Topics in Medicinal Chemistry 10

Gert Fricker
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Anne Mahringer *Editors*

The Blood Brain Barrier (BBB)

Springer

10
Topics in Medicinal Chemistry

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Editors

The Blood Brain Barrier (BBB)

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Preface

Since its discovery by Paul Ehrlich in the late nineteenth century, the blood–brain barrier has been the object of intensive research. It is formed by brain capillary endothelial cells and represents a dynamic interface that separates the brain, the most critical organ in our body, from the blood circulation. It protects the central nervous system (CNS) from potentially harmful xenobiotics and metabolites, while simultaneously regulating transport of essential molecules and maintaining a stable environment within the brain. Together with pericytes, astrocytes and neurons, the capillary endothelial cells form the so-called neurovascular unit, which is regulated by extremely complex signaling cascades. Unfortunately, the blood–brain barrier also prevents most therapeutic agents from reaching their target in the brain, which is why effective treatment of CNS diseases such as Alzheimer´s disease, Parkinson's Disease, Depression, Epilepsy or brain tumors, including brain metastases from peripheral tumors, remains to be one of the big challenges in modern medicine.

This volume of "Topics in Medicinal Chemistry" is a compilation of the latest research concerning new developments in the blood–brain barrier field. Seven internationally acknowledged research groups have contributed chapters, detailing their findings in this exciting and challenging area of biomedical research. Their works cover a broad range of topics including general structure and function of the blood–brain barrier, modes to study the blood–brain barrier in vivo, active transport systems, drug delivery across the barrier by colloidal carriers or ultrasound as well as alterations of the barrier at various disease states. From these chapters the complexity of the blood–brain barrier becomes apparent and they also illustrate which enormous efforts still lie ahead of us before we obtain a complete understanding of this fascinating area.

It has been a great pleasure for us to act as editors for this volume and we thank all authors who contributed. In addition, we hope that the volume might stimulate others to enter this research area and help to clarify the manifold unresolved questions.

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Contents

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The Blood–Brain Barrier: An Introduction to Its Structure and Function

Anne Mahringer, Melanie Ott, and Gert Fricker

Abstract The blood-brain barrier (BBB) formed by the brains microvascular system is impermeable for most therapeutically used compounds and overcoming this barrier remains to be one of the big challenges in modern medicine. It is composed of highly specialized endothelial cells, which are surrounded by pericytes and a basal membrane. Together with nearby astrocytes and neurons they constitute the so-called neurovascular unit, which restricts substance transfer from blood to brain and vice versa and maintains the cerebral ion homeostasis. Chapters of this book describe the discovery of the BBB, its evolutionary development as well as the cellular and molecular mechanisms, which underlay its structure and function in health and disease. The organization of tight junctional complexes or specific transport processes at the BBB will be addressed as well as methods to investigate BBB function in vitro and in vivo. Changes in the barrier function under several diseases conditions such as stroke or inflammation will be discussed as well as approaches to overcome the barrier by colloidal carriers or ultrasound.

Keywords Blood–brain barrier, Neurovascular unit, Morphology, Transporter

Contents

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The brain is the most critical organ in our body, which requires a very well-balanced ion homeostasis. It is extremely sensitive to a large variety of chemicals, which include potentially toxic metabolites or constituents of our daily food intake without being toxic to other parts of the body. Therefore, it is obvious that the central nervous system (CNS) needs special protection, which is set up by the brain capillaries, the so called blood–brain barrier (BBB). This microvessel network operates as a dynamic regulator of ion balance, a mediator of nutrient transport, and an impediment to harmful molecules. This barrier also represents a major obstacle to the development of CNS drugs. Approximately 98% of small molecule and all large molecule drugs, e.g., recombinant peptides or anti-sense-agents are normally excluded from the brain [1, 2]. Hence, the understanding of the morphology of the BBB as well as the molecular and cellular mechanisms that determine its function is an inevitable prerequisite for successful drug delivery to the brain. Here, we review the BBB from a historical perspective and discuss the current knowledge about the components of that barrier and their integrated function.

1 The BBB: A Historical Perspective

The first experiments indicating the existence of the barrier were performed in 1885 by the German immunologist Paul Ehrlich. He observed that a peripherally administered dye stained animal organs but failed to color brain tissue [3]. The initial interpretation of this finding was based on different binding affinities [4]. Subsequent pharmacological studies by Bield and Kraus [5] and Lewandowsky provoked the existence of a barrier at the level of cerebral vessels (1900), especially when Lewandowsky was studying the limited permeation of potassium ferrocyanate into the brain. This barrier was named “blood–brain barrier” by Goldmann [6]. Goldmann, a student of Ehrlich, also performed staining experiments with dogs and rabbits where he demonstrated a clear, exclusive staining of the choroid plexus after injection of water soluble dyes into the peripheral circulation, whereas the surrounding brain tissue and cerebrospinal fluid (CSF) remained colorless [7], thus confirming observations of other scientists in the years before [5, 8–10]. He also found that after sub-arachnoidal injection the brain was stained except the choroid plexus and concluded that the plexus epithelium was the very barrier preventing the transfer of dye into the brain [6]. However, the Russian physiologist Lina Stern observed that some test compounds could be found selectively in the brain and in the

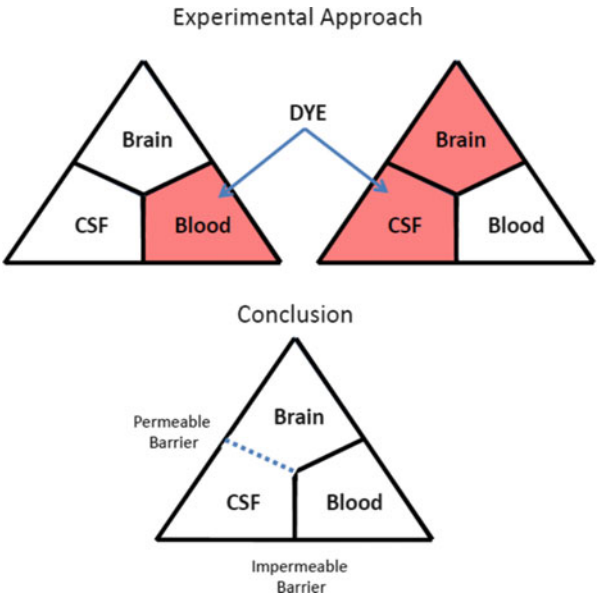

Fig. 1 Diffusion of the dye Trypan blue from the cerebrospinal fluid (CSF) into the brain. Trypan blue was injected into the blood and into the CSF, respectively. Brain, CSF, and blood were analyzed (modified from [23])

