autoimmune disease targeting the central nervous system (CNS). Blood–brain barrier (BBB) breakdown and enhanced immune cell infiltration into the CNS parenchyma are early hallmarks of CNS lesion formation. Therapeutic targeting of immune cell trafficking across the BBB has proven a successful therapy for the treatment of MS, but comes with side effects and is no longer effective once patients have entered the progressive phase of the disease. Beyond the endothelial BBB, epithelial and glial brain barriers establish compartments in the CNS that differ in their accessibility to the immune system. There is increasing evidence that brain barrier abnormalities persist during the progressive stages of MS. Here, we summarize the role of endothelial, epithelial, and glial brain barriers in maintaining CNS immune privilege and our current knowledge on how impairment of these barriers contributes to MS pathogenesis. We discuss how therapeutic stabilization of brain barriers integrity may improve the safety of current therapeutic regimes for treating MS. This may also allow for the development of entirely novel therapeutic approaches aiming to restore brain barriers integrity and thus CNS homeostasis, which may be specifically beneficial for the treatment of progressive MS.

Keywords

Adhesion molecules · Arachnoid barrier · Blood–brain barrier · Blood–cerebrospinal fluid barrier · Glia limitans · Immune cell trafficking · Multiple sclerosis · Permeability · Tight junctions

1 Introduction

Multiple sclerosis (MS) is considered an autoimmune disorder affecting the central nervous system (CNS) of young adults. If untreated in its early stages, MS leads to irreversible disability (Sospedra and Martin 2016). MS presents as a heterogeneous disease in its clinical presentation, its underlying mechanisms, and in its response to treatment. Several subtypes of the disease are known, but the predominant form is relapsing-remitting multiple sclerosis (RRMS), which occurs in 80–85% of patients (Noseworthy et al. 2000). RRMS patients present with episodes of neurological dysfunction followed by periods of stabilization, and this form can evolve into secondary progressive MS (SPMS) associated with increased disability. Furthermore, 10–15% of patients develop primary progressive MS (PPMS) characterized by a constant progression of disability from the onset (Noseworthy et al. 2000). This heterogeneity of MS in its clinical presentation suggests a complex interplay of mechanisms leading to the development of the disease.

Increased immune cell trafficking into the CNS and focal breakdown of the endothelial blood–brain barrier (BBB) are early hallmarks of MS pathogenesis (Lassmann 2018). The etiology of MS remains, however, unknown and MS presents

as a multifactorial disease caused by the interplay of environmental risk factors and a complex genetic background with now more than 230 disease susceptibility loci characterized in genome-wide association studies (GWAS) based on single nucleotide polymorphisms (SNPs) (Axisa and Hafler 2016). Many MS-associated genetic variants may include genes coding for molecules regulating functions of the immune system, which is consistent with the concept that MS is a T-cell mediated autoimmune disease targeting the CNS. Further support for a T-cell mediated autoimmunity in MS is derived from an animal model, experimental autoimmune encephalomyelitis (EAE) (summarized in Steinman and Zamvil (2006)), where CD4⁺ neuroantigen-specific T cells infiltrate the CNS and cause inflammation, demyelination, and blood–brain barrier breakdown, thus CNS pathology resembling that of relapsing-remitting MS. Lesion formation in MS is associated with focal BBB dysfunction as visualized by gadolinium-enhanced magnetic resonance imaging (MRI), and occurs early in the pathogenesis of MS (Kermode et al. 1990). The pathological findings in brain sections derived from MS patients after autopsy indicate the decreased and interrupted immunostaining for tight junction proteins associated with the leakage of serum fibrinogen, thus reflecting the increased permeability of the BBB (Kirk et al. 2003). Post-mortem studies also suggest abnormalities in the inflammatory state of CNS endothelial cells at the level of CNS capillaries and venules as upregulated expression of adhesion molecules like intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 is observed accompanied with infiltration of leukocytes (Alvarez et al. 2011a; Sospedra and Martin 2005). Taken together, this suggests that BBB abnormalities are continuously observed over all stages of MS and are thus critically involved in MS pathogenesis.

2 The Brain Barriers (Fig. 1)

Under physiological conditions the endothelial, epithelial, and glial brain barriers maintain CNS homeostasis by protecting the CNS parenchyma from the constantly changing milieu in the bloodstream.

2.1 The Endothelial Blood-Brain Barrier (BBB)

The endothelial BBB is established by brain microvascular endothelial cells, which inhibit free paracellular diffusion of water-soluble molecules by complex tight junctions that interconnect the endothelial cells (Tietz and Engelhardt 2015). The tight junctions in CNS microvessels are composed of an intricate complex of transmembrane (claudins, occludin, and junctional adhesion molecules (JAM)) and intracellular scaffolding proteins (e.g., zonula occludens (ZO)-1 and ZO-2), which are linked to the actin cytoskeleton (Fig. 1) (reviewed in Tietz and Engelhardt (2015)). Claudin-5 is essential to establish tight junctions, since claudin-5-deficient mice do not survive due to BBB leakiness (Nitta et al. 2003). Occludin is another important tight junction protein; however, it is not essential to establish tight

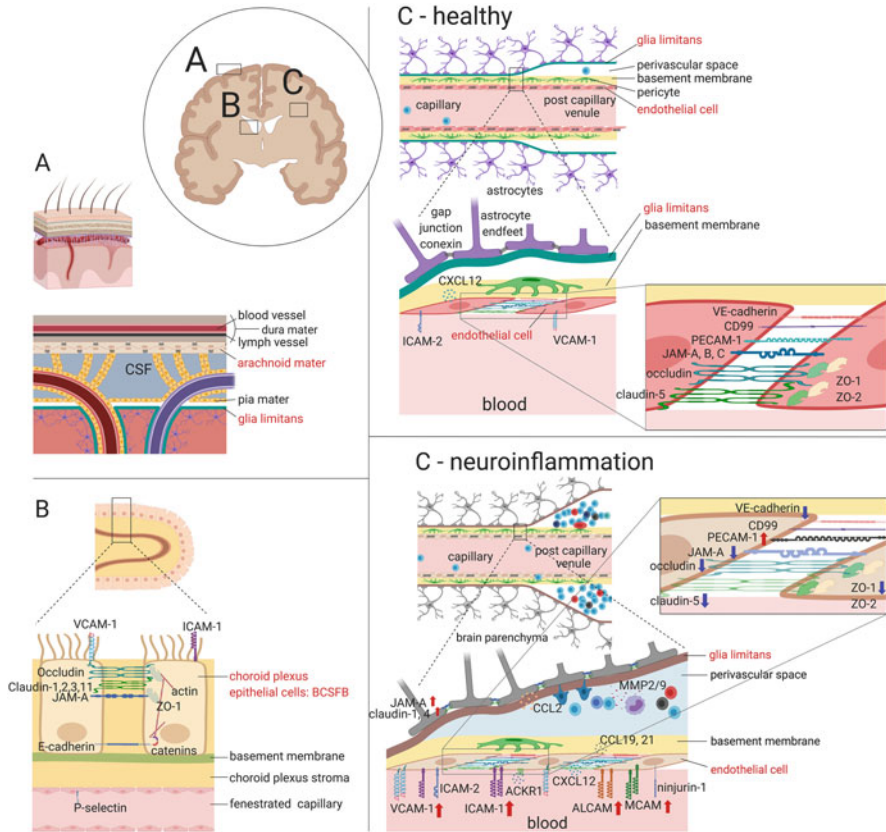


Fig. 1 The brain barriers. (a) The barriers at the surface of human brain. The brain is lined by three layers of meninges: the dura mater, the arachnoid mater, and the pia mater. The dura mater contains blood and lymph vessels, but exists outside of the CNS. The actual blood–CSF barrier is formed by arachnoid mater. In addition, the brain surface is covered by additional barrier glia limitans, which are formed by parenchymal basement membrane and astrocyte endfeet. (b) The blood–CSF barrier at the choroid plexus. The capillary of the choroid plexus is fenestrated and does not form barrier. The choroid plexus epithelial cell forms the barrier between the CSF-filled ventricle space and the choroid plexus stroma. (c) The blood–brain barrier. While transport of solutes occurs at the level of capillary, immune cells extravasate at the site of post-capillary venules. BBB-forming endothelial cells express complex tight junction molecules and adherens junction molecules. Together with extremely low pinocytotic activity, the BBB tightly controls solute diffusion. In the healthy condition, brain endothelium expresses few adhesion molecules and limits immune cell trafficking to effector and central memory T cells, which reach the CSF-filled perivascular space. Further entry into the CNS parenchyma is prevented by the glia limitans. Upon inflammation like MS, the endothelial tight junction and adherens junction molecules are disrupted and show BBB leakiness. In addition, brain endothelium upregulates adhesion molecules like ICAM-1, VCAM-1, ALCAM, MCAM, and ninjurin-1, which induce increased number of immune cells extravasating. The perivascular space is enlarged in neuroinflammation and immune cells further breach the glia limitans upon MMP activity to reach the CNS parenchyma

junctional integrity as occludin knock-out mice are born with no gross phenotype (Saitou et al. 2000). JAM family proteins regulate cell polarity, leukocyte migration, and BBB stability (Garrido-Urbani et al. 2014). The endothelial BBB also possess the adherens junction VE-cadherin, which keeps neighboring cells attached by homophilic interactions. VE-cadherin is linked to the actin cytoskeleton via other molecules, e.g. α - and β -catenin. Furthermore, platelet and endothelial cell adhesion molecule (PECAM)-1 and CD99 are highly expressed at cell-to-cell junctions, but localized outside of organized tight and adherens junctions. Combined with an extremely low pinocytotic activity, which inhibits transcellular passage of molecules across the BBB, these morphological peculiarities establish the physical permeability barrier of the BBB (Engelhardt and Sorokin 2009). At the same time the BBB establishes a functional barrier, in which expression of specific transporters and enzymes in brain microvascular endothelial cells ensures that nutrients pass into the CNS and toxic metabolites are removed from the CNS. Another important feature of brain microvasculature is a denser luminal coverage of the glycocalyx compared to cardiac and pulmonary vessels (Ando et al. 2018). The glycocalyx plays key role in the regulation of endothelial permeability and leukocyte adhesion (Curry and Adamson 2012; Schmidt et al. 2012). The precise role of the glycocalyx in BBB maintenance has remained elusive, since it is difficult to preserve glycocalyx in ex vivo tissues as well as in in vitro cultured endothelium. It has been shown that a dense glycocalyx network on the luminal surface of brain endothelium prevents diffusion of large molecules (40-kDa and 150-kDa dextran), but not of small molecules like sodium fluorescein (376 Da) and AlexaFluor dyes (643 Da) (Kutuzov et al. 2018).

Importantly, the biochemically unique BBB characteristics are not intrinsic to brain microvascular endothelial cells, but rather rely on continuous crosstalk with pericytes and astrocytes surrounding the CNS microvessels (Engelhardt and Sorokin 2009; Liebner et al. 2018). The morphogenic Wnt/ β -catenin and hedgehog pathways are involved in induction and maintenance of BBB characteristics (Engelhardt and Liebner 2014). Canonical Wnt/ β -catenin signaling is specifically activated in CNS vessels during development and blockade of this signal in vivo leads to CNS specific formation of hemorrhagic vascular malformations in mice (Daneman et al. 2009; Stenman et al. 2008; Vanhollebeke et al. 2015). In addition, BBB maturation is enhanced by endothelial specific stabilization of β -catenin in vivo, while inactivation of β -catenin causes significant downregulation of tight junction proteins (Liebner et al. 2008). Recent developments of stem cell technology further support the importance of canonical Wnt signaling in induction of the BBB phenotype in endothelial cells in vitro (Cecchelli et al. 2014; Qian et al. 2017). Furthermore, Wnt/ β -catenin activity is also involved in the long-term maintenance of the CNS blood vessel network since Wnt/ β -catenin signaling persists until adulthood in the vasculature component of the BBB and activation or inhibition of canonical WNT signaling can induce or reverse the barrier state in mature CNS endothelium (Moro et al. 2012; Zhou et al. 2014). In addition, the Hedgehog pathway is involved in brain angiogenesis and BBB maturation (Alvarez et al. 2011b). Astrocytes were shown to secrete Sonic hedgehog (Shh), which by engaging Hedgehog receptors on brain

endothelial cells during embryonic development and adulthood promotes BBB formation and maintenance (Alvarez et al. 2011b). Astrocytes also secrete retinoic acid (RA) and angiopoietin-1 (Ang-1), which have been shown to induce or support barrier properties of brain endothelium (Michinaga and Koyama 2019; Mizze et al. 2014). Several in vitro studies have also shown a role of astrocytes in the induction of mature tight junctions by direct contact with the blood vessels or by secretion of soluble factors (Arthur et al. 1987; Canfield et al. 2017; Tao-Cheng et al. 1987; Wolburg et al. 1994).

Another characteristic feature of CNS microvessels is the high number of pericytes embedded in the endothelial basement membrane, when compared to blood vessels in peripheral organs (Allt and Lawrenson 2001; Zlokovic 2008). Recruitment of pericytes to CNS microvessels is mediated by endothelial secretion of platelet-derived growth factor (PDGF)-B recruiting PDGF receptor- β expressing pericytes to the vascular wall (Armulik et al. 2010). Mice deficient for PDGF-B lack microvascular pericytes during embryonic development and develop microaneurysms in CNS blood vessels (Lindahl et al. 1997) underscoring a role for pericytes in stabilizing CNS microvessels already during development. Viable pericyte-deficient mouse mutants show increased BBB permeability due to endothelial transcytosis (Armulik et al. 2010). In addition, PDGF-B and thus appropriate localization of pericytes is involved in inducing polarized expression of the water channel aquaporin 4 (AQP4) on astrocyte endfeet (Munk et al. 2019). Pericytes also regulate the endothelial major facilitator super family domain containing 2a (*Mfsd2a*) expression. *Mfsd2a* is selectively expressed in BBB and *Mfsd2a*-deficient mice showed increased CNS endothelial cell vesicular transcytosis and, thus, a leaky BBB (Ben-Zvi et al. 2014). When cultured in vitro, brain endothelial cells rapidly lose their unique barrier characteristics, which can be re-introduced or maintained by co-culture with astrocytes and/or pericytes further underscoring the need for molecular cues of cells other than the endothelial cells from maintaining BBB characteristics in CNS microvascular endothelial cells (Canfield et al. 2017; Cecchelli et al. 2014; Mossu et al. 2018). Taken together these data show that the unique BBB phenotype in CNS microvascular endothelial cells relies on the continuous cross talk with cellular and acellular elements surrounding the CNS endothelium.

2.2 Glia Limitans

The glia limitans forms a semi-permeable barrier at the surface of the brain and around blood vessels. The glia limitans, composed of the parenchymal basement membrane and astrocyte endfeet (Engelhardt et al. 2016), has been shown to be the ancestral brain barrier of vertebrates (Bundgaard and Abbott 2008). In the healthy CNS, astrocyte endfeet are connected by gap junctions allowing for the exchange of second messengers between the astrocytes, while not hindering the diffusion of small molecules across the glia limitans.

2.3 Choroid Plexus and Blood-Cerebrospinal Fluid Barrier (BCSFB)

In addition to the endothelial BBB, the epithelial cells surrounding the choroid plexuses that extend into all four ventricles form a blood–cerebrospinal fluid barrier (BCSFB) (Engelhardt and Sorokin 2009). The BCSFB regulates the passage of solutes and, in all probability, also immune cell entry into the cerebrospinal fluid (CSF). In contrast to BBB-forming endothelial cells, the capillaries in the choroid plexus stroma are fenestrated allowing for free diffusion of blood-borne molecules into this tissue. The actual barrier, which inhibits the paracellular diffusion of water-soluble molecules across the BCSFB, is found at the level of the apical tight junctions between the choroid plexus epithelial cells. The choroid plexus epithelial cells express unique parallel tight junction strand proteins (claudin-1, -2, -3, and -11, occludin, and JAM-A), scaffolding proteins (ZO-1, -2, -3), and the adherens junction protein, E-cadherin. In addition to their barrier function, due to the expression of unique metabolite transporters at the membrane surface, choroid plexus epithelial cells are considered to be the major players in the production of the CSF (Wolburg and Paulus 2010).