cerebrospinal fluid after i.v. administration in contrast to others and called this phenomenon "barrière hématoencéphalique" [11]. Some years later Spatz and colleagues suggested the concept of two separate CNS barriers: the BBB and the blood–liquor barrier [12–15]. In 1929, H. Foertig wrote the first scientific paper entitled "Die Bluthirnschranke" or "the blood–brain barrier," which was a rather provocative term at that time [16]. Broman [17] also argued that the barrier function of the BBB was localized to the capillary endothelial cells but not to the astrocytic end feet. He also claimed that the BBB showed defects in brain diseases and demonstrated a transient opening or disruption of the BBB after intracarotid arterial administration of hypertonic solutions [17]. Friedemann postulated in 1942 that electrochemical properties of injected compounds influence the distribution behavior within the CNS. Accordingly, capillaries are permeable for uncharged and positively charged compounds, but impermeable for negatively charged compounds [18]. In 1946, August Krogh speculated about active transport mechanisms when he was thinking of the presence of nutrient supply across the endothelial cells or of the BBB as a selective impermeable obstacle [19]. Even though the exact nature of the barrier was subject to many controversies until the 1960s, the introduction of the electron microscope revealed the presence of extracellular fluid in the cortex [20] and the localization of the barrier function within the endothelial cells of the brain capillaries [21, 22] confirming that the endothelium is indeed the principal anatomical site of the barrier (Fig. 1).

In 1971, Oldendorf demonstrated BBB permeability to sugars, amines, amino acids, and neurotransmitters by the use of radiolabeled substances [24]. Freeze fracture analysis indicated that the tight junctions between endothelial cells form complex net-like anastomoses around the endothelial cells, which restrict the passage

Table 1 Milestones in the development of blood–brain barrier research

Year	Discoveries and concepts
1885	Systemic application of a blue dye stained all organs except the brain and the spinal cord [3]
1898	Systemically administered bile acids were not neurotoxic but intracerebrally injected bile acids showed neurotoxicity [5]
1900	Postulation of a barrier between blood circulation and neural tissue to describe the phenomenon [8]
1913	Intrathecal administration of trypan blue results in staining of the brain tissue, whereas intravenous application does not. Definition of the concept of the blood–brain barrier [6]
1921–1922	"Barrière hématoencéphalique" was characterized as a cerebral blood vessel compartment, whereas the choroid plexus epithelium was semipermeable, facilitating the flow of substances from the blood into the CSF [30, 31]
1941	Intracarotid arterial administration of hypertonic solutions caused a transient opening or disruption of the blood–brain barrier explaining the mechanisms behind observed defects at the blood–brain barrier in brain diseases [17]
1942	Friedemann postulated in 1942 that electrochemical properties of injected compounds influence the distribution behavior within the CNS. Thereby, capillaries would be permeable for uncharged and positively charged compounds, but impermeable for negatively charged compounds [18]
1950s	Electron microscopy could not detect an extracellular fluid compartment in the gray matter, which was considered as an explanation for the failure of tracers to enter the brain. Later, this turned out to be an artifact in 1960s
1960s	The presence of extracellular fluid in the cortex was determined by further electron microscopy studies on "freeze-substituted" tissue [20]
1967	Fine structural localization of the blood–brain barrier, demonstration of tight junctions [22]
1969	Visual proof of junctions between endothelial cells [21]
1971	Blood–brain barrier permeability to sugars, amines, amino acids, and neurotransmitters proven by radiolabeled substances [24]
1978	Description of the passage of substances in extracellular fluids from brain to CSF along the CSF "bulk flow" gradient; "sink effect" that removes substances from the brain
1982	Observation of extremely high transendothelial electrical resistances [28]
1980s	Studies in molecular biology of the blood–brain barrier. Cloning and sequencing of glucose transporter gene [29]
1990s	Importance of ABC transporters for barrier function becomes obvious [32]
2000s	Signaling cascades of transporters [33]

of macromolecules and also of low-molecular-weight substances down to a diameter of 10–15 Å [25, 26]. In addition, this cell layer exhibits a very high transendothelial electrical resistance between approximately 1,400 and 1,900 Ohm $\times$ cm^2 [27, 28]. Furthermore, the endothelial cells are surrounded by pericytes, astrocytic foot-processes, and a basal membrane. In the late 1980s molecular biology techniques emerged and first studies at the BBB were performed, resulting in cloning and sequencing of the glucose transporter gene Glut1 [29]. Table 1 gives a short summary about the development in the past 100 years of BBB research.

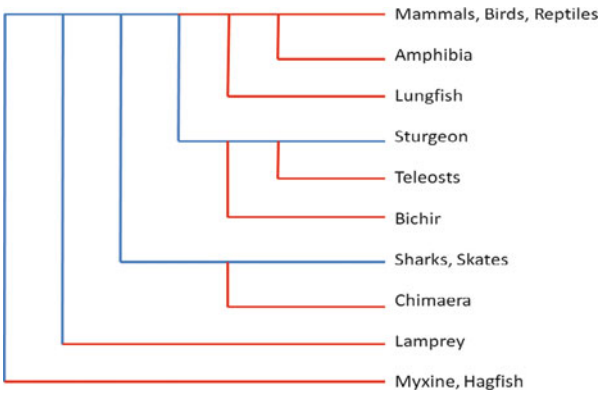

Fig. 2 Phylogenetic development of the blood–brain barrier (*red*: blood–endothelial brain barrier; *blue*: blood–glial barrier; from [35])

2 Evolutionary Development of a BBB

The BBB developed during evolution with the increasing complexity of neural tissue. Many invertebrates do not have a distinct barrier but only a leaky endothelium. Insects, crustaceans and cephalopods have a glial BBB [34].

A careful examination of the BBB of different species of evolutionary old fish gives evidence that ancestral vertebrates also had a glial barrier. About 400–500 million years ago apparently all vertebrates had a glial barrier, which has repeatedly been replaced by an endothelial barrier during evolution [35]. In elasmobranches (sharks, skates, rays) and sturgeons the BBB is set up by perivascular astrocytes. In most vertebrates including tetrapods (amphibia, reptiles, birds, and mammals), the barrier is formed by the brain microvessel endothelium [36], suggesting that once the neural tissue became larger and more complex during evolution the endothelium became tight enough to take on the barrier role (Fig. 2).

3 Anatomic Principles of the BBB

The primary element of the barrier is formed by the endothelial cells of brain microvessels, which pervade the brain with a total length of approximately 600 km, a mean distance of 40 μm and a capillary surface area available for molecular transport of about 20 m^2 [37]. It has been suggested that nearly every neuron in human brain is supplied by its own capillary [38]. A morphometric analysis of the mouse cortical vasculature indicates that perfused capillaries (4–8 μm in diameter) and small arterioles and venules (10–60 μm in diameter) occupy between 3–4% and 4–6% of the brain volume, respectively (Fig. 3). This correlates well with in vivo

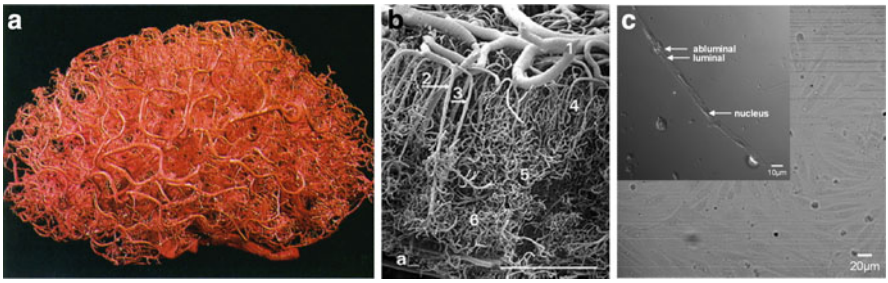

Fig. 3 Brain capillary network. (**a**) Plastinate of the blood–brain barrier network isolated from an adult human brain (adapted from [46]). (**b**) Electron microscope picture showing blood vessels in adult human cortex: pial vessels (*1*), long (*2*) and middle (*3*) cortical arteries, superficial (*4*), middle (*5*), and deep (*6*) capillary zone, scale bar 0.86 mm (adapted from [47]). (**c**) Confocal microscope picture of an isolated porcine brain capillary (*insert*) and picture of a monolayer of cultured porcine brain capillary endothelial cells (PBCECs)

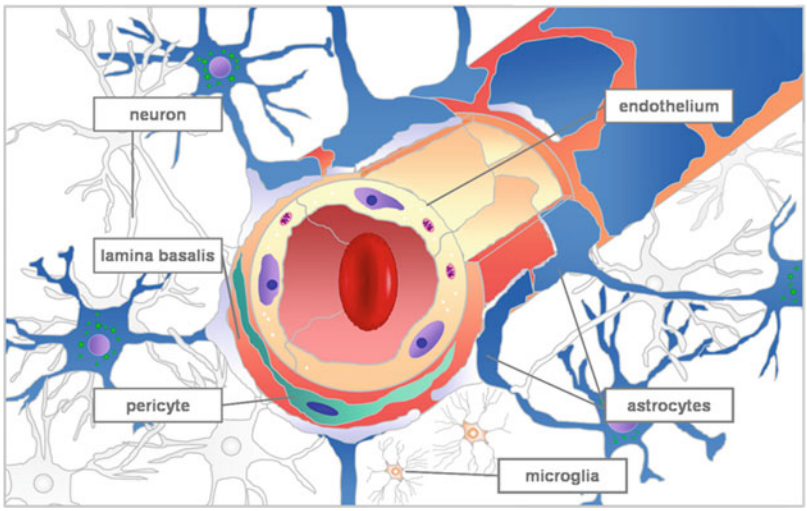

Fig. 4 Cross-section of a brain microvessel: endothelial cells surround the blood lumen and are ensheathed by the basal lamina containing pericytes. Astrocytic perivascular endfeet are attached to the basal lamina and are in contact with microglia and neuronal brain tissue

measurements of the blood volume in the gray matter in human brain determined by magnetic resonance imaging [39].

Brain capillaries exhibit some fundamental differences compared to peripheral capillaries. Whereas peripheral capillaries are fenestrated with gaps up to 50 nm wide the endothelial cells of brain capillaries are closely connected to each other by tight junctions and zonulae occludentes [40, 41]. In addition, the number of mitochondria is about five to ten times higher than in cells of peripheral microvessels,

indicating a high metabolic activity [42–44]. Furthermore, the cells exhibit a very low pinocytotic activity [45].

Five major components form the barrier – brain capillary endothelial cells, which make the actual barrier, pericytes, and the foot processes of astrocytes. Endothelial cells and pericytes are embedded into and surrounded by a basal membrane, and all these components are in close interaction with neurons (Fig. 4). Together the whole morphological framework is named the "neurovascular unit". In the following the distinct components of this unit will be discussed in more detail.

4 Pericytes

About 20% of the endothelial cells are directly covered by pericytes at their abluminal membrane [48, 49]. These cells, which are also named Rouget-Cells [50], belong to the vascular smooth muscle cell (VSMC) lineage [51]. They are contractile, responding to several vasoactive stimuli [52, 53] and appear to regulate brain capillary blood flow through contraction and relaxation [53]. It has been suggested that pericytes encircle 30–70% of the capillary wall. They are linked to the endothelial cells by gap junctions, focal adhesion plaques, and the so-called peg-and-socket-invaginations, exhibit macrophage-like activity [54], and help to regulate the endothelial cells [55]. Pericyte cytoplasma contains a relatively high number of lysosomes and the cells are able to take up macromolecular compounds, which are otherwise degraded by macrophages [56, 57]. Recent studies demonstrated that pericytes release various growth factors and angiogenic molecules, which regulate microvascular permeability and angiogenesis [58]. The interaction between pericytes and endothelial cells appears to be modulated by several ligand-receptor systems [59]. Hori et al. [60] showed that Angiopoietin-1 released from pericytes induces occludin expression via the Tie-2 receptor. The cells may also be implicated in endothelial differentiation by TGFβ, S1P (sphingosine-1-phosphate) or PDGF release via the respective receptors as well as anti-apoptotic mechanisms [61, 62]. Recent findings suggest that pericytes may be involved in the development of neuropathological alterations in several CNS diseases such as hypertension, diabetes, multiple sclerosis, CNS tumor formation, Alzheimer´s disease, or central nervous infections [51, 59, 63–66]. For example, it was postulated [67] that pericytes are more permissive for human cytomegalovirus replication compared to endothelial cells and that pericytes could serve as amplification reservoirs for HCMV. Recently, Yemisci et al. [68] demonstrated that pericytes contracted during acute ischemia and remained unchanged despite a reopening of the artery in a mouse focal ischemia model. Contracted pericytes induced narrowing of capillary lumen, which entrapped erythrocytes and clogged microcirculation. Thus, ischemia/reperfusion-induced injury to pericytes may be a major mechanism that negatively affects tissue survival by limiting oxygen and substrate delivery. Amyloid deposits have been detected within degenerating pericytes in the brains of patients with Alzheimer's disease [69, 70]. LRP (low density lipoprotein-related receptor)-mediated degradation of Amyloid-β (Aβ) in pericytes lowers Aβ levels in perivascular spaces ([71, 72], reviewed by [73]). Thus, it may be speculated that pericyte dysfunction plays also a

role in impaired Aβ-peptide clearance in Alzheimer's disease and initiates secondary neurodegenerative changes [74].