2.4 Additional Brain Barriers

Additional barriers surround the brain and spinal cord in the form of blood–leptomeningeal barriers (BLMB). The CNS is lined by three layers of meninges: the dura mater, the arachnoid mater, and the pia mater. The blood vessels localized in the dura mater that comprises the outermost layer connected to the skull and spinal column do not form a barrier between the periphery and the CNS. Instead, the arachnoid mater localized below the dura mater forms a true blood–CSF barrier between the dura mater (technically located outside of CNS) and the CSF-filled subarachnoid space (Engelhardt et al. 2017). The cells forming the arachnoid barrier are connected by tight junctions and express efflux pumps similar to the epithelial cells of the choroid plexus of the BCSFB and the endothelial cells of the BBB (Alcolado et al. 1988; Nabeshima et al. 1975; Yasuda et al. 2013). The pia mater covers arteries and veins in the subarachnoid space and is reflected from the surface of the CNS, where the blood vessels penetrate into the CNS parenchyma. At the same time the pia mater continues around the arteries diving into the CNS parenchyma. The pia mater is neither a barrier for small solutes nor actively migrating immune cells, but may restrict passage of erythrocytes (Hutchings and Weller 1986) unless there is massive pathology as observed in subarachnoid hemorrhage (Siler et al. 2014).

3 Cell Trafficking Across Brain Barriers

3.1 Immune Cell Trafficking Across the BBB

In the absence of neuroinflammation, the BBB appears to limit immune cell trafficking to effector and central memory T cells, which can reach the CSF compartments to ensure CNS immune surveillance (Engelhardt et al. 2017). Importantly, the segments of the microvasculature mediating immune cell trafficking versus regulating transport of solutes are not identical. Whilst solute transport is localized to CNS capillaries, immune cell trafficking occurs at the level of CNS post-capillary venules (Engelhardt and Ransohoff 2012). To reach the parenchyma, immune cells first need to breach the endothelial BBB and endothelial basement membranes, and then encounter antigen-presenting cells in the perivascular space. Entry into the CNS parenchyma requires further local protease digestion of the glia limitans (Agrawal et al. 2006).

The migration of T cells across the brain endothelium is a multi-step process regulated by the sequential interaction of different signaling and adhesion molecules on BBB endothelium and on immune cells (Fig. 2, reviewed in Engelhardt and Ransohoff (2012)). This multi-step cascade has been deeply studied in CD4⁺ T cells in the context of EAE and is less well-described during non-inflammatory conditions. The sequential molecular mechanisms used by other immune cell types (e.g., CD8⁺ T cells, B cells or myeloid cells) to cross the BBB are not yet fully understood (Alvarez et al. 2015a; Ifergan et al. 2011a; Kooij et al. 2014b; Martin-Blondel et al. 2015; Rudolph et al. 2016), monocytes (Alvarez et al. 2015a; Ifergan et al. 2011b; Lyck et al. 2016; Seguin et al. 2003; Veenstra et al. 2017; Williams et al. 2015), and dendritic cells (DCs) (Jain et al. 2010; Sagar et al. 2012a, b; Zozulya et al. 2007).

Under normal immune surveillance, α 4-integrin/VCAM-1 interaction mediates the capture of activated T cells on non-inflamed CNS endothelium (Vajkoczy et al. 2001). Similar mechanisms are observed in the blood–retina barrier (BRB), which resembles the BBB, where activated T cells but not naïve T cells were able to migrate through BRB without rolling or reducing shear stress within the venules (Xu et al. 2003). α 4-integrin/VCAM-1 interaction further mediates subsequent G protein-dependent adhesion strengthening of T cells to microvascular VCAM-1 (Vajkoczy et al. 2001). Lymphocyte function-associated antigen (LFA)-1 is involved in neither the capture nor the adhesion steps, but rather mediates T-cell transendothelial migration (Laschinger et al. 2002).

Once inflammation is ongoing, cell adhesion molecules on the endothelial BBB are upregulated allowing for an increased number of T cells extravasating. In brief, circulating T cells are first captured and subsequently roll along the luminal aspect of CNS endothelium allowing them to slow down. These first steps are mediated by interaction of P-selectin glycoprotein ligand (PSGL)-1 on T cells and P-selectin on BBB. Rolling on the endothelial surface allows T cells to interact with chemokines displayed on proteoglycans on the luminal surface of the endothelial cells via their specific G-protein coupled receptors (GPCR). Chemokine binding to its GPCR

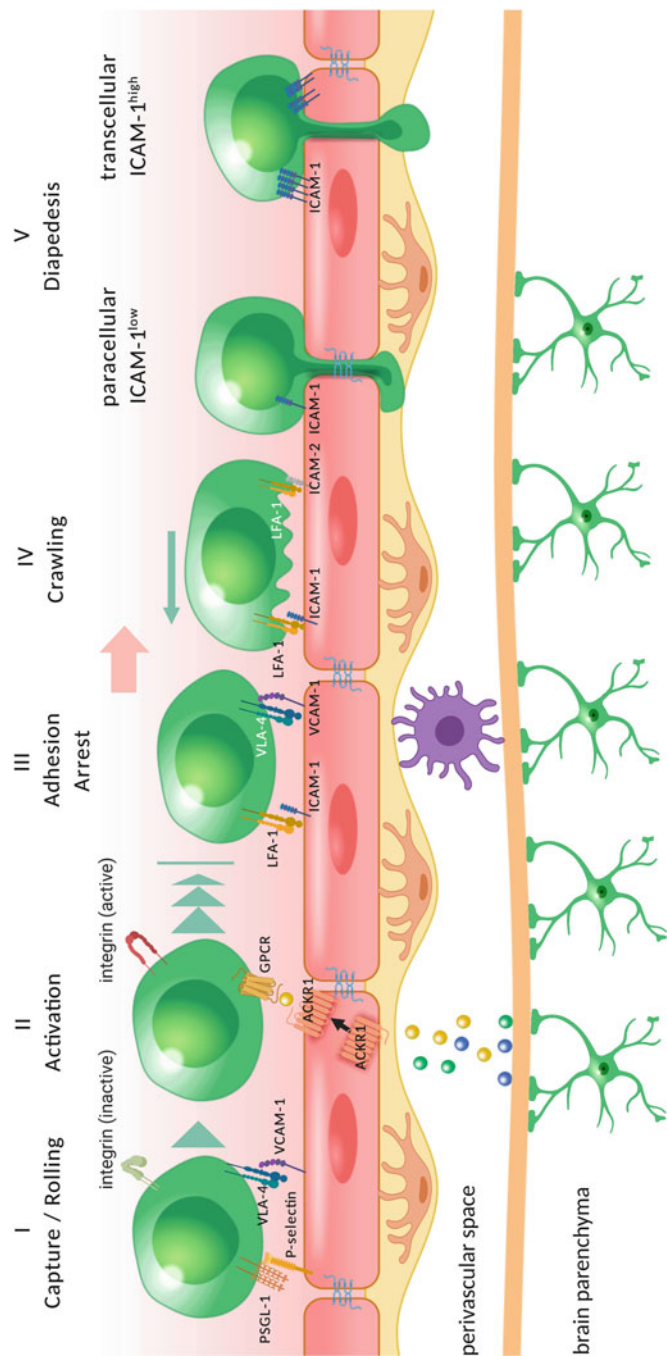


Fig. 2 Molecular mechanisms involved in T-cell trafficking across the blood-brain barrier. Endothelial P-selectin is upregulated upon neuroinflammation and binds to T-cell PSGL-1, which mediates T cell rolling during neuroinflammation. ACKR1 shifts the chemokine from the abluminal side to luminal surface of endothelium, which induces chemokine-mediated activation of T-cell integrins. G-protein-dependent integrin activation on T cells triggers their arrest on the BBB in a LFA-1/ICAM-1- and VLA-4/VCAM-1-dependent manner. Subsequent T cell polarization and crawling is mediated by LFA-1/ICAM-1 and LFA-1/ICAM-2 interactions. T cell diapedesis across the BBB occurs either via the paracellular route, where the T cells migrate through the junctional complexes between two adjacent BBB endothelial cells, or via the transcellular route, where the T cell crosses the endothelial cell body by forming a pore. Upon inflammation and increased endothelial surface ICAM-1 expression, T cells favor transcellular diapedesis

induces an inside-out activation of adhesion molecules of the integrin family on the surface of the T cell. This activation causes unfolding of the extracellular portion of the integrin to form a high-affinity receptor and only integrins with increased affinity for endothelial ligands of the immunoglobulin superfamily (ICAM-1, ICAM-2, VCAM-1) are able to mediate the arrest of the T cells to the BBB endothelium. Activated very late antigen (VLA)-4 ($\alpha 4\beta 1$ -integrin) on the T cell interacts with endothelial VCAM-1, which mediates T cell arrest under flow conditions (firm adhesion). This step is also mediated by interaction of LFA-1 ($\alpha L\beta 2$ -integrin) and ICAM-1. Subsequent T cell polarization and crawling against the direction of blood flow is mediated by LFA-1 engagement of endothelial ICAM-1 and ICAM-2. Interestingly, the commonly observed phenomenon for crawling of T cells for an extended period against flow is unique to CNS barrier-forming endothelium (Bartholomaeus et al. 2009; Steiner et al. 2010). Extensive crawling of T cells on the luminal BBB allows them to find sites permissive for diapedesis. This can occur either via the paracellular route, where T cells migrate through junctional complexes between two adjacent BBB endothelial cells or via the transcellular route, where the T cell crosses the endothelial cell body by forming a pore (Carman 2009). A predominantly paracellular migration occurs when junctions are intact and endothelial cell surface levels of ICAM-1 are low, however, once inflammation is ongoing with impaired tight junctions and endothelial cell surface ICAM-1 levels are upregulated, T cells favor the transcellular diapedesis pathway (Abadier et al. 2015; Lutz et al. 2017; Wimmer et al. 2019).

3.2 Immune Cell Trafficking Across the Glia Limitans

The glia limitans provides a barrier for migrating immune cells, which in the absence of neuroinflammation do not enter the parenchyma (Engelhardt and Sorokin 2009). This may be due to the fact that the molecular composition of the parenchymal basement membrane of the glia limitans is distinct from that of the endothelial basement membrane. The most prominent proteins establishing the parenchymal basement membrane are laminin $\alpha 1$ and $\alpha 2$ (Baeten and Akassoglou 2011; Hannocks et al. 2018), which are not involved in the binding of encephalitogenic CD4⁺ T cells (Sixt et al. 2001).

Under neuroinflammatory conditions, such as during MS or its animal model EAE, immune cells penetrate the glia limitans and infiltrate the CNS parenchyma. In EAE, this is directly associated with the onset of the neurological symptom (Agrawal et al. 2006; Owens et al. 2008). In contrast to transendothelial migration, which is regulated by adhesion molecule and integrin interaction, penetration of glia limitans needs focal gelatinase (enzymatic) activity. In murine EAE, T lymphocytes use the matrix metalloproteinase (MMP)-2 and MMP-9 activity of perivascular macrophages, which cleave dystroglycan, an extracellular matrix receptor anchoring astrocyte endfeet to the parenchymal basement membrane, to penetrate the parenchymal basement membrane (Agrawal et al. 2006).

3.3 Immune Cell Trafficking via the Choroid Plexus

The choroid plexus has been suggested as an alternative T cell entry site into the CNS. It was proposed that T cells cross the fenestrated capillaries of the choroid plexus in a P-selectin dependent manner to reach the choroid plexus stroma (Kivisakk et al. 2003), where antigen-presenting cells are located (Meeker et al. 2012). To reach the CSF, immune cells would need to then cross the choroid plexus epithelium, which expresses adhesion molecules such as ICAM-1 and VCAM-1 and chemokines such as CCL20 (Engelhardt and Sorokin 2009). However, as expression of ICAM-1 and VCAM-1 is strictly localized to the apical, i.e. the CSF facing, surface of the choroid plexus epithelium their role in T cell migration across the BCSFB remains to be shown. The C-C motif chemokine ligand 20 (CCL-20) and the cell-surface proteoglycan syndecan-1 expressed by choroid plexus epithelial cells were suggested to mediate the migration of CCR6⁺ IL-17-producing T helper (Th) 17 cells into the CNS via the choroid plexus (Reboldi et al. 2009); however, the precise molecular mechanisms on how immune cell migrate from the choroid plexus stroma into CSF-filled ventricles are not yet defined.

3.4 Immune Cell Trafficking Across Additional Barriers

Two-photon imaging studies have provided evidence for T cells to extravasate across the walls of leptomeningeal – most probably subpial – veins and venules, from where they reach the subarachnoid space (Schlager et al. 2016). In the absence of neuroinflammation their entry into the CNS parenchyma is prohibited by the glia limitans. It has been proposed that T cells can reach the dura mater lymphatics from the subarachnoid space, however, these studies have ignored the barrier characteristics of the arachnoid mater (Louveau et al. 2015, 2018). Recent studies have shown another possible anatomical route that myeloid cells (Herisson et al. 2018) or acute lymphoblastic leukemia cells (Yao et al. 2018) migrate into the brain and spinal cord surface using the blood vessels directly connecting the bone marrow and the brain or spinal cord surface. Such routes would also require the immune cells to cross the arachnoid barrier through yet to be elucidated mechanisms.

4 Mechanisms of Brain Barriers Impairment in MS

It is well-established that endothelial BBB breakdown is observed in MS. BBB breakdown in MS patients is determined by contrast-enhanced MRI from the extravasation and subsequent tissue accumulation of intravenously injected paramagnetic contrast agents, typically gadolinium (Gd)-based media (Liebner et al. 2018). From the clinical observation of contrast-enhanced MRI, it is widely accepted that BBB leakage occurs early during the disease. It is tightly associated with a first clinically isolated syndrome and later with the relapse phase of RRMS and therefore useful for both diagnosis and evaluation of disease activity. Clinical MRI performed

in standard of care practice detects fewer gadolinium-enhancing lesions in progressive MS, however, when increasing the dose of applied gadolinium contrast agent to triple the normal dose, Gd-enhancing lesions are also observable in PPMS patients (Filippi et al. 1995). This finding highlights the limits of sensitivity of clinical MRI in detecting BBB dysfunction. To date, detection of BCSFB or arachnoid barrier dysfunction by MRI is much less developed, although recent advancements with 3 Tesla Gd post-contrast T2-weighted fluid-attenuated inversion recovery (FLAIR) MRI have shown enhancement in the leptomeningeal compartment in progressive MS patients (Absinta et al. 2015). Current contrast-enhancing MRI techniques also do not allow one to distinguish the precise mechanisms underlying the extravasation of the paramagnetic contrast agents from the bloodstream across the BBB. This could occur paracellularly through impaired tight junctions or transcellularly due to increased vesicular transport across the BBB endothelium (Hawkins et al. 1992). In addition, the resolution of current MRI does not allow one to distinguish whether dysfunction occurs at the BBB and/or the glia limitans. Thus, although the development of contrast-enhancing MRI technology has provided significant advancement in visualizing BBB dysfunction in vivo and has improved the management of MS patients, it does not enable an improved understanding of the precise cellular and molecular mechanisms underlying BBB impairment during MS. One must continue to rely on histological and immunostaining approaches, mainly of post-mortem brain tissue, to advance our understanding of the mechanisms underlying brain barrier dysfunction in MS. Here, we summarize known mechanisms of endothelial, epithelial, and glial brain barriers impairment in MS pathogenesis.

4.1 BBB

MS post-mortem brain tissues show reduced or interrupted staining of ZO-1 (Alvarez et al. 2011a, 2015b; Kirk et al. 2003; Leech et al. 2007; Plumb et al. 2002), occludin (Alvarez et al. 2011a, 2015b; Plumb et al. 2002), claudin-5 (Alvarez et al. 2015b), VE-cadherin (Alvarez et al. 2011a, 2015b), JAM-A (Padden et al. 2007), and α -catenin (Alvarez et al. 2015b) suggesting that BBB tight and adherens junctions are impaired in MS. Furthermore, post-mortem brain tissue of MS patients showed fibrinogen and IgG staining outside of blood vessels, underscoring the leakage of these serum components across an impaired BBB (Vos et al. 2005).