5 Astrocyte: Endothelium Interactions

Astrocytes appear to be an important component in the development and/or maintenance of BBB characteristics [75]. Co-culture of brain endothelial cells with astrocytes [76, 77] or with astrocyte-conditioned media [78] has been demonstrated to improve BBB characteristics in vitro. These observations are supported by in vivo studies showing loss and restoration of barrier integrity after a temporary focal loss of astrocytes [79]. Further on, a dynamic bidirectional Ca^{2+}-signaling occurs between neurons, astrocytes, and the endothelium, for which two mechanisms have been proposed – an intracellular IP3 (inositol-trisphosphate)- and gap junction-dependent pathway and a pathway involving extracellular diffusion via gap junctions and purinergic transmission [80–82] – which might play a role in the regulation of microvascular permeability [83]. Co-culture experiments with endothelial cells and astrocytes showed that TGFβ produced by astrocytes downregulates tissue plasminogen activator (tPA) and anticoagulant thrombo-modulin (TM) expression in cerebral endothelial cells [84], which might be relevant at intracerebral bleeding or intraventricular hemorrhage. Glial cell-derived neurotrophic growth factor (GDNF), a member of the TGFβ group, seems to be involved in postnatal maturation of brain microvessels [85].

Vice versa, endothelial cells produce leukemia-inhibiting factor (LIF), which plays a role in the induction of astrocyte differentiation [86]. When neonatal mouse astrocytes were co-cultured with a mouse endothelial cell line an alteration of the astrocytes from confluent monolayers to elongated multicellular columns occurred [87]. In addition, aquaporin-4 expression was upregulated in astrocytes under co-culture conditions [88].

6 Neurons

It is obvious that the cerebral microcirculation needs to be responsive to the nearby brain tissue. Very early reports suggest that brain activity imposes the transfer of oxygen and nutrients from the circulation into activated regions through a "neurovascular coupling" process [89]. Although the intracellular pathways involved in neurovascular coupling are not fully understood, a large number of data indicate that diverse mediators released in response to neuronal glutamate influence the microcirculation. A recent study shows that neuronal activity drives localized transport of serum insulin-like growth factor-I across the BBB (which may help to explain distinct observations such as proneurogenic effects of epileptic seizures, rehabilitation upon neuronal stimulation, and modulation of blood flow in response to brain activity) [90]. Transplantation experiments gave strong evidence that BBB characteristics of capillary endothelial cells depend on their neural environment

[91]. Although neurons are not directly structurally involved in the formation of the BBB, there is evidence that microvascular endothelium and/or associated astrocytic foot processes underlie innervation by noradrenergic [92, 93], serotonergic [94], cholinergic [95, 96], GABA-ergic [97], and other neurons [98]. In Alzheimer´ s disease a significant loss of cholinergic innervation of cortical microvessels has been observed, which might explain why the disease is associated with an impaired cerebrovascular function [95].

7 Basal Membrane

Endothelial cells and pericytes are embedded into the basal membrane consisting mainly of laminin, collagen type IV, proteoglycans, heparan sulfate, fibronectin, and other extracellular matrix proteins [99]. This membrane, which is 30–40 nm thick, appears to have a direct impact on the endothelium via interaction of laminin and other matrix proteins with endothelial integrin receptors [100] and the regulation of endothelial tight junction protein expression by matrix proteins [101, 102]. Consequently, disruption of this extracellular matrix is strongly associated with increased BBB permeability in pathological states [103, 104]. In addition, cell–matrix interactions can stimulate a number of intracellular signaling pathways (reviewed in [105]).

8 Junctional Complexes at the BBB

The BBB is characterized by exceptionally high electrical resistances being indicative for very tight intercellular connections. Three types of junctions are found: Adherens junctions [106], tight junctions [41, 107, 108], and possibly gap junctions [80, 109–111].

Adherens junctions mediate the mutual adhesion of endothelial cells and play a role in setting up cellular polarity and contact inhibition during vascular growth [109, 112]. They are formed by vascular endothelial (VE)-cadherin, a Ca^{2+}-dependent protein that mediates cell–cell adhesion [113]. At its intracellular site VE-cadherin binds to β-catenin and plakoglobin, which is then linked via α-catenin, α-actinin, and vinculin to the actin cytoskeleton [114–116].

Closely associated with the adherens junction proteins are those of the tight junctions suggesting that both junctional types are interspersed at the BBB. Tight junctions are mainly responsible for the high transendothelial resistances at the BBB. They are also composed of transmembrane proteins that form the primary lining linked via accessory proteins to the actin cytoskeleton [107]. Tight junction proteins include occludin, junctional adhesion molecule (JAM)-1, and the claudins.

Occludin is a 60 kDA transmembrane protein, which spans the cell membrane four times with a short cytoplasmic *N*-terminus and a long carboxy-terminal cytoplasmic domain. It is highly expressed in tight junctions of the BBB, but not in endothelial tight junctions of non-neuronal tissues [117, 118]. Its role in tight junction formation and maintenance has not been completely clarified.

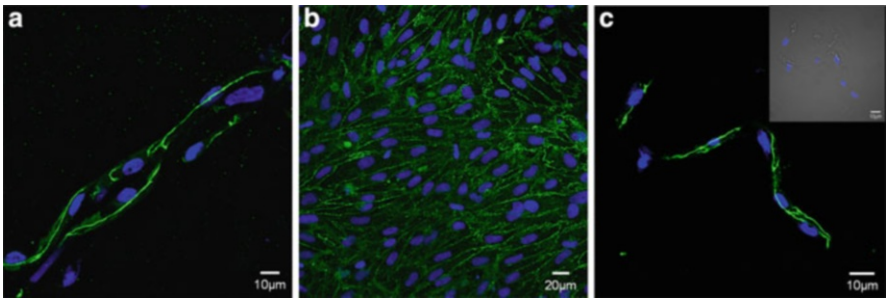

Fig. 5 Immunostaining for occludin in PBCs (**a**), PBCECs (**b**), and RBCs (**c**). (**a**) Occludin staining of a PBC shows localization along cell–cell contacts (*green*), nuclei (*blue*) were counterstained with DAPI. (**b**) Occludin revealed a belt-like staining along PBCEC contacts in cell culture confirming its membrane localization (*green*). (**c**) RBC stained for occludin (*green*) demonstrates expression along the plasma membrane of adherent cells (insert with transmitted light picture and stained nuclei)

For example, transfection of insect cells devoid of endogenous tight junctions with occludin cDNA demonstrated that occludin is not sufficient to form tight junction strands, suggesting that occludin is rather needed for regulation than for establishing BBB properties [41]. However, occludin seems to interact with claudins in a heterophilic manner and is recruited into the long strands formed by coexpression of claudin-1 and claudin-2 [119, 120]. A consistent staining for occludin along cell–cell contacts of porcine (PBC) and rat brain capillaries (RBC) as well as of cultured brain endothelial cells is seen in Fig. 5.