Brain tissue sections from progressive MS patients also show, although less prominently than acute MS patients, a reduced and abnormal staining for tight junction and adherence junction molecules (e.g., ZO-1, claudin-5, occludin, VE-cadherin) when compared to other neurological disorder controls (Kirk et al. 2003; Leech et al. 2007; Plumb et al. 2002). Notably, BBB abnormalities characterized by an impaired junctional integrity visualized by staining for ZO-1 and serum protein leakage across the BBB are also not limited to active lesions, which are characterized by the presence of macrophages and activated microglia throughout the lesion area. The same BBB abnormalities can also be detected in inactive lesion characterized by the almost complete lack of macrophages and

activated microglial cells, as well as in normal appearing white matter (NAWM) (Leech et al. 2007; Plumb et al. 2002; Vos et al. 2005). Considering these pathological data, it is obvious that BBB impairment occurs even in situations beyond the active and acute lesions that can be visualized as gadolinium-enhancing regions by MRI imaging.

Interrupted staining of ZO-1 in brain microvascular endothelial cells was abundantly found in MS brain tissues in capillaries ($<10\text{ }\mu\text{m}$) as well as in venules and arterioles ($11\text{--}50\text{ }\mu\text{m}$) (Kirk et al. 2003), suggesting a causal role for diffusible inflammatory mediators but not for direct effects of infiltrated inflammatory cells, which occurs at the level of post-capillary venules. Interestingly, the predominant location of interrupted staining pattern for ZO-1 highlighting junctional BBB disturbances is different depending on MS subtypes: it is more frequent in chronic white matter lesions in PPMS, while more frequent in normal appearing gray matter (NAGM) in SPMS (Leech et al. 2007; Plumb et al. 2002). These data suggest that the pathomechanisms underlying the disruption of BBB integrity might be different depending on the MS phenotype and disease course.

Several pro-inflammatory cytokines and chemokines such as $\text{TNF-}\alpha$, $\text{IFN-}\gamma$, $\text{IL-1}\beta$, IL-17 , IL-22 , and CCL2 are upregulated and may be involved in BBB disruption (Baecher-Allan et al. 2018; Becher et al. 2017; Brosnan et al. 1995; Hojati 2017; Kebir et al. 2007; Perriard et al. 2015; Yong et al. 2007). Indeed, humoral factors in serum from MS patients were found to reduce claudin-5 protein levels detected by western blot in cultured human brain endothelium (Nishihara et al. 2015; Shimizu et al. 2014). Interestingly, the serum components that induce downregulation of claudin-5 are different depending on the MS subtype. Sera from MS patients collected during the relapse phase were found to induce secretion of MMP-2 and MMP-9 from cultured brain endothelial cells leading to degradation of claudin-5. While serum levels of MMP-9 and MMP-2 do not change with the disease stage of MS, serum from patients with SPMS harbors autoantibodies directed against human brain endothelium which induce the loss of claudin-5 (Nishihara et al. 2017; Shimizu et al. 2014).

BBB leakiness can also be caused by increased vesicular activity, which has been shown in the blood–retina barrier after pro-inflammatory cytokine exposure (Claudio et al. 1994) or transgenic mice lacking *Mfsd2a* (Ben-Zvi et al. 2014) or pericytes (Armulik et al. 2010). However, increased vesicular activity in the context of MS pathogenesis remains to be investigated.

During MS, the endothelial cells of the BBB change their phenotype allowing for the infiltration of additional immune cell subsets into the CNS. Post-mortem MS brain tissues show endothelial upregulation of ICAM-1 (Alvarez et al. 2015b; Sobel et al. 1990) and VCAM-1 (Alvarez et al. 2015b) in MS lesions (Alvarez et al. 2011a; Cayrol et al. 2008). Additional adhesion molecules reported to be upregulated on brain endothelial cells in MS are activated leukocyte cell adhesion molecule (ALCAM) (Alvarez et al. 2011a; Cayrol et al. 2008; Lyck et al. 2016) and melanoma cell adhesion molecule (MCAM) (Larochelle et al. 2012) and have been shown to contribute to the recruitment of myeloid cells and pathogenic Th17 cells to the CNS, respectively. Furthermore, nerve injury-induced protein (ninjurin)-1 was proposed to

be involved in the migration of myeloid cells and T cells into the CNS during in both EAE models and MS patients (Ifeghan et al. 2011b; Odoardi et al. 2012).

In addition, chemokines might play role in MS pathogenesis by increasing immune cell trafficking. CCL2 has been proposed to promote T cell and monocyte trafficking across the BBB as immunostaining for CCL2 is increased in brain endothelium in acute MS lesions (McManus et al. 1998; Subileau et al. 2009). Another chemokine, CCL19, which is constitutively expressed by brain endothelial cells, is upregulated in MS lesions and is thought to mediate the recruitment of CCR7⁺ T cells (Krumbholz et al. 2007). In addition, together with CCL19, CCL21 was found to be upregulated at the BBB during EAE and to contribute to the adhesion of encephalitogenic Th1 cells to the BBB (Alt et al. 2002).

Considering the high specialization and the polarization of the expression pattern of chemokines, the expression of a respective chemokine by brain endothelial cells does not allow one to conclude on its role in recruiting circulating cell across the BBB. To this end, it has been shown that C-X-C motif chemokine ligand 12 (CXCL12) expressed by brain endothelial cells is instead released at the abluminal side, where it traps CXCR4⁺ T cells in the perivascular space in the absence of neuroinflammation (McCandless et al. 2006). During EAE, expression of the atypical chemokine receptor 3 (ACKR3) is induced in brain endothelial cells and is suggested to reduce CXCL12 concentrations in the perivascular space as a prerequisite for T cell infiltration across the glia limitans (Williams et al. 2014). This scenario is supported by the observation that blocking ACKR3 function maintains high perivascular CXCL12 concentrations and reduces immune cell infiltration into the CNS parenchyma during EAE (Cruz-Orengo et al. 2011). In MS tissue CXCL12 is additionally detected on the luminal surface of brain endothelial cells (McCandless et al. 2008), possibly mediating the transmigration of CXCR4⁺ T cells. While all these data suggest an important role of chemokines in immune cell trafficking across the BBB in the context of MS or EAE pathogenesis, it is not yet clear how inflammatory chemokines such as CCL2 and CCL5 which are rather produced by astrocytes, i.e. behind the glia limitans, could reach the luminal side of the BBB endothelium and contribute to T-cell migration across the BBB. One possible explanation is that the atypical chemokine receptor 1 (ACKR1, also known as Duffy antigen/receptor for chemokines (DARC)) is induced at the BBB during EAE, and an increased number of ACKR1-positive microvessels can be observed in the subcortical white matter in MS brains (Minten et al. 2014). ACKR1 transports inflammatory chemokines across peripheral endothelial barriers (Pruenster et al. 2009). Deletion of ACKR1 specifically in endothelial cells in genetically modified mice leads to amelioration of EAE due to a reduced shuttling of inflammatory chemokines from the abluminal to the luminal side of brain endothelial cells (Minten et al. 2014).

Interestingly, perivascular accumulation of inflammatory cells does not necessarily correlate with visible signs of BBB breakdown. In MS patients, immune cell infiltration is observed with or without association of BBB leakage detected by fibrinogen staining. T cell and B cell infiltration in conjunction with BBB leakage is mainly observed in active demyelinating lesions as well as in the surrounding

NAWM in early stage MS. Immune cell infiltration without associated fibrinogen leakage is also already present in early phase MS, but gradually increases with disease progression and is mainly observed in perivascular and leptomeningeal spaces (Alvarez et al. 2015b; Lassmann 2018; Machado-Santos et al. 2018). Indeed, the cellular and molecular mechanisms that regulate BBB leakage versus the migration of immune cells across the BBB are distinct. It has been shown that distinct tyrosine phosphorylation sites of VE-cadherin control different functions of the peripheral endothelium. While phosphorylation of VE-cadherin Tyr731 controls leukocyte diapedesis, the phosphorylation of VE-cadherin Tyr685 regulates vascular permeability (Wessel et al. 2014). Furthermore, although ectopic endothelial cell-specific expression of the tight junction sealing protein claudin-1 failed to have any impact on immune cell trafficking across the BBB during EAE, it reduced the BBB leakiness of plasma proteins into the CNS resulting in amelioration of the chronic phase of EAE (Pfeiffer et al. 2011). Also, while depletion of PECAM-1 induced increased BBB permeability, it did not increase T-cell migration across brain endothelium but unexpectedly shifts T cell diapedesis across brain endothelium to transcellular sites *in vitro* (Wimmer et al. 2019). Based on ultrastructural observations of immune cell trafficking across the BBB during neuroinflammation, it was originally thought that the complex tight junctions of the BBB do not allow for paracellular leukocyte diapedesis as it occurs in peripheral vascular beds (Engelhardt and Wolburg 2004; Wolburg et al. 2005). However, recent reports suggest that in the absence of neuroinflammation the BBB directs T cell diapedesis to paracellular sites. Investigation of T-cell diapedesis across brain endothelium under physiological flow *in vitro* has shown that weakening brain endothelial junctions by either cytokine stimulation or lack of PECAM-1 favors transcellular T cell diapedesis (Abadier et al. 2015; Wimmer et al. 2019). These observations are supported by *in vivo* findings employing intravital microscopy in transgenic mice expressing claudin-5-GFP, where T cells were found to use the paracellular route for crossing the BBB during the onset of EAE. On the other hand, transcellular T-cell diapedesis was observed during active disease, when BBB characteristics are impaired (Lutz et al. 2017). These data underscore that mechanisms regulating BBB junctional leakiness during EAE and MS are distinct from those regulating the cellular pathway of T-cell diapedesis across the BBB.

4.2 The Glia Limitans

In the absence of neuroinflammation, immune cells in the perivascular space do not cross the glia limitans highlighting that the mechanisms required for immune cell trafficking through the BBB are distinct from those needed to enter the CNS parenchyma. In the context of EAE, it has been shown that expression of gelatinases, MMP-2 and MMP-9, in infiltrating myeloid cells and astrocytes is required to allow immune cell penetration across the glia limitans (Agrawal et al. 2006; Hannocks et al. 2019; Song et al. 2015). Mice deficient for MMP-2 and MMP-9 show massive immune cell infiltration into perivascular spaces as immune cell trafficking across

the glia limitans is abrogated and do not develop EAE (Agrawal et al. 2006), further underscoring that the glia limitans is an important barrier regulating immune surveillance of the CNS. Furthermore, recent observations have shown that the glia limitans changes upon breakdown of the endothelial BBB. Reactive mouse astrocytes *in vivo*, as well as cultured human astrocytes *in vitro*, upregulate claudin-1, claudin-4, and JAM-A upon pro-inflammatory stimulation allowing for the formation of tight junctions between astrocyte endfeet (Hornig et al. 2017). Inducible astrocyte-specific deletion of claudin-4 leads to increased leukocyte infiltration into the CNS parenchyma and exacerbation of EAE (Hornig et al. 2017). These data highlight that the glia limitans is an important barrier for immune cell infiltration within CNS parenchyma and could be involved in the pathogenesis of MS.

Immunostaining of post-mortem MS brain tissue showed increased MMP-2, MMP-7, and MMP-9 in macrophages and lymphocytes of perivascular cuffs in acute MS lesions (Anthony et al. 1997; Cossins et al. 1997; Lindberg et al. 2001; Yong et al. 2007). Clinical data suggest a correlation of MMP expression and the impairment of brain barriers as increased serum levels of MMP-9 were found to correlate with newly appearing Gd-enhanced lesions (Waubant et al. 1999). Histological data from MS patients does however support a contribution of alterations of the glia limitans in MS pathogenesis at least at the surface of the brain. SPMS brain tissue samples with meningeal B cell infiltration showed that neuronal loss in the brain cortex is accompanied by loss of the astrocytes in cortex layer (Magliozzi et al. 2010), which form the glia limitans of the brain surface.

4.3 Blood–Cerebrospinal Fluid Barrier (BCSFB) of the Choroid Plexus

Recent data has suggested that the choroid plexus is an alternative CNS entry site for T lymphocytes, especially for Th17 cells (Reboldi et al. 2009). As the epithelial cells of the choroid plexus forming the BCSFB constitutively express high levels of CCL20, while brain endothelial cells do not, it was proposed that CCR6⁺ encephalitogenic Th17 cells may penetrate the BCSFB in a CCR6/CCL20 dependent manner (Reboldi et al. 2009). The choroid plexus as the CNS entry site for Th17 cells is further supported by the observations that induction of EAE in mice with T cell-specific deletion of $\alpha 4$ -integrins prohibits entry of Th1 cells into the brain and spinal cord, but fails to inhibit Th17 entry into the brain parenchyma and is characterized by marked immune cell infiltration within the choroid plexus (Rothhammer et al. 2011). The integrin LFA-1 and the adhesion molecule MCAM were shown to be required for brain entry of $\alpha 4$ -integrin deficient Th17 cells during EAE (Breuer et al. 2018; Rothhammer et al. 2011). Although the choroid plexus is thus recognized as a potential CNS entry site for Th17 cells, the precise cellular and molecular mechanisms mediating immune cell trafficking from the choroid plexus stroma into the CSF-filled ventricular space remain to be demonstrated.

Another EAE study combining MRI using iron oxide magnetic nanoparticles and histological analysis shows that the accumulation of iron oxide particles is seen in the choroid plexus of adoptive transfer EAE mice prior to the onset of clinical signs. This accumulation of iron oxide particles is not observed in control mice (Millward et al. 2013). All these data support the notion that immune cells can infiltrate the choroid plexus stroma in EAE. At the same time, there is limited information on how this cell infiltration may induce alterations of the BCSFB in EAE or MS patients. Choroid plexus tissue from autopsies of MS patients showed decreased claudin-3 mRNA and protein expression compared to control cases without neurological disease (Kooij et al. 2014a), suggesting an impairment in BCSFB tight junctions. However, the exact role of claudin-3 at the BCSFB remains to be elucidated as EAE studies using claudin-3 deficient mouse models have provided discrepant observations on the role of claudin-3 at the BCSFB (Castro Dias et al. 2019; Kooij et al. 2014a).

Taken together, these findings highlight a role of the choroid plexus in MS pathogenesis and suggest the need for further studies focused on BCSFB dysfunction to help to understand novel pathomechanisms of MS. Since the novel gadolinium-based contrast agent Gadofluorine M was shown to sensitively detect BBB leakage compared to conventional Gd-DTPA (Bendszus et al. 2008; Stoll et al. 2009) and accumulate in the choroid plexus during EAE and correlated with disease activity (Wuerfel et al. 2010), improved MRI sequences capable of visualizing this organ may help us to further understand the involvement of choroid plexus in MS pathogenesis.

4.4 Arachnoid Barrier

There is an increased interest in the relation of meninges and MS pathogenesis since the discovery of meningeal follicles in progressive MS patients. Post-mortem brain samples from progressive MS patients show the presence of B cell aggregates characterized as ectopic follicles with germinal centers in the meninges of SPMS cases (41.4–54%), but not in PPMS patients (Magliozzi et al. 2007, 2010). This B cell accumulation seems to be observed in the subarachnoid space and is thus present at the location where CNS immune surveillance takes place. Pathological alterations of the dura mater in MS patients have not been much examined to date. This may be because it is understood that the dura mater is outside of the CNS and separated from the subarachnoid space by the arachnoid barrier.

The cells of the arachnoid barrier are connected by tight junctions expressing claudin-11 (Brochner et al. 2015; Weller et al. 2018; Whish et al. 2015). A recent study has shown downregulation of claudin-11 staining in meningeal ER-TR7⁺ cells, considered as arachnoid barrier cells, during EAE (Uchida et al. 2019). In addition, T-cell infiltration in the spinal cord parenchyma occurred at the regions where meningeal claudin-11 staining was decreased (Uchida et al. 2019). If ER-TR7⁺ cells include arachnoid mater cells, these findings suggest that if the

arachnoid mater is impaired in MS, this might lead to alterations in the drainage of CSF as well as immune cell trafficking across this barrier.

Antigen as well as cells (CD4⁺ T cells (Goldmann et al. 2006), monocytes (Kaminski et al. 2012), and dendritic cells (Hatterer et al. 2006)) from the CNS have been shown to drain to the cervical lymph nodes (de Vos et al. 2002; Harling-Berg et al. 1989, 2002; Hochwald et al. 1988; Widner et al. 1988; Yamada et al. 1991). Previous reports have shown that antigen-presenting cells injected into the CSF but not into the CNS parenchyma can reach deep cervical lymph nodes in rats (Hatterer et al. 2006), and macrophages containing myelin debris are observed in cervical lymph nodes of MS patients (van Zwam et al. 2009b). These findings support the notion that CNS immune surveillance is maintained in the CSF drained CNS spaces and it is possible that CNS antigen carrying antigen-presenting cells promote epitope spreading, and thus propagate or suppress the disease process. If there is arachnoid barrier dysfunction in MS patients, CNS antigens and potentially also antigen-presenting cells might directly reach lymphatic vessels localized in the dura mater and contribute to MS pathology. Since ablation of dural lymphatics or inhibition of lymphatic growth near cribriform plate, as well as surgical removal of CNS-draining lymph nodes, was shown to ameliorate EAE (Hsu et al. 2019; Louveau et al. 2018; van Zwam et al. 2009a), alterations in CSF drainage pathways established by arachnoid barrier dysfunction might contribute to MS pathogenesis.