JAM-1 is a member of the IgG superfamily and appears to mediate the early attachment of adjacent cell membranes [121]. It is a transmembrane immunoglobulin-like molecule composed of a single membrane-spanning chain with a large extracellular domain [122], which co-distributes with tight junctions components. JAM-2 and JAM-3 being related to JAM-1 are also present in endothelial tissues and lymphatic cells, but not epithelia [123, 124]. Interestingly, in West Nile virus infections endocytosis of JAM-1 occurs, which ultimately results in lysosomal degradation of the protein. Understanding this process might offer a basis for revealing the mechanism of viral neuroinvasion [125]. Apparently it is also involved in leukocyte extravasation during acute inflammation [126].

Amongst 24 known claudins [123, 124] claudin-1, claudin-3, claudin-5, and claudin-12 are expressed at the BBB. They appear to be essential for barrier formation and maintenance (Fig. 6). A comparison of microvessels from different human glioblastoma multiforme showed a loss of claudin-1 expression in most of the tumor tissues, suggesting that the increase in microvascular permeability in human gliomas, which contribute to the symptoms of brain edema, is a result of a dysregulation of junctional proteins [127]. However, other studies failed to detect claudin-1 at the BBB [128, 129]. On the other hand, claudin-1, which was integrated into BBB tight junctions by transient transfection of endothelial cells, reduced BBB leakiness for both a small molecular tracer as well as endogenous plasma proteins. Claudin-1 induced sealing of BBB tight junctions during experimental autoimmune

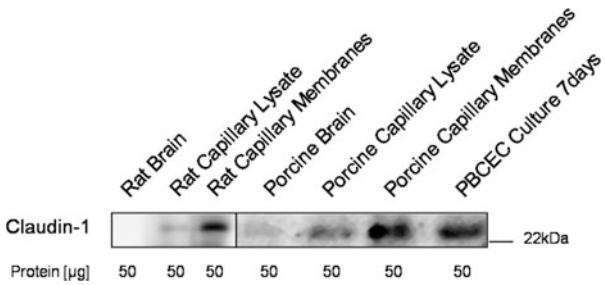

Fig. 6 Western blot for claudin-1 in rat brain homogenate, rat brain capillaries, and rat brain capillary membrane fraction as well as in brain, brain capillaries, membrane fraction of brain capillaries and in the membrane fraction of cultured endothelial cells derived from pig. It shows enhanced expression of the tight junction protein in the plasma membrane of brain endothelial cells in rat and pig. In vitro cell culture conditions did not influence expression levels

encephalomyelitis (EAE; a model for multiple sclerosis) and was found to significantly ameliorate the chronic phase of EAE in two independent transgenic mouse lines [130].

In brains of mice with EAE a selective loss of claudin-3 immunostaining from tight junctions of venules was seen, whereas the localization of the other tight junction proteins remained unchanged [129]. A similar finding was made in altered cerebral microvessels of human glioblastoma multiforme resulting in a compromised BBB. From these observations it was concluded that claudin-3 is a central component determining the integrity of BBB tight junctions in vivo.

Further on, in the brains of claudin-5-deficient mice a size-selective loosing for molecules <800 Da was observed [131]. In immortalized mouse brain capillary endothelial cells a significant decrease of claudin-12 expression was determined when cells were exposed to pathophysiologically high concentrations of ammonia, which is a key neurotoxin involved in neurological complications of acute liver failure [132].

The tight junctional proteins are linked to the cytoskeleton by the submembranous components ZO-1, ZO-2, ZO-3/p130 and the peripherally tight junction associated proteins 7H6 and cingulin (for a detailed review, see [108]).

Although not traditionally considered as a tight junction protein, the actin cytoskeleton in brain endothelial cells plays also a critical role in modulating BBB permeability [133].

A group of proteins, which have considerable impact on tight junctional integrity in diverse disease states, are matrix metalloproteinases (MMPs). Under normal conditions the expression of MMPs in the adult brain is very low. However, clinical and experimental studies give evidence that several MMPs such as MMP-2, MMP-3, MMP-7, or MMP-9 are upregulated and activated after ischemic stroke and neurodegenerative disorders (for review, see [134, 135]). They are expressed by various cell types including endothelial cells, microglia, neurons, and astrocytes and are synthesized and secreted as inactive pro-enzymes that subsequently are proteolytically cleaved and activated.

9 Transport Proteins at the BBB

Due to its tight barrier properties the BBB has to be passed via the transcellular route. Only few small polar compounds including water, glycerol, or urea diffuse across tight junctions.

However, lipophilicity hardly correlates with BBB permeability. More than 98% of small molecules do not cross the barrier nor do large molecules including recombinant proteins or monoclonal antibodies [1, 2]. Some cerebral nutrients such as glucose or amino acids pass the BBB via carrier-mediated mechanisms such as facilitated diffusion or active transport processes. Glucose is transported following its concentration gradient by the GLUT1transporter. Other transporters in the BBB belong to the family of solute carrier proteins (SLC), such as the monocarboxylate transporters MCT-1 and MCT-2 (SLC16a1/2), which transport short-chain monocarboxylic acids (e.g., lactate, pyruvate or mevalonate). SLC7 transports cationic amino acids (arginine, lysin, and ornithine). For mice it has been shown that the thyroid-transporters SLC16a2 and SLCO1c1, the sulfate transporter SLC13a4, the L-ascorbic acid transporter SLC23a2, the amino acid transporter SLC 38a3, and the folate transporter SLC19a1 are also highly expressed in the BBB [136].

The most interesting export proteins for drug transport across the BBB are the primary active, ATP-dependent ones, which represent a major defense mechanism of the brain: P-Glycoprotein (P-gp, ABCB1), the Mdr1 gene product, was the first of these export pumps being identified at the BBB [137, 138]. It is of particular relevance, since it recognizes a multitude of diverse substrates and it is subject of complex signaling cascades ([123, 124, 139, 140]) regulating its expression and function. One cascade is triggered by tumor necrosis factor-α, which signals through TNF-R1 (tumor necrosis factor-α receptor 1) resulting in the release of endothelin-1. Endothelin-1 itself signals through the ET_B receptor which alters P-gp expression and function through nitric oxide synthase and protein kinase CβI [123, 124, 141–144]. A second pathway is activated by glutamate, which acts via the *N*-methyl-*D*-aspartate receptor, cyclooxygenase-2, and the prostaglandin E2 receptor EP1 to up-regulate P-gp expression and activity [145–149]. A third type of cascades involves activation of orphan or nuclear receptors including pregnane xenobiotic receptor (PXR), aryl hydrocarbon receptor (AhR), the glucocorticoid receptor (GR), and the constitutive androstane receptor (CAR) to regulate expression of xenobiotic eliminating systems [123, 124, 150–155].