5 Therapeutic Strategies Targeting Brain Barriers

5.1 Current MS Therapies Directly Targeting Brain Barriers

A number of therapeutic strategies used for the treatment of MS exert direct or indirect effects on brain barriers.

5.1.1 BBB Stabilizing Effects

Glucocorticoids are mainly used short term and at high doses for the therapy of acute relapses in patients with RRMS. In addition to the immunosuppressive effects of glucocorticoids, MRI studies of acute lesions in RRMS patients have shown that high dose (1,000 mg/day, 4–8 days) intravenous methylprednisolone can restore BBB function as shown by fewer Gd-enhancing lesions, which correlated directly with clinical improvement (Burnham et al. 1991). In vitro studies have supported a role for glucocorticoids in increasing BBB tightness, as addition of glucocorticoids to brain endothelial cells induces the upregulation of adherens junction proteins, such as VE-cadherin, and of tight junction proteins, such as occludin and claudin-5 (Forster et al. 2008).

Interferon beta (IFN β) is an immunomodulatory therapy widely used for the treatment of RRMS patients for more than 20 years (reviewed in Kraus and Oschmann (2006)). Although the underlying mechanisms of how IFN β ameliorates MS are not fully understood, at least part of its efficacy is related to BBB stabilization. Serial MRI scans from IFN β -treated MS patients have shown an early reduction

in the appearance of Gd-enhancing lesions within a few weeks after starting the therapy when compared with placebo treated MS patients (Pozzilli et al. 1996). A direct effect of IFN β -1a in stabilizing BBB characteristics is provided by the observation that IFN β decreases paracellular permeability of brain endothelium in vitro and even prohibits pro-inflammatory cytokine-induced BBB leakage of these brain endothelial cells (Kraus et al. 2004, 2008).

Fingolimod. Fingolimod is an agonist of sphingosine-1-phosphate (S1P) receptors 1, 3, 4, and 5 and is used for the treatment of RRMS. The major mechanism by which fingolimod prevents relapses in RRMS patients is suggested to be due to sequestration of lymphocytes in lymph nodes by inhibition of their egress into lymph (Hla and Brinkmann 2011; Mehling et al. 2011). However, fingolimod also has effects on CNS cells, including brain endothelium, since these cells express S1P receptors. Fingolimod upregulates claudin-5 mRNA and protein in cultured human brain endothelium and prevents disruption of barrier characteristics exerted by MS sera in these brain endothelial cells (Nishihara et al. 2015). Furthermore, fingolimod reduces the death of human brain microvascular endothelial cells induced by high concentrations of pro-inflammatory cytokines (Spampinato et al. 2015). Additional effects of fingolimod on brain endothelium are inhibition of upregulation of VCAM-1 on cultured human brain endothelium induced by MS sera (Nishihara et al. 2015). Gene expression analysis of EAE CNS tissue samples has confirmed that fingolimod downregulates vascular adhesion molecules such as ICAM-1, VCAM-1, and P-selectin on the BBB endothelium, which will eventually reduce immune cell entry into the CNS (Foster et al. 2009).

Other disease modifying drugs: Laquinimod, which is a derivative of the immunomodulator linomide (quinoline-3-carboxamide), also has been shown to act directly on the brain endothelium by increasing junctional staining of ZO-1 and transendothelial electrical resistance (TEER) of human primary brain microvascular endothelial cells in vitro (Luhder et al. 2017). Laquinimod was shown to reduce *ICAM-1* and *ALCAM* mRNA expression in brain endothelium in vitro, which resulted in reduced migration of Th1 and Th17 cells across the brain endothelial cells (Luhder et al. 2017). Dimethyl fumarate was shown to inhibit the cytokine-induced upregulation of E-selectin, VCAM-1, and ICAM-1 on human umbilical vein endothelial cells (Vandermeeren et al. 1997), suggesting it could exert a similar effect on brain endothelium. Vitamin D, one of the environmental factors of MS, also has a direct BBB enhancing effect. Treatment with 1 α ,25-(OH) $_2$ D $_3$ prevents BBB disruption caused by both RRMS and SPMS sera through upregulation of ZO-1 and claudin-5, and downregulation of cell adhesion molecules, ICAM-1 and VCAM-1 in human culture brain endothelium in vitro (Takahashi et al. 2017).

5.2 Current MS Therapies Indirectly Targeting the Brain Barriers

Natalizumab. Natalizumab is a humanized monoclonal antibody that selectively blocks the α 4-integrin subunit of the VLA-4 and inhibits VLA-4 mediated interaction of autoaggressive T cells with endothelial VCAM-1 on the BBB, thus indirectly

targeting the BBB. Natalizumab is a highly efficient treatment inhibiting T cell entry into the CNS in patients with RRMS (Miller et al. 2003).

6 Future Directions for Direct or Indirect Therapeutic Targeting of the Brain Barriers for Treating MS

6.1 Direct Targeting Approaches

Breakdown and/or impairment of brain barriers are hallmarks of the pathogenesis of MS. Thus, an attractive option for treating MS is to stabilize the integrity and immune function of the brain barriers. A rapid stabilization of the BBB after a relapse may be a promising strategy to limit inflammation and CNS damage. Furthermore, stabilization of the BBB during the clinically stable phase of RRMS or during progressive MS could have potential to reduce disease progression by re-establishing brain homeostasis. Previous reports support this hypothesis: induction of claudin-1 in BBB endothelium was found to reduce BBB leakiness in EAE and to ameliorate chronic EAE (Pfeiffer et al. 2011). Interestingly, BBB developmental pathways are thought to have possible protective mechanisms to stabilize BBB under neuroinflammation, as it has been reported that Shh expression by reactive astrocytes is increased in MS lesions (Alvarez et al. 2011b). Human recombinant Shh has barrier-enhancing properties, since Shh increases TEER and reduces the permeability of cultured human brain endothelium in vitro (Alvarez et al. 2011b). Another interesting molecule is retinoic acid (RA). MS brain tissue showed that reactive astrocytes in both acute and chronically active MS lesions express the RA-producing enzyme retinaldehyde dehydrogenase 2 (RALDH2) (Mizee et al. 2014). RA reduced inflammation-induced adhesion molecule expression and decreased the permeability in cultured endothelial cell in vitro (Mizee et al. 2014). In vitro modeling of the BBB from stem cell sources further supports the barrier-enhancing capacity of RA (Lippmann et al. 2014).

The mechanisms regulating brain barrier integrity in MS pathogenesis are far less understood compared to mechanisms for immune cell trafficking across brain barriers. Almost all immunomodulatory therapies have shown no therapeutic efficacy for the progressive form of MS. Further studies addressing the mechanisms contributing to continuous BBB dysfunction and prohibiting the re-establishment of CNS homeostasis during MS are needed. This may enable development of novel drugs targeting BBB stabilization for the treatment of progressive MS patients.

6.2 Indirect Targeting Approaches

Natalizumab showed remarkable efficacy in preventing clinical and radiological relapses of MS patients and proved that immune cell trafficking across the BBB is a useful target for the treatment of MS. Unfortunately, the use of this drug is associated with the development of progressive multifocal leukoencephalopathy

(PML) caused by JC virus infection of oligodendrocytes and astrocytes, which suggests that natalizumab eventually interferes with CNS immune surveillance. Thus, there is an unmet need to identify disease-relevant pathogenic T cell subsets and to investigate potential differences in the molecular mechanisms used by each T cell subset for crossing the BBB. It is widely accepted that Th1 and Th17 cells are important targets for MS pathogenesis. Recent studies have shown the importance of a unique T cell subset referred to as Th17.1 or Th1*, characterized by a mixed Th1 and Th17 phenotype (expression of both signature transcription factors and cytokines, T-bet and ROR γ T, and IFN- γ and Th17, respectively) (Dhaeze et al. 2019; van Langelaar et al. 2018). Th1* cells were predominantly found in the CSF of MS patients during early disease and have been shown to have a higher migratory capacity to breach human brain endothelium *in vitro* compared to the classical Th17 subset (van Langelaar et al. 2018). Another Th subset expressing the transcription factor Eomes was shown to be increased in the peripheral blood and in CSF during the progressive state of MS (Raveney et al. 2015). Eomes-expressing Th cells show cytotoxic characteristics by expression of granzyme B and IFN- γ (Oki 2018; Raveney et al. 2015). Different Th cells subsets express different patterns of chemokine receptors and studies in EAE showed that different Th subsets use different routes and molecular mechanisms to enter the CNS (Glatigny et al. 2011; Pierson et al. 2012; Reboldi et al. 2009; Rothhammer et al. 2011). Hence, it is likely that these different human T cell subsets use different cellular and molecular mechanisms to cross the brain barriers. Thus, future studies addressing the cellular and molecular mechanisms by which these different Th cell subsets cross the BBB and BCSFB are key to develop treatment strategies. This will allow for preferentially blocking CNS recruitment of pathogenic T cells, while leaving the migration of protective immune cell subsets into the CNS unaffected.

7 Conclusions and Outlook

There is an increasing number of MS therapies targeting immune cell trafficking or even depleting circulating immune cells. Although highly effective, these treatments are associated with rare but severe side effects like PML, suggesting that the current therapeutic strategies also target immune cells mediating immune surveillance of the CNS. The next generation of MS drugs targeting immune cell infiltration across brain barriers need a higher specificity, allowing for selective prevention of CNS entry of pathogenic immune cells, while leaving CNS entry of immune cells required for CNS surveillance unaffected. A therapy for progressive MS is also urgently needed. While at this stage of the disease there is no longer an increased immune cell entry into the CNS, there still exists an impairment of brain barrier function. The recent insight that the mechanisms regulating vascular permeability and immune cell migration across the endothelium are different may provide a novel platform for specific stabilization of brain barrier integrity. Despite significant advances in our understanding of BBB dysfunction in MS, progress of precisely understanding brain barriers dysfunction in MS is hampered due to the lack of tissue sample availability

during the disease. Thus, a large part of our knowledge of brain barriers dysfunction is derived from the EAE model or post-mortem MS tissues. Little is known about dynamic changes of the BBB or other brain barriers in individual MS patients. Future studies looking for imaging biomarkers indicating brain barrier dysfunction in live patients could improve our knowledge of BBB dynamics during the entire course of MS. These kind of studies should include the choroid plexus and the arachnoid barriers, which have been understudied and may represent novel sites to make unprecedented observations of the role of brain barrier alterations in MS patients. GWAS data may give further hints to consider targets on BBB in MS patients. There has been no direct link between genetic influence on the BBB and MS elucidated thus far, except for ALCAM, however GWAS data shows potential genetic influence with MS susceptibility and BBB function (Damotte et al. 2014; Spencer et al. 2018). The studies addressing MS-specific BBB alterations are allowing an improved understanding of MS pathogenesis and the hope for better treatment options for RRMS and progressive MS.

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Glossary

Activated leukocyte cell adhesion molecule (ALCAM) ALCAM is a member of the immunoglobulin gene superfamily and is upregulated on inflamed brain endothelium. ALCAM binds to CD6 in a heterophilic manner or to ALCAM in a homophilic manner. ALCAM contributes to the recruitment of myeloid cells to the CNS.

CD99 CD99 is a heavily O-glycosylated type I transmembrane protein, which localizes to endothelial cell-to-cell contacts and mediates immune cell trafficking across the brain endothelium in vitro.

Intercellular adhesion molecule-1 (ICAM-1) ICAM-1 is a member of the immunoglobulin gene superfamily and is observed on endothelial cells of the BBB and the BLMB, as well as on the choroid plexus epithelium of the BCSFB. LFA-1/ICAM-1 interaction mediates firm adhesion and subsequent T cell polarization and crawling on the inflamed endothelium. Endothelial cell surface ICAM-1 expression levels determine the T cell diapedesis pathway (T cells favor transcellular diapedesis across ICAM-1 high endothelium, while T cells use paracellular route when endothelial ICAM-1 levels are low).

Intercellular adhesion molecule-2 (ICAM-2) ICAM-2 is a member of the immunoglobulin gene superfamily, which binds to T-cell LFA-1 and mediates T cell crawling on the luminal surface of inflamed microvascular endothelial cells.

Lymphocyte function-associated antigen-1 (LFA-1) LFA-1 is also known as $\alpha\text{L}\beta\text{2}$ -integrin. T-cell LFA-1 binds to endothelial ICAM-1 and ICAM-2.

Melanoma cell adhesion molecule (MCAM) MCAM is an adhesion molecule of the immunoglobulin superfamily, which is upregulated on inflamed brain

endothelium and also expressed by Th17 cell population. MCAM binds to laminin-411 and mediates pathogenic Th17 cell recruitment to the CNS.

Nerve injury-induced protein-1 (ninjurin-1) Ninjurin-1 is a 22 kDa membrane protein, which is upregulated in inflamed brain endothelium and involved in the migration of myeloid cells and T cells into the CNS during EAE and MS.

Platelet and endothelial cell adhesion molecule-1 (PECAM-1) PECAM-1 is a type 1 transmembrane glycoprotein of the immunoglobulin superfamily of cell adhesion molecules. PECAM-1 is highly enriched at interendothelial junctions outside of organized adherens junctions and tight junctions. PECAM-1 mediates neutrophil and monocyte diapedesis across the vascular wall. *PECAM-1* deficient mice show increased BBB permeability. T cells favor transcellular diapedesis across an in vitro BBB model derived from *PECAM-1* deficient mice.

P-selectin P-selectin mediates PSGL-1-dependent T cell rolling, irrespective of Ag specificity, in inflamed leptomeningeal brain vessels. P-selectin also mediates T cell migration across the fenestrated capillaries of the choroid plexus to reach the choroid plexus stroma.

P-selectin glycoprotein ligand-1 (PSGL-1) PSGL-1 is a homodimeric sialomucin, which binds to L-, E-, and P-selectin. To serve as a selectin ligand, the protein backbone of PSGL-1 must be decorated with specific carbohydrate side chains.

Vascular cell adhesion molecule-1 (VCAM-1) VCAM-1 is a member of the immunoglobulin gene superfamily and is observed on endothelial cells of the BBB and the BLMB, as well as on the choroid plexus epithelium of the BCSFB. VLA4/VCAM-1 interaction mediates T cell firm adhesion on inflamed CNS endothelium. α 4-integrin/VCAM-1 interaction mediates the capture of T cell and subsequent G protein-dependent adhesion strengthening of T cells to the microvascular in non-inflamed endothelium (at least in mice).

Very late antigen-4 (VLA-4) VLA-4 is also known as α 4 β 1-integrin. The α 4-integrin subunit of the VLA-4 is targeted by the humanized monoclonal antibody natalizumab.

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Epilepsy and Alterations of the Blood–Brain Barrier: Cause or Consequence of Epileptic Seizures or Both?

Wolfgang Löscher

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Abstract

The blood–brain barrier (BBB) is a dynamic, highly selective barrier primarily formed by endothelial cells connected by tight junctions that separate the circulating blood from the brain extracellular fluid, thereby preserving a narrow and stable homeostatic control of the neuronal environment. The endothelial cells lining the brain microvessels are under the inductive influence of neighboring cell types within the “neurovascular unit” including astrocytes and pericytes. In addition to the morphological characteristics of the BBB, various specific transport systems, enzymes, and receptors regulate the molecular and cellular traffic across the barrier. Furthermore, the intact BBB prevents many macromolecules

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and immune cells from entering the brain. This changes dramatically following epileptogenic brain insults; such insults, among other BBB alterations, lead to albumin extravasation and diapedesis of leukocytes from blood into brain parenchyma, inducing or contributing to epileptogenesis, which finally leads to development of spontaneous recurrent seizures and epilepsy. Furthermore, seizures themselves may cause BBB disruption with albumin extravasation, which has been shown to be associated with activation of astrocytes, activation of innate immune systems, and modifications of neuronal networks. However, seizure-induced BBB disruption is not necessarily associated with enhanced drug penetration into the brain, because the BBB expression of multidrug efflux transporters such as P-glycoprotein increases, most likely as a “second line defense” mechanism to protect the brain from drug toxicity. Hopefully, a better understanding of the complex BBB alterations in response to seizures and epilepsy can lead to novel therapeutic intervention to prevent epileptogenesis and the development of other detrimental sequelae of brain injury.

Keywords

Albumin · Antiepileptic drugs · Epileptogenesis · P-glycoprotein · Tight junctions

1 Introduction

The blood–brain barrier (BBB) is a dynamic and complex barrier essential for the normal function of the central nervous system (CNS), playing a crucial role in the maintenance of a strict extracellular environment around synapses and axons (Ballabh et al. 2004; Abbott et al. 2010; Obermeier et al. 2013). The BBB is formed by the endothelial cells lining the brain capillaries (Fig. 1). Endothelial cell tight junctions limit the paracellular flux of hydrophilic molecules and macromolecules across the BBB, while nutrients including glucose and amino acids enter the brain via specific membrane transporters. In addition to endothelial cells, the BBB is constituted by the capillary basal or basement membrane, composed of collagen type 4, elastin, fibrillin, laminin, fibronectin and extracellular matrix proteins, and pericytes embedded within the basal membrane (Fig. 1). Furthermore, the glia limitans, formed by astrocytic end-feet that surround the endothelial cells, adds to the barrier properties of the BBB. There has been a long-standing interest in how the brain regulates its own blood supply. In this context, the concept of the *neurovascular unit* (NVU) emerged to emphasize the unique neurovascular coupling that controls cerebral blood flow and other functions of blood vessels in the brain (Iadecola 2017).

For drugs (or xenobiotics), the BBB largely functions as a diffusion barrier, thus limiting drug penetration through the BBB to small (<500 Da), lipophilic, and uncharged compounds (Abbott et al. 2010). Additional physicochemical parameters of drugs that affect BBB permeability include hydrogen bond donors and acceptors and the polar surface area of the drugs (Seelig 2007). As a consequence, >98% of all small molecule drugs do not cross the BBB (Pardridge 2007). Antiepileptic drugs

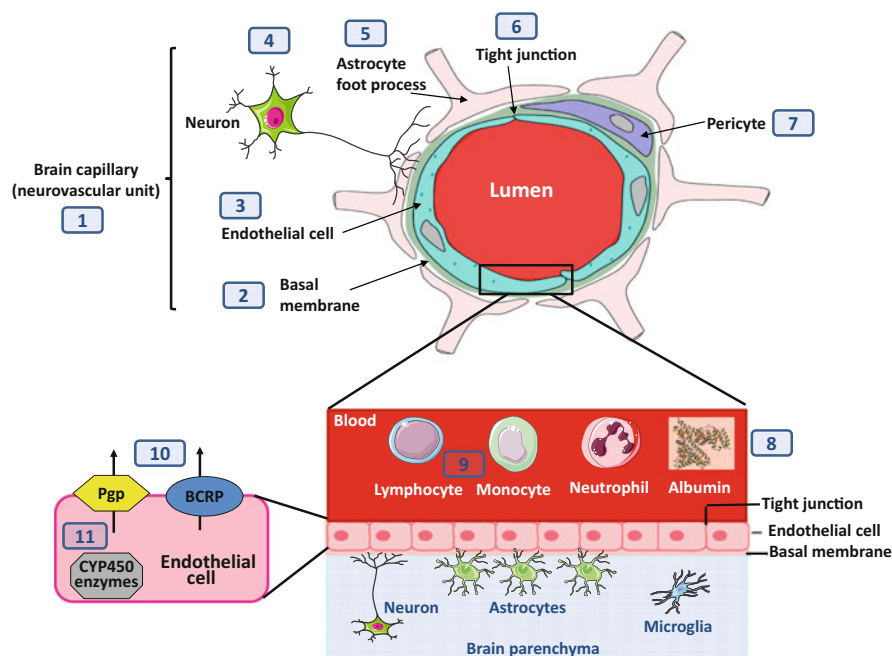


Fig. 1 A schematic view of the various morphological and biochemical constituents of the blood–brain barrier (BBB) and how they relate to alterations in response to seizures or epilepsy. As described in the text, seizures or processes underlying seizures may affect brain capillaries (also termed neurovascular unit) (1) by thickening of the basal membrane (2), alterations of the endothelial cells that form the barrier (3), of neurons that are involved in the neurovascular unit (4), of astrocyte foot processes (5) that form a second barrier, termed the glia limitans, of tight junctions (6) between the endothelial cells, and of pericytes (7). In addition, epileptogenic brain injury typically leads to extravasation of albumin (8) and blood-born immune cells (8) into the brain parenchyma. Possibly as a “second line defense” mechanism, BBB disruption is associated with increased expression of efflux transporters such as Pgp and BCRP at the apical site of BBB endothelial cells (10) and with increased activity of drug-metabolizing CYP450 enzymes in the endothelial cells (11). Modified from Löscher and Friedman (2020)

(AEDs) are typically small, lipophilic, and uncharged and can thus easily access the CNS by passive diffusion (Löscher et al. 2011). In addition to passive diffusion, few drugs may use carrier-mediated active transport at the BBB to penetrate into the brain parenchyma (Abbott 2013). One example is the short branched-chain fatty acid valproate, an AED that is subject to active transport at the BBB (Frey and Löscher 1978), mediated by a medium-chain fatty acid transporter (Adkison and Shen 1996).

However, it has to be noted that the BBB (or NVU) is not uniform throughout the brain (Wilhelm et al. 2016). At the level of circumventricular organs (CVOs, such as the area postrema, the subfornical organ, the organum vasculosum laminae terminalis, the pineal gland, the posterior pituitary, the intermediate lobe of the pituitary gland, the median eminence, and the subcommissural organ) capillaries are more permeable, containing fenestrations and discontinuous tight junctions.

Although various compounds, including xenobiotics, can enter these regions from the blood, they do not provide direct passage of blood-borne substances to the rest of the brain due to the presence of diffusion barriers (Wilhelm et al. 2016). Both the outer basement membrane and also astrocytes and tanocytes are considered alternative barriers to prevent diffusion of blood-derived molecules outside the sensory CVOs (Wilhelm et al. 2016).

In addition to the morphological basis of barrier function, particularly tight junctions between brain capillary endothelial cells, a number of ABC energy-dependent efflux transporters (ATP-binding cassette transporters), including P-glycoprotein (Pgp; MDR1; ABCB1), provide a transport barrier in brain capillary endothelial cells by actively pumping many potentially toxic lipophilic compounds back into the blood, which, however, also restricts the brain entry of many therapeutically used drugs (Löscher and Potschka 2005a, b). Furthermore, drug-metabolizing cytochrome (CYP)P450 enzymes are present in brain capillary endothelial cells and are thought to add to the metabolic component of the BBB (Ghosh et al. 2011).

The existence of a physical interface between the CNS and the vascular capacity was first suggested by observations, often ascribed to Ehrlich (1885), that injection of a hydrophilic dye into the blood circulation stained peripheral organs but not the spinal cord and the brain. In 1921, Lisa Stern postulated a barrier between blood and neuronal tissue, coining the term blood–brain barrier (*barrière hématoencéphalique*).

The first indication that epileptic seizures may compromise the functionality of the BBB stems from experiments in the 1950s, in which seizures induced by pentylenetetrazole (PTZ) led to the presence of a hydrophilic dye (Evans Blue or Geigy-Blau 536) in the brain parenchyma, whereas this dye did not penetrate through the BBB in controls (Bauer and Leonhardt 1955; Bauer and Lenhardt 1956). Subsequent studies using BBB tracers such as Evans Blue, Horse Radish Peroxidase or fluorescein showed that BBB disruption in several (limbic) brain regions of animals occur within 5–30 min after acute seizures that are induced using PTZ, bicuculline, pyridoxine or hyperthermia (van Vliet et al. 2015). Furthermore, BBB disruption is also evident in humans after traumatic brain injury (TBI), stroke, brain infections, status epilepticus (SE) and in patients with temporal lobe epilepsy (TLE) as well as in animal models of TLE, such as the kainate-, pilocarpine- or electrical stimulation-induced post-SE models (van Vliet et al. 2015). This led to the concept that epileptogenic brain insults and seizures induce a non-specific “opening” of the BBB that may allow drugs that normally do not penetrate through this barrier to now enter the brain. This, however, is not true. Instead, BBB alterations in response to seizures or epilepsy are highly specific and may result in complex functional consequences such as albumin extravasation and drug resistance. Furthermore, BBB alterations in response to brain injury may be involved in the generation of seizures and epilepsy. In the following I will review morphological and functional alterations in BBB integrity in response to seizures and brain injury and critically discuss the pathophysiological impact of such alterations. An extended version of this chapter has recently been published (Löscher and Friedman 2020).

2 Morphological Alterations of the BBB in Epilepsy

BBB cells are not fixed in a post-mitotic state but rather react to various pathological and rheological signals to stimulate angiogenesis, leading to the formation of new microvessels and vascular remodeling (Marchi and Lerner-Natoli 2013). Aberrant cerebrovascular angiogenesis has been found in epileptic foci of patients with drug-resistant TLE and in experimental models of TLE (Marchi and Lerner-Natoli 2013). The aberrant angiogenesis was associated with loss of tight junctions and increased permeability to immunoglobulines G (Rigau et al. 2007). Electron microscopy studies showed increased pinocytotic activity, tight junction aberration, and a thickening of the capillary basement membrane in epileptic brains (Cornford and Oldendorf 1986; Cornford 1999). In addition to pinocytosis, pericyte-microglia clustering was observed at the cerebrovasculature in the epileptic brain, which may contribute to BBB dysfunction (Giannoni et al. 2018; Klement et al. 2018). Furthermore, astrocytes undergo morphological alterations in epilepsy and these structural changes are accompanied by modified cellular function that may affect the BBB (Jabs et al. 2008; Coulter and Steinhauser 2015).

3 Why Do Seizures Alter the Morphology and Functionality of the BBB?

The mechanism of seizure-induced BBB alterations is not fully understood. Reactive oxygen species (ROS), matrix metalloproteinases (MMPs), angiogenic factors, inflammatory cytokines, autoantibodies, leukocyte adhesion, and immune cell extravasation have been discussed in this regard (Obermeier et al. 2013). Furthermore, the seizure-induced increase in brain glutamate levels may lead to barrier dysfunction (Xhima et al. 2016). A recent study suggests that glutamate released during seizures increases MMP-2 and MMP-9 protein expression and activity levels at the BBB, which results in reduced levels of tight junction proteins and consequently barrier leakage (Rempe et al. 2018). In the neurovasculature, MMPs likely affect barrier integrity by digesting and remodeling the extracellular matrix surrounding brain capillaries and by degrading tight junction proteins that seal the endothelium (Lischper et al. 2010; Feng et al. 2011). A leaky barrier contributes to seizure genesis through a positive feedback loop, in which seizures drive barrier leakage leading to more seizures, thereby promoting epilepsy progression. Thus, barrier leakage is both a consequence and a trigger of seizures and epilepsy, which will be discussed in detail below.

4 Impairment of BBB Function in Epilepsy, Leading to Increased Brain Uptake of Xenobiotics and Albumin

As described above, early studies with hydrophilic dyes (e.g., Evans Blue) on seizure-induced impairment of BBB function indicated a non-specific “opening” of the BBB that may allow various drugs to enter the brain (Clarke and Gabrielsen 1989; Duncan and Todd 1991; Carvey 1998). This, however, is not true, but the “leakiness” is highly specific and mainly restricted to increased brain uptake of highly protein-bound hydrophilic dyes (e.g., Evans blue), hydrophilic drugs or contrast agents (e.g., gadolinium), which normally do not enter the brain (Löscher 2016). Indeed, the increase of most (if not all) dyes in brain parenchyma is mainly a consequence of the fact that such dyes are highly bound to plasma albumin and that prolonged seizures induce extravasation of albumin through a transcellular pathway and, hence, increased extravasation of albumin-bound dyes such as Evans Blue into the brain parenchyma, whereas the brain: plasma ratio of small molecules that are not bound to plasma albumin is most often not changed or even decreased (Löscher et al. 2011; Kang et al. 2013). Albumin extravasation has not only been observed in patients with TLE (and animal models of TLE), but also in focal lesions associated with drug-resistant epilepsy such as in focal cortical dysplasia, tuberous sclerosis complex, gangliogliomas and vascular malformations (van Vliet et al. 2015). Tight junctions are only disrupted by very severe brain insults, so terms such as “BBB opening,” “BBB damage” or “BBB breakdown” in response to seizures is generally misleading, but the “leakiness” is thought to be mediated, at least in part by enhanced micropinocytosis through endothelial cells, while the tight junctions remain intact (Löscher et al. 2011; Löscher 2016). Surprisingly, only relatively few studies examined whether prolonged, severe seizures alter the BBB permeability to commonly used drugs, such as AEDs.

In a study performed by my group (Potschka et al. 2011), which was prompted by earlier observations that extracellular brain levels of the AED phenytoin are *lower* in kindled than non-kindled rats (Potschka and Löscher 2002), microdialysis experiments were performed in amygdala-kindled rats and electrode-implanted, non-kindled rats with the microdialysis probe located directly adjacent to the stimulation/recording depth electrode in the basolateral amygdala (BLA). Penetration of phenytoin to the extracellular fluid in the focus region (the stimulated BLA) was investigated at different time points in relation to seizure activity elicited in kindled rats. Access of phenytoin to the kindled focus proved to be similar in kindled rats 2 h or 14 days following a single generalized seizure compared to non-kindled, electrode-implanted control rats. When a single generalized seizure was elicited 10 min following phenytoin administration, average phenytoin brain dialysate levels were *lower* (up to 44%) than those of control animals. During self-sustained SE, which was induced by 30 min of electrical stimulation of the amygdala and lasted 3 h, phenytoin access to the site of seizure initiation tended to be lower in the early phase following drug administration, but reached the control level 2 h later. BBB disruption was demonstrated by enhanced brain uptake of Evans Blue. These data clearly demonstrate that seizure-induced alterations in BBB integrity and function do not

increase brain levels of phenytoin in dialysates of the affected brain region, but rather decrease the free (non-protein bound) concentration of phenytoin in the extracellular compartment.

Marchi et al. (2009) studied the effects of impaired BBB integrity on brain distribution of hydrophilic (deoxyglucose and sucrose) and lipophilic (phenytoin and diazepam) molecules. They tested the hypothesis that lipophilic and hydrophilic drug distribution is differentially affected by BBB damage. In vivo BBB disruption was performed in rats by intracarotid injection of hyperosmotic mannitol. Drugs were measured and correlated with brain water content and protein extravasation. BBB disruption resulted in extravasation of serum albumin and radiolabeled drugs. Total drug permeability increase was greater for lipophilic than hydrophilic compounds. However, BBB disruption markedly *reduced* the amount of free phenytoin in the brain, which is comparable to our microdialysis findings with phenytoin after seizure-induced BBB disruption (Potschka et al. 2011). Marchi et al. (2009) concluded that after BBB disruption, drug binding to protein is the main controller of total brain drug accumulation. Osmotic BBB disruption increased serum protein extravasation and reduced free phenytoin brain levels. Thus, two studies independently found that the functionally relevant free concentration of the AED phenytoin is reduced after BBB disruption (Marchi et al. 2009; Potschka et al. 2011), which is in contrast to the often suggested notion that BBB disruption should increase brain penetration of AEDs and various other drugs. Indeed, BBB “opening” to large molecules such as albumin does not necessarily imply BBB “opening” to small molecules or ions (Kang et al. 2013). In line with this concept, analysis of various AEDs in brain extracellular fluid, brain tissue, cerebrospinal fluid, and serum of patients with intractable epilepsy did not indicate any increase in brain AED levels as a result of BBB impairment (Rambeck et al. 2006). Duncan and Todd (1991) already argued that the state of the BBB should have little effect on brain tissue levels of AEDs because these are highly lipid soluble in contrast to hydrophilic dyes and thus hardly affected by BBB impairment, which is substantiated by our experimental and clinical data (Rambeck et al. 2006; Potschka et al. 2011).