Recent studies using a fluorescent labeled construct of P-gp indicate that the export pump is not organized as a single molecule within the endothelial membrane but forms clusters of several proteins close together [156]. Interestingly, expression and function of P-gp may be altered at pathological conditions, e.g., Alzheimer´s diseases or drug resistant epilepsy [157–160].

Yet, P-gp is not the only important contributor to the selective barrier: Breast cancer resistance protein (Bcrp; ABCG2) is another efflux pump at the BBB [161, 162], which has a partially overlapping substrate specificity with P-gp and also significantly restricts xenobiotic permeability in the brain. It is also target of several signaling pathways,

e.g., 17β-estradiol induces the down-regulation of Bcrp on transcriptional and translational levels via the activation of the estrogen receptor β in the BBB [163]. Moreover, – similar to P-gp – its expression is induced by activation of nuclear receptors such as CAR, PXR or AhR [154, 155].

In addition to P-gp and Bcrp multidrug resistance related proteins, Mrps, are expressed at the BBB. However, there is still considerable discussion about the extent of expression, involvement in drug transport across the BBB and subcellular localization [164]. Mrp1, Mrp2, Mrp4, and Mrp5 appear to be localized at the luminal surface of the BBB, although significant species differences have been observed. MRP1/Mrp1 has been detected in cow and human, Mrp2 has been observed in rat, but not in cow or human species, MRP4/Mrp4 was seen in mouse, cow, and human and MRP5/Mrp5 has been detected in human and cow [165–170]. Mrp1, Mrp3, Mrp4, and Mrp5 are also found on microglia and astrocytes.

ABCA1 and ABCA2 appear to be involved in lipid and cholesterol homeostasis in the brain and in brain capillary endothelial cells [171, 172]. Recently it has been suggested that at least ABCA1 may play a role in the cerebral clearance of Amyloid B and is thus likely to be involved in the pathogenesis of Alzheimer's disease, too (for review, see [173]).

A comprehensive discussion about expression, signaling cascades and function of the ABC efflux pumps is found in the chapter by D. Miller (ABC Transporter) of this book.

Members of the above-mentioned solute carrier family (SLC) include also transporters for organic cations (OCTs/*SLC22A1-3*, OCTNs/*SLC22A4-5*) and organic anions (organic anion transporters, OAT/*SLC22A6-8, 11,* and organic anion transporting polypeptides, OATP/*SLCO*/*SLC21*).

From the SLC22 family OAT3, OCTN2, and RST are expressed in the BBB. Oat3 recognizes a broad variety of substrates, including amphiphilic organic anions such as estradiol-17β-glucuronide (E217βG), estrone sulfate, and dehydroepiandrosterone sulfate, hydrophilic organic anions, such as benzylpenicillin, indoxylsulfate, homovanillic acid or PAH (para aminohippuric acid), and the organic cations ranitidine and cimetidine (for review, see [174]).

Renal-specific transporter (RST) is a mouse homolog of the human urate transporter (URAT1) with 74% identity at the amino acid level and has been identified in brain capillary endothelial cells. However, its precise localization remains to be clarified.

Octn2/OCTN2 has been characterized as a sodium-dependent carnitine transporter. It is involved in brain uptake of carnitine, and it was found that functional loss of Octn2 is associated with a decreased brain concentration of acetyl-carnitine [175].

The SLCO/SLC21 family comprises 14 members in human and rodents, whereof Oatp1a4, Oatp1a5, and Oapt1c1 are expressed in brain capillaries [174]. Immunofluorescence studies indicate that Oatp1a4 is expressed both on the luminal and abluminal membrane of brain capillaries [176]. It also recognizes multiple substrates including cardiac glycosides (digoxin, ouabain), bile acids, steroid conjugates, some peptides, as well as some cations (e.g., *N*-(4,4-azo-n-pentyl)-21-deoxyajmalinium, *N*-methyl-quinidine, *N*-methyl-quinine and rocuronium; for review, see [174]). RT-PCR studies indicate also expression of Oatp1a5 in the

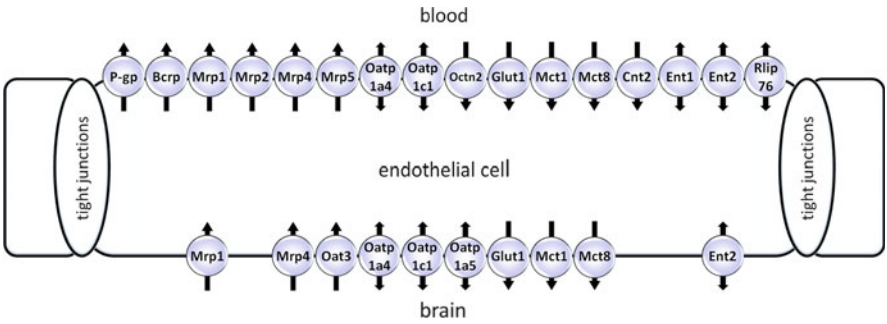

Fig. 7 Localization of transport proteins at the blood–brain barrier. Transporters for amino acids as well as small inorganic molecules are not shown. P-gp (p-glycoprotein, Abcb1), Bcrp (Breast cancer resistance protein, Abcg2), Mrp (Multidrug resistance protein, Abcc1/2/4/5), Oatp (Organic anion transporting polypeptide, Slco1a4/1a5/1c1), Octn (Organic cation transporter, Slc22a5), Glut (glucose transporter, Slc2a1), Cnt (Concentrative nucleoside transporter, Slc28a2), Ent (Equilibrative nucleoside transporter, Slc29a1/2), Rlip (Ral-binding protein) [180]

BBB [177]. The human OATP1A2 has structural similarity to the rodent Oatp1a4 and Oatp1a5 and exhibits also a very broad substrate specificity, but its precise membrane localization at the human BBB is yet unclear [178].

24S-Hydroxycholesterol (24S-OH-chol) is a major cerebral cholesterol metabolite and the elimination mechanism of 24S-OH-chol from the brain is one of the key issues for understanding cerebral cholesterol homeostasis (Fig. 7). Studies with *Xenopus laevis* oocytes expressing rat Oatp2 exhibited significant transport of [$^{(3)}$H] 24S-OH-chol suggesting that Oatp2 might be responsible for the 24S-OH-chol elimination from brain to blood [179]. Moreover, other relevant transporters include equilibrative (es, ei) and concentrative (N2, N3) nucleoside transporters (ENT,CNT) that cover the demands of cerebral nucleosides needed as precursors of nucleic acid synthesis [181].

10 Cytotic Processes at the BBB

The BBB contains several receptors being responsible for the passage of large molecules, such as the transferrin receptor (TfR), insulin receptor (IR), insulin-like growth factor 1 receptor (IGF1R), LDL receptor, leptin receptor (OBR), low density lipoprotein-related receptor 1 (LRP1), or the receptor of advanced glycation end products (RAGE). The latter two are of particular interest in the pathogenesis of Alzheimer‘s disease as they are involved in the cerebral homeostasis and clearance of Aβ [182]. In general, these receptors may provide targets for the brain directed delivery of drugs, which under normal circumstances do not cross the BBB, including large biopharmaceuticals. Recombinant proteins, enzymes, and monoclonal antibodies can be re-engineered for transport across the human BBB with the molecular Trojan horse technology either by direct coupling to antibodies versus a distinct receptor or by packing them into a colloidal carrier, such as nanoparticles or

liposomes, which are surface modified with receptor-directed antibodies or antibody fragments. A detailed discussion of delivery options and targeted receptors is given by Jones and Shusta [183].

In addition to specific receptor internalization there are two other pathways across the BBB mediated by caveolae or plasmalemmal vesicles and clathrin-coated pits/vesicles (for review, see [184]). The caveolae-mediated permeation across endothelial cells is also known as bulk-phase or fluid-phase transcytosis, which is independent of interactions between the transported molecules and the caveolar vesicle membrane. It is under debate to what extent this mechanism plays a role at the BBB because of the relatively low occurrence of caveolae in brain capillaries [185]. The density of clathrin-coated pits/vesicles at the BBB appears to be much higher [186]. Because of the negative surface charge of the clathrin-coated pits, only very few of the plasma proteins can be transcytosed randomly within the fluid phase of clathrin-coated vesicles. However, this pathway is of interest for transport of positively charged molecules including artificially cationized proteins, such as albumin [187], when electrostatic interactions occur between the positively charged moieties of the proteins and negatively charged membrane surface regions on the endothelial cells [188].

Another option for drug delivery to the CNS offer cell-penetrating peptides, which are quite heterogeneous in size (10–27 amino acid residues), but they all possess positive charges. Cell-penetrating peptides derived from natural proteins include the transcription-activating factor Tat, penetratin, and the so-called Syn-B vectors as well as engineered short peptides like the homoarginine vectors, transportan or sequence signal-based peptide (SBP) and fusion sequence-based peptide (FBP) [184]. The exact mechanisms, by which these peptides are internalized and carry their payload, are still under discussion and may be different for the distinct peptides, but several studies indicate a crucial role of basic residues in the translocating ability of these molecules [189–192].

11 Outlook

Since its discovery about 100 years ago the BBB has become immensely important and rapidly experiences increasing attention from different scientific disciplines. In order to proceed it is important to better understand the communication between cells of the neurovascular framework under various physiological and pathophysiological conditions and to explore how distinct components of the BBB are linked to each other and how their expression and function is regulated. Although significant achievements have been made in the past 10 years, a lot of open questions remain to be answered. For example, it is still not yet completely clear how tight junction molecules assemble, how they are regulated in health and CNS diseases, and how they interact with several mediators, neurotransmitters, or medications. In addition, transporters and receptors including their signaling cascades become more and more interesting as targets to ameliorate CNS drug delivery and brain protection. Besides, drug delivery systems which are able to pass the BBB and to release their

load within the CNS for treatment of neurological diseases have been developed and proven to be successful in animal studies. Nevertheless, further research of the basic mechanisms underlying the BBB should help to identify new approaches to the rational treatment of CNS-related diseases.

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In Vivo Approaches to Assessing the Blood–Brain Barrier

Margareta Hammarlund-Udenaes

Abstract Methods for in vivo assessment of blood-brain barrier (BBB) transport are presented, with their advantages and disadvantages. The methods described are brain uptake index, the i.v. injection technique, in situ brain perfusion, brain efflux index, % injected dose, microdialysis, CSF sampling and positron emission tomography, and the combinatorial mapping of unbound drug partitioning across the BBB. The methods are put into a pharmacokinetic context by delineating the type of readings that they give, be it the rate of transport across the BBB or the extent of transport of total drug (unbound and bound), or of the unbound drug.

Keywords Brain uptake index, i.v. injection technique, In situ brain perfusion, Brain efflux index, Microdialysis, CSF sampling, Positron emission tomography, Fraction unbound in the brain, Brain homogenate method, Brain slice technique, Volume of distribution of unbound drug in the brain

Contents

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Abbreviations

%ID	Percentage of the injected dose
A	Capillary surface area (also denoted S in the literature)
AUC	Area under the concentration–time curve
BBB	Blood–brain barrier
BCRP	Breast cancer resistance protein
BCSFB	Blood–cerebrospinal fluid barrier
BEI	Brain efflux index
BUI	Brain uptake index
C_{blood}	Concentration of drug in blood
C_{brain}	Concentration of drug in brain devoid of blood
$C_{injectate}$	Concentration of drug in the injection solution
C_{plasma}	Concentration of drug in plasma
CL_{act_efflux}	Active efflux clearance at the BBB (sum of all processes contributing to active efflux)
CL_{act_uptake}	Active uptake clearance at the BBB (sum of all processes contributing to active uptake)
CL_{bulk_flow}	Clearance caused by bulk flow of fluid from brain ISF to CSF
CL_{in}	Influx clearance i.e. the net influx given all transport processes at the BBB
$CL_{metabolism}$	Clearance caused by metabolism in the BBB or brain parenchyma
CL_{out}	Efflux clearance, i.e., the net efflux given all transport and metabolism processes from the brain ISF
$CL_{passive}$	Passive clearance (permeability surface area product) across the BBB being the same in both directions
CSF	Cerebrospinal fluid
$C_{tot,brain,ss}$	Total brain concentrations at steady state (whole brain minus capillary blood)
$C_{tot,plasma,ss}$	Total plasma concentrations at steady state
$C_{u,brainISF}$	Concentration of drug in brain ISF
$C_{u,plasma}$	Unbound drug concentration in plasma
F	Blood flow
$f_{u,brain}$	Fraction of unbound drug in whole brain homogenate
$f_{u,plasma}$	Fraction of unbound drug in plasma
ICF	Intracellular fluid
ISF	Interstitial fluid
J_{in}	Rate of influx to the brain
J_{out}	Rate of efflux from the brain
K_{in}	Transfer constant at the BBB (a clearance term)