However, severe BBB impairment may result in entry and accumulation in neurons and the neuronal interstitium of several toxic molecules from blood, including fibrinogen, thrombin, hemoglobin, iron-containing hemosiderin, free iron, plasmin (an extracellular matrix degrading enzyme), and environmental toxins (Montagne et al. 2016). Furthermore, magnetic resonance (MR) contrast agents containing the heavy metal gadolinium are used in association with MR imaging (MRI) in routine clinical practice to detect and quantify BBB leakage (Montagne et al. 2016; van Vliet et al. 2017). Under normal circumstances, gadolinium-based contrast agents, such as gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA), do not cross the intact BBB. This may either be a result of their physicochemical (hydrophilic or polar) properties or binding of gadolinium-based contrast agents to plasma albumin (Elst et al. 2001; Kang et al. 2013). However gadolinium-based contrast agents extravasate from blood into brain tissue even when the BBB is only partially compromised (McDonald et al. 2017). A recent study using a gadolinium-based contrast agent (gadobutrol) in patients with chronic

epilepsy provided evidence for the occurrence of BBB dysfunction, which is temporally and anatomically associated with epileptic seizures (Rüber et al. 2018). However, recent evidence has suggested that gadolinium appears to accumulate even among patients with normal brain tissue and no history of intracranial pathology, indicating that current thinking with regard to the permeability of the BBB is greatly oversimplified (McDonald et al. 2017).

In addition to increased brain uptake of toxic molecules from blood, BBB dysfunction can result in an imbalance of ions, transmitters, and metabolic products in the interstitial fluid, causing abnormal neuronal activity, which may be involved in the generation of seizures (Obermeier et al. 2013). However, it is important to note that BBB disruption does not always lead to immediate seizures (van Vliet et al. 2015). This is further supported by the fact that BBB disruption not only occurs in the epileptic brain, but is also observed in various other neurological disorders (e.g. multiple sclerosis, Alzheimer's disease, and cerebral ischemia). Although seizures may occur in these diseases, the risk to develop epilepsy is not as high as after TBI, stroke or SE, indicating that BBB leakage by itself is not always a cause of seizures. Furthermore, apart from BBB dysfunction, there are multiple other brain alterations that can cause seizures and epilepsy (Klein et al. 2018), so the relative role of the BBB in ictogenesis and epileptogenesis should not be overestimated.

5 Increased Efflux Transport at the BBB in Epilepsy, Leading to Decreased Brain Uptake of Antiepileptic Drugs: a Role in Drug Resistance?

Drugs required to act in the brain, including AEDs, have generally been made lipophilic, and are thus able to cross the brain endothelium via the lipid membranes. However, such lipophilic drugs are potential substrates for efflux carriers of the BBB, particularly Pgp, which is predominantly located on the endothelial luminal membrane of the BBB. It is estimated that up to 50% of drug candidates may be substrates for Pgp (Abbott et al. 2002). In several neuropathological conditions, including epilepsy, the BBB is disturbed, either as a result of pathology of the endothelium or adjoining cells responsible for barrier induction and maintenance (see above). During epileptic seizures, there may be transient BBB disruption in the epileptogenic zone. There is evidence that under such pathological conditions, “second line defense” mechanisms in brain capillary endothelial cells and perivascular glia may be up-regulated, including expression of Pgp and other drug efflux transporters (Löscher and Potschka 2005a, b). This may be involved in the mechanisms of AED resistance in epilepsy, which affects about 30% of all patients and is a major problem in epilepsy therapy (Löscher and Schmidt 2011).

Tishler et al. (1995) were the first to describe an increased expression of the drug efflux transporter Pgp in brain capillary endothelial cells of patients with drug-resistant seizures and suggested that this overexpression may restrict brain penetration of AEDs such as phenytoin. This led to the “transporter hypothesis” of refractory epilepsy (Fig. 2), suggesting that increased expression of efflux

The transporter hypothesis of refractory epilepsy

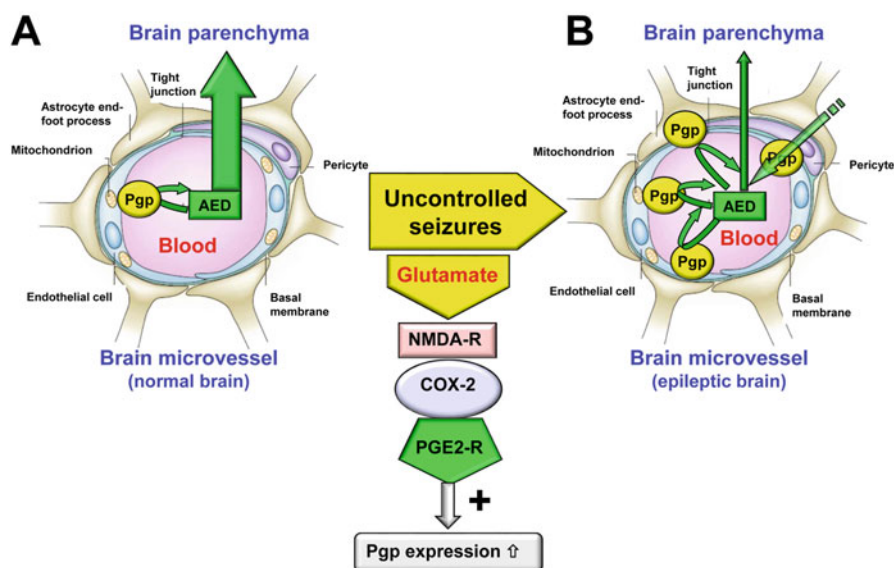


Fig. 2 Schematic illustration of the transporter hypothesis of drug-resistant (refractory) epilepsy (modified from Löscher and Friedman 2020). About 30% of all patients with epilepsy do not respond to current antiepileptic drugs (AEDs) and are thus drug resistant (Löscher and Schmidt 2011). Drug resistance is associated with increased morbidity and mortality (Laxer et al. 2014). Thus, understanding mechanisms of drug resistance is important for developing more effective therapies. The transporter hypothesis is one of the several hypotheses to explain why patients do not adequately respond to treatment with AEDs. Based on the transporter hypothesis, patients with drug-resistant epilepsy have an increased expression of efflux transporters at the BBB in affected brain regions, leading to reduced penetration of AEDs across the BBB. Indeed, preclinical experiments have shown that sustained seizure activity leads to induction of efflux transporters such as P-glycoprotein (Pgp) at the apical membrane of brain capillary endothelial cells that form the BBB (Löscher and Potschka 2005a, 2005b; Tang et al. 2017). As shown in A, in the absence of seizures (in the nonepileptic brain) AEDs easily penetrate through the BBB by diffusion, because they are small, lipophilic, and non-ionized and only relatively weak substrates of Pgp (Löscher et al. 2011). For instance, based on in vivo data with chemical inhibition of Pgp in rats and genetic knockout in mice, it has been calculated that the normal, constitutive expression of Pgp at the BBB restricts brain penetration of the AED phenytoin by about 40–50% (Löscher et al. 2011). As shown in B, following seizures, Pgp is overexpressed in the endothelial cells, so the AED fraction bound to Pgp in the endothelial cells increases and the drugs are transported back into the blood, thereby reducing brain levels of these drugs in affected brain regions. For instance, for phenytoin it has been shown in vivo that Pgp can affect up to about 70–80% of brain drug uptake under these conditions (Löscher et al. 2011). In addition, AEDs that are already in the brain parenchyma undergo enhanced efflux from the brain, which is mediated by Pgp. This cannot be simply counteracted by increasing the dose, because Pgp is only overexpressed in epileptogenic or focal brain tissue, so increasing the dose would lead to toxic brain levels in other regions. By inhibiting Pgp, e.g., by tariquidar, the increased AED efflux and the associated drug resistance can be reversed in preclinical animal models of epilepsy, and, anecdotally, in patients with drug-resistant seizures (Löscher et al. 2011, 2020). The mechanisms underlying the Pgp increase in response to seizures (Miller et al. 2008) are also illustrated. The increase in Pgp resulting from enhanced glutamate release in response to seizures can be inhibited by NMDA receptor antagonists or COX-2 inhibitors or PGE2 receptor

transporters in brain capillaries of epileptogenic tissue restricts AEDs to reach their brain targets (Löscher and Potschka 2005a, b). There is now a large body of evidence that multidrug transporters such as P-gp, BCRP, MRP1, and MRP2 and their genes are overexpressed in capillary endothelial cells and astrocytes in epileptogenic brain tissue that has been surgically resected from patients with medically intractable epilepsy (Tang et al. 2017). The expression of multidrug transporters in astroglial end-feet covering the blood vessels found in epileptogenic brain tissue might represent a “second barrier” under these conditions. The increased expression of Pgp induced by frequent seizures is thought to be a result of a complex signaling cascade (Fig. 2), including seizure-induced glutamate release, which through stimulation of NMDA receptors leads to an induction of cyclooxygenase-2 (COX-2), which then, via prostaglandin E2 (PGE2) receptors, increases the expression of Pgp (Miller et al. 2008). In line with this hypothesis, the seizure-induced increase of Pgp can be inhibited by NMDA receptor antagonists and COX-2 inhibitors and PGE2 receptor antagonists (Bankstahl et al. 2008; Bauer et al. 2008; Zibell et al. 2009; Pekcec et al. 2009).

Consistent with the transporter hypothesis, animal experiments have shown that (1) rats with spontaneous recurrent seizures not responding to AEDs (AED nonresponders) exhibit higher expression of Pgp at the BBB than AED responders, (2) various AEDs are transported by rodent Pgp, (3) overexpression of Pgp is associated with lower brain levels of AEDs in rodents, and (4) inhibition of Pgp by the selective Pgp inhibitor tariquidar counteracts resistance to AEDs in a rat model of TLE (Löscher et al. 2011). Indeed, several widely used AEDs, which have been made lipophilic to penetrate into the brain, are substrates for both rodent and human Pgp and other drug efflux transporters (Löscher et al. 2011, 2020; Zhang et al. 2012).

The transporter hypothesis has also been investigated clinically. By using positron emission tomography (PET) and the PET ligand (*R*)-[¹¹C]verapamil with and without the Pgp inhibitor tariquidar in 14 pharmacoresistant patients, eight seizure-free patients, and 13 healthy controls, Feldmann et al. (2013) substantiated the association between Pgp overactivity in some regions of the brain and pharmacoresistance in TLE, thus providing the first in vivo proof-of-concept of the transporter hypothesis in humans. In a large study on post-mortem brains from patients with drug-sensitive or drug-resistant chronic epilepsy and controls, Liu et al. (2012) found highly localized overexpression of Pgp in the epileptogenic hippocampus of patients with drug-resistant epilepsy (on the vascular endothelium and end-feet of vascular glia, forming a “double cuff”) and concluded “our findings show that the expression of Pgp is compatible with the inherent assumptions of one

Fig. 2 (continued) antagonists (Löscher et al. 2011, 2020). It is important to note that it is not likely that drug resistance is mainly due to one mechanism such as the mechanism illustrated here, but rather several resistance mechanisms have been postulated which may even occur in concert in the same patient (Löscher et al. 2013; Tang et al. 2017). Furthermore, the transporter hypothesis of drug-resistant epilepsy has been questioned, mainly because some studies did not detect any transport of AEDs by Pgp (see text)

current hypothesis of multidrug resistance.” Thus, pharmacological overcoming Pgp overactivity could provide a potential treatment strategy, as demonstrated in animal models (Brandt et al. 2006; Löscher et al. 2011); however, despite some anecdotal clinical reports, no controlled trials on this strategy have been performed until now. At least in part, this is due to the unexpected toxicity of add-on treatment with Pgp inhibitors in clinical cancer treatment trials. This toxicity was due to the increased penetration of cytotoxic chemotherapeutic drugs into normal tissues as a result of Pgp inhibition (Chung et al. 2016). This would not be a problem with AEDs, because these drugs are not cytotoxic.

In addition to Pgp, some AEDs are transported by breast cancer resistance protein (BCRP, ABCG2), e.g., lamotrigine (Römermann et al. 2015). This is important, because BCRP is significantly higher expressed at the human BBB than Pgp, whereas the opposite is true for the rodent BBB (Uchida et al. 2011). In contrast to Pgp and BCRP, most AEDs seem to be not transported by human multidrug resistance proteins (MRPs, ABCCs), such as MRP1, MRP2, and MRP5, which are overexpressed in drug-resistant epilepsy (Luna-Tortós et al. 2010).

However, at present, aspects of the transporter hypothesis are still controversial, and further research is needed to determine the clinical relevance of efflux transporter overexpression at the BBB (Tang et al. 2017; Löscher et al. 2020). Indeed, the transporter hypothesis of drug-resistant epilepsy has been questioned mainly because some studies did not detect any transport of AEDs by Pgp (Löscher et al. 2011; Tang et al. 2017). In this respect it is important to note that most AEDs are only weak substrates of Pgp, so modified *in vitro* transport assays are needed to demonstrate significant Pgp-mediated transport of AEDs (Löscher et al. 2011, 2020; Zhang et al. 2012). Despite the low affinity of most AEDs to Pgp *in vitro*, significant overexpression of Pgp may still restrict AED access to epileptic neurons *in vivo* as demonstrated, for instance, for phenytoin. For the latter drug, it has been shown *in vivo* that Pgp can affect up to about 70–80% of brain drug uptake under these conditions in epileptic rodents (Löscher et al. 2011). Furthermore, studies utilizing PET/MR imaging demonstrated increased Pgp transport activity in patients with drug-resistant epilepsy and that seizure reduction after surgery leads to a decrease in Pgp overactivity (Feldmann et al. 2013; Bauer et al. 2014; Shin et al. 2016; Ilyas-Feldmann et al. 2020). Together, these patient data suggest that an optimal outcome after epilepsy surgery is associated with a reduction in Pgp transport activity and that Pgp overexpression could serve as a surrogate marker for drug-resistant epilepsy (Tang et al. 2017).

In addition to increased expression of efflux transporters such as Pgp in BBB endothelial cells and astroglia, cytochrome P450 enzymes known to be responsible for the metabolism of several AEDs (CYP3A4, CYP2C9, CYP2C19, CYP2A6, and CYP2E1) were demonstrated to be elevated in brain endothelial cells isolated from temporal lobe resections of drug-resistant epileptic subjects (Ghosh et al. 2011). This indicates that increased metabolism of AEDs at the level of the BBB may add to drug resistance. Overall, these data suggest that alterations of the BBB in epilepsy decrease rather than increase drug penetration into brain parenchyma, thus arguing against the concept of a “leaky” BBB, at least with regard to drug distribution.

6 Impairment of Barrier Function of the BBB in Epilepsy, Leading to Invasion of Inflammatory Cells Into the Brain

Clinical and experimental evidence indicates that inflammatory processes contribute to the pathophysiology of several types of epilepsy (Ravizza and Vezzani 2018). However, the respective contribution of brain resident and peripheral immune cells to epileptogenesis is only incompletely understood. Under healthy conditions, peripheral immune cells are restricted from CNS entry by the presence of the BBB (Prinz and Priller 2017). However, when the BBB is impaired following brain injury or during CNS diseases such as epilepsy, peripheral adaptive and innate immune cells, including monocytes, neutrophils, T cells, and B cells, can enter the CNS, where they execute distinct cell-mediated effects which might be either neuroprotective or neurotoxic or both (Zattoni et al. 2011; Prinz and Priller 2017). Indeed, trafficking of blood-born immune cells over the BBB into the CNS represents a key process in neuroinflammation and consists of a well-defined and regulated multi-step cascade involving consecutive steps of adhesive interactions between the leukocytes and the endothelium. During the initial contact with the activated endothelium leukocytes roll along the endothelium via a loose bond which is mediated by selectins. Subsequently, leukocytes are activated by chemokines presented on the luminal endothelial surface, which results in the activation of leukocyte integrins and the firm leukocyte arrest on the endothelium. After their firm adhesion, leukocytes make use of two transmigration processes to pass the endothelial barrier, the transcellular route through the endothelial cell body or the paracellular route through the endothelial junctions and enter the perivascular space (Imhof and Dunon 1997; Man et al. 2007; Langer and Chavakis 2009; Rossi et al. 2011). Within the perivascular space, the invading immune cells interact with other perivascular immune cells and are further guided towards the brain parenchyma by chemoattractants, such as chemokines and cytokines. Interestingly, before the multi-step paradigm described above, immune cells may become activated in the periphery (e.g., in the lung) to reprogram their gene-expression profile, characterized by downregulation of their activation program and upregulation of cellular locomotion molecules together with chemokine and adhesion receptors (Odoardi et al. 2012).

Once invading leukocytes have crossed the glia limitans (the basement membrane laid down by astrocytes) by an enzyme-mediated process (Engelhardt et al. 2016), they reach the brain parenchyma and are further activated to produce cytokines, resulting in massive immune cell recruitment and clinical disease (Kawakami and Flugel 2010). This inflammatory response subsequently further promotes BBB damage “from the inside..” Astrocytic and microglial IL-1 β , VEGF or both have been suggested to promote enhanced BBB permeability (Obermeier et al. 2013). Fingolimod, which impairs T cell migration to the CNS, was shown to exert anti-seizure and anti-epileptogenic effects in experimental TLE, indicating that brain invading T cells and cytokines released by these cells are involved in both ictogenesis and epileptogenesis (Pitsch et al. 2019).

There is evidence that leukocytes may also facilitate seizures and epileptogenesis from outside the BBB, notably without entering the brain parenchyma (Fabene et al.

2008). In a mouse model of pilocarpine-induced epilepsy, it has been demonstrated that the BBB endothelium displays an activated phenotype after a seizure and that increased *Icam-1*, *Vcam-1*, *E-selectin*, and *P-selectin* expression promote leukocyte rolling and arrest at the luminal surface of the cerebral vessel (Fabene et al. 2008). Remarkably, when leukocyte-endothelial interactions were inhibited, the number of recurrent seizures and the extent of BBB damage were reduced. Furthermore, this approach prevented the development of epilepsy. These findings suggest that it may be possible to develop selective therapeutics that inhibit leukocyte-endothelial interactions in the periphery, thereby preventing disease initiation or progression within the brain without the need to deliver drugs across the BBB.

In addition to T cells and neutrophils, peripheral monocytes are known to enter the brain after TBI and other epileptogenic brain insults and contribute to neuronal injury (Corps et al. 2015; Minogue 2017). Indeed, recently, brain invasion of peripheral monocytes have been implicated in epileptogenesis. Varvel et al. (2016) demonstrated that infiltrating monocytes promote brain inflammation and exacerbate neuronal damage after SE in mice. Importantly, inhibiting monocyte recruitment reduced albumin extravasation and attenuated neuronal damage. We demonstrated that suppressing monocyte recruitment prevents hippocampal neurodegeneration in a mouse model of viral encephalitis-induced epilepsy (Käufer et al. 2018). Brain resident cells such as microglia and astroglia contribute to the proinflammatory effects of brain invading immune cells (Prinz and Priller 2014, 2017). Overall, inflammatory processes, including brain invasion of inflammatory cells through a disturbed BBB, contribute to the generation of acute and chronic epileptic seizures and associated brain pathology.

7 Impairment of Barrier Function of the BBB Following Brain Injury: Role in the Development of Epilepsy?

In addition to immune cell migration through the impaired BBB, albumin extravasation has been suggested to play a role in epileptogenesis (Heinemann et al. 2012; Vezzani et al. 2013). Indeed, almost 40 years ago, BBB disruption was proposed to cause seizures, as osmotic opening of the BBB resulted in epileptic seizures in rats (Fieschi et al. 1980). This finding was supported by a more recent clinical study in which the BBB was transiently opened in patients to treat brain tumors, which resulted in focal motor seizures that were related to albumin extravasation (Marchi et al. 2007). Albumin extravasation is found after several, if not all, epileptogenic brain insults, including TBI and SE (Friedman et al. 2009). Following extravasation, albumin is present in the parenchyma, but can also be taken up or bound to neurons, astrocytes, and microglial cells. Albumin can enter astrocytes via transforming growth factor beta (TGF- β) receptors. This is followed by a downregulation of inward rectifying potassium (Kir 4.1) channels and water (aquaporin 4; AQP4) channels in these astrocytes. As a result, the buffering of extracellular potassium is reduced, which facilitates NMDA-mediated neuronal hyperexcitability and eventually induces epileptiform activity (Heinemann et al. 2012). Furthermore, albumin

can induce transcriptional changes involving TGF- β signaling as the most prominent pathway. This can lead to long lasting alterations in the brain, such as brain inflammation, which is suggested to be an important mechanism that can contribute to epileptogenesis (Vezzani et al. 2013). Several studies show that brain inflammation can directly affect synaptic signaling, plasticity, reduce inhibitory transmission, affect BBB permeability and thereby lower the seizure threshold and promote epileptogenesis (van Vliet et al. 2015). The angiotensin II type 1 receptor antagonist, losartan, blocks brain TGF- β signaling and prevents epilepsy in different models of epileptogenesis (Bar-Klein et al. 2014, 2017). Interestingly, using gadolinium-DPTA to assess BBB impairment in patients following TBI, revealed increased BBB permeability in 76.9% of patients with post-traumatic epilepsy compared with 33.3% of patients without epilepsy ($p = 0.047$), indicating a correlation between disrupted BBB and abnormal neuronal activity in patients with TBI (Tomkins et al. 2008).

We recently explored the spatiotemporal evolution of extravasation of albumin and illuminated associated responses of the NVU contributing to early epileptogenic neuropathology (Bankstahl et al. 2018). For this purpose, we applied translational in vivo MRI (using gadolinium-DPTA as a contrast agent) and complementary immunohistochemical analyses in the widely used rat pilocarpine post-SE model of TLE. The observed rapid BBB leakage affected epileptogenesis-associated brain regions, peaked between 1 and 2 d post-SE, and rapidly declined thereafter, accompanied by cerebral edema generally following the same time course. At the peak of BBB leakage, serum albumin colocalized with NVU constituents, such as vascular components, neurons, and brain immune cells. Surprisingly, astroglial markers did not colocalize with albumin, and AQP4 was clearly reduced in areas of leaky BBB, indicating a severe disturbance of astrocyte-mediated endothelial-neuronal coupling. In addition, a distinct adaptive reorganization process of the NVU vasculature apparently takes place at sites of albumin presence, substantiated by reduced immunoreactivity of an endothelial marker (biotinylated *Solanum tuberosum* lectin) and changes in a vascular basement membrane marker (laminin). Taken together, degenerative events at the level of the NVU, affecting vessels, astrocytes, and neurons, seem to outweigh reconstructive processes. Considering the rapidly occurring BBB leakage and subsequent impairment of the NVU, our data support the necessity of a prompt BBB-restoring treatment as one component of rational therapeutic intervention to prevent epileptogenesis and the development of other detrimental sequelae of epileptogenic brain insults such as SE.

8 Conclusions

The BBB is a highly selective, semipermeable interface regulating the passage of ions and larger molecules into the brain parenchyma and thereby playing a crucial role in maintaining strict neuronal homeostasis. The association between epilepsy and impairment of the BBB has long been suggested (Jasper 1970), leading to the concept that BBB dysfunction represents a hallmark of seizures and epilepsy.

However, epilepsy research has faced a crucial question since then: whether BBB impairment is a cause or a consequence of epileptic seizures or both (Friedman and Heinemann 2012). More recent data from brain imaging indicate that BBB dysfunction with albumin extravasation into brain parenchyma is the commonality of epileptogenic brain insults and may be suited as a biomarker of epileptogenesis in both animal models and patients (Heinemann et al. 2012; Friedman et al. 2014). Albumin extravasation into brain parenchyma can induce epileptogenesis and can sustain or even aggravate the epileptic condition. It is important to note that BBB dysfunction is generally locally restricted (focal) and transient. The consequence of seizure- or injury-induced BBB disruption for drug distribution into the brain is more complex because, as outlined in this chapter, BBB dysfunction is compensated, at least in part, by focal upregulation of efflux transporters such as Pgp, BCRP, and MRPs, which may reduce functionally relevant free (unbound) drug concentrations in epileptogenic brain regions. Hopefully, a better understanding of the complex BBB alterations in response to seizures and epilepsy can lead to novel treatment strategies. A rapidly acting BBB-restoring treatment would be one component of rational therapeutic intervention to treat drug-resistant seizures and prevent epileptogenesis and the development of other detrimental sequelae of brain injury (Bankstahl et al. 2018). Currently, the only applicable and most widely used therapeutic approach has been to improve BBB integrity by glucocorticosteroid treatment (Obermeier et al. 2013). Indeed, the beneficial effect of add-on glucocorticosteroids for treatment of drug-resistant seizures is associated with restoration of BBB function (Marchi et al. 2012). Similarly, the efficacy of natalizumab in drug-resistant epilepsy may be a result of preventing BBB-leukocyte interaction (Marchi et al. 2012). Other approaches that potentially qualify for therapeutic BBB repair are currently under investigation, but in most cases their clinical implementation is still far from reality.

Further research in this field will hopefully lead to clinically usable treatment options for neurological complications associated with BBB breakdown.

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Novel Treatment Approaches for Brain Tumour from a Blood–Brain Barrier Perspective

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Abstract

For a chemotherapeutic agent to be effective, it must conquer the presence of blood–brain barrier (BBB), which limits the penetration of drugs into the brain.

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Tumours in the brain compromise the integrity of BBB and result in a highly heterogeneous vasculature, known as blood–brain tumour barrier (BBTB). In this chapter, we firstly highlight the cellular and molecular characteristics of the BBB and BBTB as well as the challenges aroused by BBB/BBTB for drug delivery. Secondly, we discuss the current strategies overcoming the challenges in invasive and non-invasive manners. Finally, we highlight the emerging strategy using focused ultrasound (FUS) with systemic microbubbles to transiently and reversibly enhance the permeability of these barriers for drug delivery.

Keywords

Blood–brain barrier (BBB) · Blood–brain tumour barrier (BBTB) · Focused ultrasound (FUS) · Microbubbles

1 BBB Basics

An adequate supply of blood, as well as the functional and structural integrity of the central nervous system (CNS) blood vessels that efficiently deliver oxygen and nutrients, are critical to proper neuronal functioning of the brain (di Giovanna et al. 2018). The complete function of the neurovascular unit (NVU) in the CNS is necessary for preventing macromolecules and unwanted compounds from penetrating the brain, protecting the CNS from potentially neurotoxicant complexes. This protective barrier known as the blood–brain barrier (BBB) is a cellular barrier that maintains the homeostasis in ionic composition for adequate synaptic transmission. The main BBB component is the brain capillary endothelial cells (ECs), the interplay with the surrounding cells such as pericytes, astrocytes, microglia, neurons, and the extracellular matrix compose the NVU (van Tellingen et al. 2015; Hansen 1985).

Pericytes, as vascular mural cells, uniquely reside in the basement membrane of brain capillaries and contribute both to the maintenance of BBB integrity, as well as the induction of barrier properties during development (Obermeier et al. 2013; Sweeney et al. 2016). Astrocytic endfeet compose a complex network surrounding the basal lamina of cerebral capillaries, providing biochemical support to the endothelium as well as supplying cellular connection to neurons, to play a key role in the maintenance of barrier properties. Brain capillary ECs are closely connected by tight junctions (TJs), which enormously restrict brain entry of molecules across the paracellular path except for certain extremely small or gaseous molecules (e.g. water, oxygen, carbon dioxide) as well as tiny highly hydrophobic/liposoluble molecules, that can freely traverse the BBB through passive diffusion (Abbott et al. 2006). Brain capillary ECs display transcytosis at exceptionally low rates as compared with peripheral ECs, which greatly hampers the vesicle-mediated transcellular transport of solutes. The tense paracellular and transcellular barrier establishes the polarization of brain ECs with abluminal and luminal membrane portions. The movement of solutes between the bloodstream and brain parenchyma can therefore be delicately controlled based on the characteristics of regulated cellular transport as

well as the physicochemical properties of solutes, such as electrical charge and lipid solubility (Zahraoui et al. 2000). The tight junction-associated proteins expressed on the adjacent region between brain capillary ECs include occludin (di Giovanna et al. 2018; van Tellingen et al. 2015; Hansen 1985), claudins (di Giovanna et al. 2018; Hansen 1985; Yu et al. 2005), and junctional adhesion molecules (JAMs) (Wolburg and Lippoldt 2002; Ballabh et al. 2004; Abbott et al. 2010; Wolburg et al. 2009). Claudin-5 and occludin, playing a key role in limiting paracellular diffusion, are connected via zonula occludens (ZO) proteins to tight junction-associated proteins (e.g. Cingulin and 7H6) that linked to the myosin/actin cytoskeletal system, contributing to modification of tight junction properties (Yu et al. 2005; Stamatovic et al. 2008).

Furthermore, there are two major kinds of transporters expressed in brain capillary ECs. The first are highly specific transporters that mediate the uptake of essential nutrients across the BBB into the brain, as well as the elimination of specific metabolic waste into the blood from the brain. Many of these transporters appertain to the solute carrier (SLC) superfamily, such as SLC2A1 (glucose), SLC16A1 (pyruvate, lactate), and SLC7A1 (cationic amino acids) (Zlokovic 2008; Daneman and Prat 2015).

The second are efflux transporters, located at the luminal surface and transport a broad array of lipophilic molecules, that could passively diffuse through the cell membrane of ECs, back into the blood, and thus act as ‘gatekeepers’ to control brain entrance of these substrates. A variety of adenosine triphosphate (ATP) binding cassette efflux transporters, including P-glycoprotein (P-gp; also known as ABCB1), breast cancer resistance protein (BCRP; also known as ABCG2), and multi-drug resistance-associated proteins (MRPs; also known as ABCCs), utilize the energy of ATP hydrolysis to transport their substrates against their concentration gradient (Ha et al. 2007). Therefore, active ATP binding cassette (ABC) efflux transporters play a significant role in reducing or preventing neurotoxic side effects of drugs.

Moreover, brain capillary ECs express a range of different receptor-mediated transport systems, such as the transferrin receptor that are involved in the uptake of required nutrients. Taken together, the combination of physical restriction properties (TJs, low transcytosis rate) and transport barrier properties (specific transports facilitating solute flux, enzymes metabolizing molecules), as well as specific transporters for delivery of essential nutrients, allows the brain ECs to delicately regulate CNS homeostasis (Daneman and Prat 2015).

The unique barrier properties of BBB also offer challenges for drug delivery to the CNS and confer chemo-resistance to brain tumours. Apart from hampering paracellular transport, a wide range of anticancer agents are unable to cross the BBB via transcellular diffusion, which is principally owing to the presence of active efflux transporters (AETs). P-gp, a 170 kD transmembrane protein encoded by the ABCB1 gene is well known. Nearly 60% of all marketed anticancer drugs could be recognized by P-gp and subsequently pumped back into the blood circulation from the tumour cells, leading to lower drug accumulation and diminished therapeutic efficacy (van Tellingen et al. 2015; Ambudkar et al. 1992). For certain drugs, such as vemurafenib, pronounced overlapping functions of P-gp and BCRP in reducing drug

brain accumulation have been documented (Durmus et al. 2012). Hence, the development of potential approaches to improve anti-tumour drug delivery and overcome tumour resistance to chemotherapy has become a major issue in the treatment of brain tumours (especially glioblastomas (GBM)).

2 BBB and BBTB

Impaired BBB functions can be observed in different neurological disorders including multiple sclerosis, epilepsy, stroke, Alzheimer's disease (AD), and brain cancer (Obermeier et al. 2013; Abbott et al. 2006; de Vries et al. 2012). Physically disruption of the BBB is evident from the higher drug accumulation within the extravascular extracellular space in nearly all GBM (Cao et al. 2006). The BBB is destroyed with tumour progression, leading to a vasculature referred to as the blood–brain tumour barrier (BBTB). The BBTB is characterized by heterogenous pericyte coverage (Dubois et al. 2014) and shrinkage of astrocyte endfeet, that can be physically displaced from brain capillary ECs by invading glioma cells (Watkins et al. 2014), all of which can diminish the barrier functions of the CNS NVU.

The BBTB, generally regarded as leakier than the BBB, consist of three different capillaries: (1) discontinuous capillaries; (2) continuous fenestrated capillaries, and (3) continuous non-fenestrated capillaries (Blasberg et al. 1987; Groothuis 2000; Schlageter et al. 1999). In low-grade gliomas, the function of the BBTB can remain mostly unchanged akin to the BBB under health conditions (Machein et al. 1999). However, neuroimaging including positron emission tomography (PET) and magnetic resonance imaging (MRI) have demonstrated major functional alterations of the normal NVU leading to BBTB disruptions particularly in high-grade brain tumours such as GBM (Dhermain et al. 2010; Sarkaria et al. 2018). Moreover, T cell subpopulations and peripheral blood monocytes observed in brain tumour tissues suggest compromised permeability of the NVU to circulating immune cells (Ratnam et al. 2019). The elevated metabolic demands of high-grade glioma cause hypoxic zones that stimulate increased expression of vascular endothelial growth factor (VEGF) and neovascularization, resulting in abnormal vasculature and dysfunctional BBTB (Hardee and Zagzag 2012; Plate et al. 2012).

Generally, the tumour bulk in GBM consists of three main components: (i) the necrotic central area where the healthy tissue is entirely replaced by tumour cells and an enhanced vascular permeability is present, (ii) the proliferative/angiogenic forehead, and (iii) the brain adjacent to tumour, where the neoplastic cells infiltrate into healthy brain tissue and the BBB remains undamaged (van Tellingen et al. 2015; Woodworth et al. 2014; Drean et al. 2016). Enhanced permeability and retention (EPR) effects resulted from a leaky BBTB were ever regarded as an important reason leading to the accumulation of therapeutic drugs in the tumour tissues (Hendricks et al. 2015). However, the invasive nature of GBM motives widespread proliferation of high-grade glioma cells, which extends away from the tumour core with a disrupted BBTB. These invading tumour cells favour infiltrating to otherwise normal

brain areas with an intact BBB as well as with less conspicuous EPR effect. Typically, this includes the regions that do not exhibit enhancement on post-gadolinium T1 weighted MRI (la Fougere et al. 2011). A leaky BBTB does not contribute much to drug accumulation in all tumour sites attributed to the less dysfunctional BBB area that exists in the whole BBB/BBTB system in GBM. As a result, both in high-grade and low-grade glioma the BBTB and the BBB barrier represent a huge hindrance in the treatment of glioma by hampering sufficient drug delivery (Juillerat-Jeanneret 2008; Korfel and Thiel 2007).

Furthermore, despite exhibiting non-uniform permeability, the BBTB preserves certain significant features of the BBB such as expression of AETs in ECs and tumour cells (Arvanitis et al. 2020). Also, overexpression of the drug efflux transporters, especially P-gp can be found in ECs of the BBB/BBTB in GBM (van Tellingen et al. 2015; Leweke et al. 1998; Takamiya et al. 1997). Within the tumour core, the expression P-gp, having a protective role, is significantly decreased and ascribed to a compromised BBTB, whereas P-gp activity is retained at the tumour edges where the BBB is intact (Deeken and Loscher 2007). Taken together, the BBB/BBTB prevents the entry of a variety of anticancer agents into the tumour infiltration zone, posing an additional challenge to curative treatment of GBM.

3 Current Chemotherapeutic Drugs for Brain Tumour

Primary brain and CNS cancers that affect both children and adults are diagnosed in all anatomic regions of the CNS, including the brain, the meninges, spinal cord, and cranial nerves. They represent a high morbidity and mortality worldwide (Global Burden of Disease Cancer 2017). GBM, characterized by high recurrence and poor prognosis, is the most malignant primary brain tumour in adults, whereas medulloblastoma is the most common paediatric brain cancer. However, brain metastases, frequently arising from non-small-cell lung cancer (40% to 50%), breast cancer (15% to 25%), and melanoma (5% to 20%) are the most common brain malignancy (Eichler et al. 2011). The prognosis of patients with GBM remains poor despite adjuvant chemotherapy and radiotherapy after aggressive brain surgery. The 5-year survival rate is less than 5% and the median survival is approximately 12–15 months (Stupp et al. 2005).

During primary brain tumour progression and the development of brain metastasis, the tumour vasculature becomes increasingly heterogeneous. Consequently, the limited chemotherapeutic uptake and the heterogeneous permeability can be identified (Lyle et al. 2016). Some drug delivery platforms have been employed in primary and metastatic brain tumours, including nanoparticle drug delivery, chemically mediated BBTB permeability, efflux pump inhibitors, and intraventricular delivery (Hendricks et al. 2015). However, the BBTB still prevents the uptake and absorption of several drugs from entering the brain parenchyma.

With the BBB intact, hydrophobic molecules with molecular weight <500 Da (smaller than 1 nm) diffuse transcellularly into the neuroparenchyma, whereas small hydrophilic compounds can enter the brain via the paracellular route (Banks 2009).

However, the multi-drug resistance ABC transporters are affinitive to many therapeutic compounds and decreasing the delivery of some potential drugs crossing the BBB as well as BBTB.

Over the past 10 years, the role of chemotherapy in glioma management has changed substantially such that chemotherapy plays a role in the management of almost all newly diagnosed gliomas (WHO grade II-IV). This is based on several prospective studies that disclosed survival benefit after combined chemoradiotherapy with temozolomide (TMZ) in glioblastoma (WHO grade IV) or after PCV chemotherapy, which includes procarbazine, CCNU (lomustine) and vincristine, in diffuse low-grade (WHO grade II) and anaplastic (WHO grade III) glioma (Taal et al. 2015).

To date, TMZ is the most commonly used and the food and drug administration (FDA) approved chemotherapy drugs for GBM treatment (Fernandes et al. 2019). TMZ, a cytotoxic alkylating agent belonging to the group of triazene compounds, has the same active metabolite as dacarbazine (DTIC). The small prodrug (temozolomide, MW: 194 Da) can cross the BBB easily due to its lipophilicity (Brada et al. 1999). In *in vivo* rat models, maximum plasma and brain TMZ concentrations were observed almost simultaneously, that is, the near T_{max} for both cases, suggesting good BBB/BBTB penetration. Nevertheless, the ABCB1 gene expression levels at the capillary ECs of BBB/BBTB had an impact on TMZ uptake by the brain, directly indicating that TMZ is a substrate for ABCB1 (Reyderman et al. 2004; Goldwirt et al. 2014). By contrast, the influence of ABCG2 on TMZ penetrating the BBB/BBTB is inconsequential.

Nitrosourea alkylating agents such as CCNU, BCNU (carmustine), ACNU (nimustine), and fotemustine have been explored early on for their efficacy against brain tumours because of their superior penetration across the BBB (Walker et al. 1980). CCNU is utilized as a part of the combination PCV regimen, which was generally applied in the 1990s, especially for oligodendroglial tumours (Taal et al. 2015). In contrast to TMZ, CCNU revealed a lower brain/plasma ratio in rats (22–41% *v.s.* 20%) even though it does not participate in the substrate spectrum of ABC transporters (Drean et al. 2016; Azad et al. 2015). The PCV regimen is becoming used less commonly because of the simpler dosing schedule and a more favourable side-effect profile of TMZ.

4 Current Strategies for Overcoming the BBB/BBTB

4.1 Convection-Enhanced Delivery by Direct Injection

A straightforward approach to overcome the BBB/BBTB is by direct injection of the therapeutic agents into the tumour. Direct infusion of the drug via a cannula to the brain parenchyma is used to bypass the BBB/BBTB and designate the bulk convective flow of the drug in the interstitial space. The distribution of the drug is determined by diffusion as well as the rate and the duration of the injection (Allhenn et al. 2012). Although several clinical trials have been completed in patients with

glioblastoma, the improvement in survival remains insufficient (Jahangiri et al. 2017). A more promising technique is convection-enhanced delivery (CED), which involves a minimally invasive surgical exposure of the brain and then followed by placement of small diameter catheters directly into the brain tumour. Therapeutics are subsequently infused into the tumour over several hours to saturate the targeted area. Since this approach effectively bypasses the BBB, it allows macromolecules reach a high concentration within the brain tumour (Yamashita et al. 2007). Recent preclinical studies and clinical trials using CED in glioma models have evaluated a wide range of potential therapeutic agents such as carboplatin (Yang et al. 2014) and paclitaxel (Lidar et al. 2004). However, these trials have failed due to poor drug penetration and accumulation in the targeting tumour area (Mueller et al. 2011). To improve the drug penetration and accumulation, the administration protocol (for example, the placement of the catheter, infusion rate and so on) may need to be further optimized (Lonser et al. 2015).

4.2 Implantation of Wafers, Gels, and Microchips

Another direct local delivery approach is the use of wafers, which are biodegradable polymer wafers loaded with a chemotherapeutic agent implanted into the brain or tumour cavity at the time of surgery (Westphal et al. 2003). Macromolecules incorporated in biodegradable polymers are released by a combination of diffusion and polymer degradation. Microchips can provide even more control over the release. Biodegradable discs or wafers that contain the chemotherapy drug carmustine led to modest improvement in survival in patients with high-grade glioma (Chowdhary et al. 2015) but were also associated with high complication rates (Bregy et al. 2013). Currently, preclinical investigations with injectable gel-like lipid capsules and microchips have shown promising results (Masi et al. 2012; Bastiancich et al. 2016). However, the limitations of these methods include the requirement of invasive placement, increased risk of local neuronal damages along the track, and side effects such as oedema, bleeding, and infections (Bidros and Vogelbaum 2009; Kunwar et al. 2010).

4.3 Inhibition of Drug Efflux Transporters

Current biochemical modifications of existing drugs have focused on decreasing their molecular size and increasing their affinity to enhance their BBB/BBTB permeability. The increased ligand to receptor-mediated transport (RMT) could be achieved through the modification to an analog of the ligand against the specific receptor at the BBB/BBTB (Bradley et al. 2001; Gaillard et al. 2014). In addition to the modifications using the ligand to RMT, the amendment of drugs to minimize the affinity to AETs alters their admittance to the BBB/BBTB. The main targeted agents are substrates of ABCB1(P-gp) and ABCG2 (BCRP) (Oberoi et al. 2016) to yield adequate brain penetration. Hence, Elacridar and tariquidar are currently two suitable

options (Bankstahl et al. 2013). However, direct inhibition of AETs at the BBB/BBTB did not appear to have sufficient efficacy *in vivo* (Kurnik et al. 2008). This was attributable to their poor binding affinities, which requires the usage of high doses and thus results in intolerable toxicities (Bhowmik et al. 2015). Hence, developing an inhibitor with high specificity to AETs as well as suitable tolerability requires overcoming the active efflux of drugs in the future.

4.4 Non-invasive BBB/BBTB Permeability Enhancement

4.4.1 Osmotic BBB/BBTB Opening

Apart from the biochemical approaches of BBB/BBTB penetration, the other way to enhance the permeability of BBB is to use the intra-arterial infusion of the hyperosmotic solution of mannitol, causing temporary endothelial cells shrinkage with consequent opening of tight junctions and an increased BBB permeability for a period of time (Siegal et al. 2000; Rodriguez et al. 2015). This time window provides the opportunity of intra-arterial delivery of chemotherapeutics such as methotrexate and carboplatin (Gerber et al. 2014). The treatment efficacy has been reported in a phase I trial in patients with malignant gliomas after mannitol-induced BBB/BBTB disruption followed by *i.a.* administration of melphalan/carboplatin (Guillaume et al. 2010). The non-selective permeability enhancement of not only the BBTB but also the BBB causes a gross and uncontrolled influx of chemotherapeutic compounds and an increase of brain fluid with the possibility of leading to neurological toxicity, aphasia, and hemiparesis (Kemper et al. 2004).

4.4.2 Focused Ultrasound-Induced BBB Opening

The other promising technology to boost the BBB permeability is to combine low-intensity focused ultrasound (FUS) with intravenously injected microbubbles (usually 1 to 10 μm), which are gas bubbles shelled with lipids or albumin. When FUS excites systemically administered microbubbles, the bubbles expand and contract, resulting in mechanical stress to the vascular walls. This leads to endothelial cell modification with increased transcytosis activity, the formation of trans-endothelial fenestration, and the opening of the tight junctions (Sheikov et al. 2008). Besides, P-glycoprotein expression is inhibited up to 48 h after sonication, producing inhibition of drug efflux mechanisms (Aryal et al. 2017). These different mechanisms show up in order; hence BBB permeability enhancement occurs in fast and slow phases (Raymond et al. 2007). The degree of BBB permeability increase varies depending on acoustic parameters such as acoustic pressure, frequency, burst length (McDannold et al. 2008), and microbubble size as well as concentration (McMahon and Hynynen 2017). With proper FUS + MB parameters, controllable levels of increased BBB permeability can be achieved non-invasively and transcranially in the selected brain areas (Hynynen et al. 2001); the BBB permeability alteration can be restored to baseline within 6–24 h (O'Reilly et al. 2017).

Since the initial study (Hynynen et al. 2001), FUS + MB treatments have been used to successfully deliver a variety of agents across the BBB. Figure 1

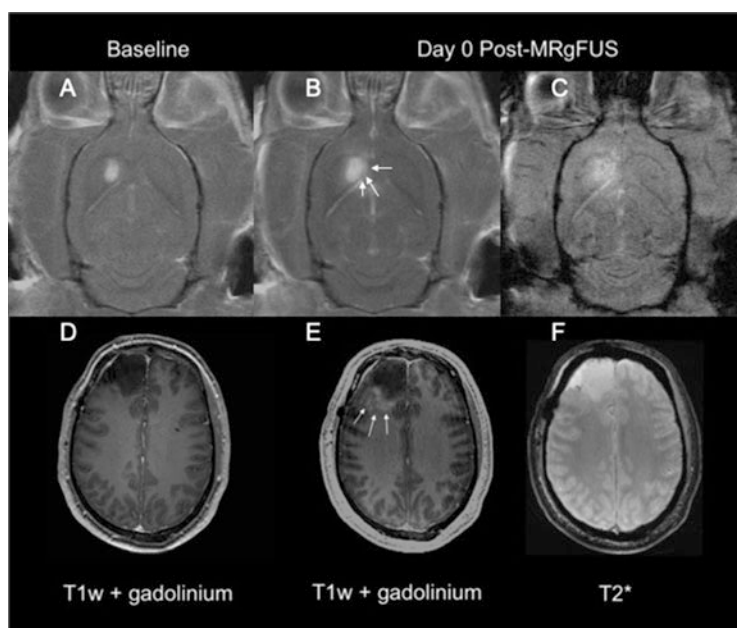


Fig. 1 MR-guided FUS is able to non-invasively open the BBB/BBTB in rat (upper panel) and human (lower panel) brain tumours. The pre-sonication contrast-enhanced T1-weighted MR images are scanned as baseline (**a** and **d**). The post-sonication contrast-enhanced T1-weighted MR images (**b** and **e**) are used to identify the BBB opening area (white arrows). T2* sequence (**c** and **f**) acquired immediately after FUS shows no evidence of microhemorrhages. Adapted with permission from Mainprize et al. *Scientific Reports* 2019

demonstrates that FUS + MB is capable of enhancing the BBB/BBTB permeability in both preclinical (rodent) and clinical studies. Chemotherapeutics delivered to the brain using FUS + MBs resulted in increased concentrations of drugs within brain tumours, reduced tumour growth, and improved survival in preclinical studies (Treat et al. 2007, 2012; Kovacs et al. 2014; Kang et al. 2010; Alli et al. 2018; Liu et al. 2014; Wei et al. 2013). In the meanwhile, clinical trials are currently taking place to test the safety and efficacy of delivering therapeutic agents across the BBB using FUS and microbubbles to treat brain diseases, such as primary brain tumours, and metastatic brain tumours (Mainprize et al. 2019), amyotrophic lateral sclerosis (ALS) (Abrahao et al. 2019), and Alzheimer's disease (Meng et al. 2019).

Currently, two clinical trials enrolled using this technology to deliver chemotherapeutics into the human brain tumours (Mainprize et al. 2019; Idbaih et al. 2019). In the first study, the complications of sonicating through the skull were overcome by an implantable device installed via a burr hole on the skull to sonicate the recurrent GBM (Carpentier et al. 2016). This study allowed the repeated sonications before routine chemotherapy. The overall survival was improved slightly but not yet significantly due to the limited area that can be covered by a single transducer (Carpentier et al. 2016). In the second study the sonications were

delivered through intact skull using a non-invasively MRI-guided phased-array FUS to deliver liposomal doxorubicin and TMZ into the tumour area and then quantify the drug concentration compared with the unsonicated area (Mainprize et al. 2019). This study demonstrated the feasibility and safety of this method on the application against human brain tumours. In the next phase repeated treatments of TMZ were delivered into brain tumours safely (Park et al. 2020). Although the method appears feasible and safe, larger phase 2 trials that are underway and eventually phase 3 trials are required before the clinical effectiveness of these methods can be judged.

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