K_{out}	Overall loss constant (a clearance term)
$K_{p,brain}$	Partition coefficient of total drug between whole brain and plasma
$K_{p,uu,brain}$	Partition coefficient of unbound drug between brain ISF and plasma
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
Mrp1	Multidrug resistance protein 1
P	Permeability
PET	Positron emission tomography
P-gp	P-glycoprotein
PA	Permeability surface area product (mL min^{-1} g $brain^{-1}$) also denoted PS
Q_{brain}	Amount of drug in brain parenchyma devoid of blood
$Q_{tot,brain}$	Amount of drug in brain parenchyma including capillary blood
V_{blood}	Physiological volume of blood in brain
V_{brain}	Effective volume of distribution in the brain
V_i	Effective vascular space in which a compound can be found including endothelial cell binding and accumulation and intravascular volume
$V_{u,brain}$	Volume of distribution of unbound drug in the brain

1 Introduction

The blood–brain barrier (BBB) is an intricate organ that is made up of the endothelial cell walls of the brain capillaries, thus extending throughout the whole brain. The length of the capillary network in one human brain is 644 km, the surface area is 20 m^2, and the distance between two capillaries is no more than 25–40 μm, while the thickness of the wall is one cell layer or 200–500 nm [1]. The function of the BBB is to control the environment of the brain by promoting the uptake of nutrients, hindering the entrance of harmful compounds, and effluxing metabolites. Much has been discovered regarding the functions of the BBB in recent years. The focus is currently on the whole neurovascular unit, which consists not only of the endothelial cells, but also encompasses the astrocytes, pericytes, basement membranes, and surrounding connections to neurons and glial cells. It is becoming clearer that all these components collaborate to maintain a tight, well-functioning system of exchange with the blood compartment [2–4].

There are several approaches to the assessment of BBB function; many involve in vitro cell culture models which allow the different mechanisms of BBB function to be studied in detail. These approaches will not be discussed in this chapter, but some references are provided for further reading [5–22].

In vivo approaches to studying the BBB involve estimation of drug concentrations in both brain and blood and include such procedures as microdialysis and the i.v. injection technique, in situ brain perfusion, and the brain efflux index (BEI) method. The concentration of unbound drug in the cerebrospinal fluid (CSF) may also be used as a replacement for that in the brain. Several review articles and book chapters have discussed in vivo methods of studying the BBB [11, 12, 23–29].

Because the reasons for studying the BBB differ, different methods are required. Either endogenous or exogenous substances can be studied. In this chapter, the focus is on the study of the rate and/or extent of transport of exogenous compounds such as drugs across the BBB. In many cases, these same methods can be used for studying endogenous compounds.

This chapter will therefore outline the available in vivo and in situ methods of assessing BBB function, listing some of their specific properties. The pharmacokinetic principles behind the BBB transport of drugs are presented first, followed by discussion of the various methods and the properties of BBB transport that each illustrates.

2 Pharmacokinetic Principles of Blood–Brain Barrier Transport

The driving force for drug transport to the brain is the concentration of unbound drug in the plasma ($C_{u,plasma}$) (Fig. 1). After administration, the drug molecules in the plasma will endeavor to achieve equilibrium with all body tissues, including the brain. Molecules will be let through the BBB from the plasma to the brain passively, will be effluxed if they are the substrates of efflux transporters like P-glycoprotein (P-gp) or breast cancer resistance protein (BCRP), or will be actively taken up into the brain by influx transporters at the BBB [30, 31]. Once in the brain interstitial fluid (ISF), the drug molecules are distributed into the intracellular fluid (ICF) and may bind specifically or nonspecifically to components of the brain parenchymal cells. The drug molecules that are present in the brain ISF are defined as being unbound. The brain ISF accounts for 19% of the total volume of brain tissue [32]. Most drug binding takes place in the intracellular compartment, as intracellular membranes provide a majority of all membranes in the brain parenchyma.

The free drug hypothesis states that only unbound drug molecules can interact with receptors. Thus, the drug molecules that are bound to plasma proteins or to components of the brain parenchyma are not pharmacologically active but act as a pool to and from which drug molecules are bound and released. Experimental evidence indicates that the brain unbound drug concentrations predict receptor binding or pharmacological effects much more reliably than the total brain or even unbound drug plasma concentrations; this was most clearly demonstrated by Watson and coworkers for dopamine D_2 receptor occupancy of antipsychotic drugs [33]. When a drug target is situated within the membranes, it is more difficult to predict whether the unbound or bound drug molecule is the active entity but, irrespective of the direct action, it is the unbound drug that equilibrates across the different compartments (Fig. 1).

Drugs can have very different affinities to brain parenchymal tissue. Thus, the total concentration of drug measured in the whole brain can differ substantially from the concentration of unbound, pharmacologically active drug. Total brain

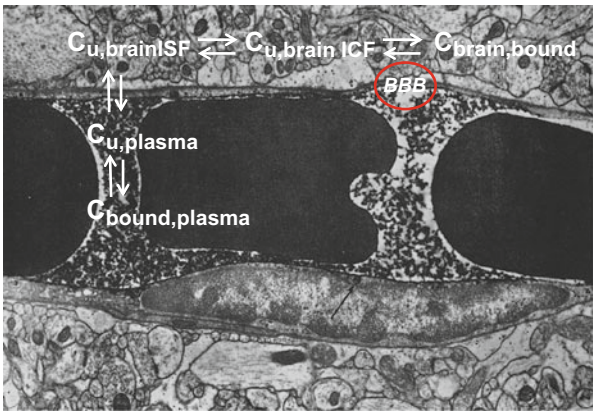

Fig. 1 Distribution of drugs in plasma and brain across the endothelial cells comprising the BBB. Further distribution takes place into the brain interstitial fluid (ISF) and brain intracellular fluid (ICF). The equilibria between the different sites are projected on an electron micrograph of a brain capillary depicting three red blood cells (*black*), endothelial cells (marked with a *red circle* at the top right and BBB), a pericyte, and brain parenchymal cells surrounding the capillary [90]. With permission from Rockefeller University Press. © 1967 Reese and Karnovsky. Originally published in The Journal of Cell Biology 34:207–217

concentrations can be 1- to 3,000-fold higher than unbound drug ISF concentrations [34]. Total brain concentrations could therefore be up to 3,000-fold higher than the actual concentration of active moiety required for therapeutic success.

The unbound drug concentrations in plasma can differ substantially from the unbound drug concentrations in the brain, and plasma concentrations are therefore not suitable for predicting the effects. The differences between plasma and brain unbound drug concentrations are the result of the active transporters in the BBB, which dramatically and substantially influence the concentrations in the brain but are not yet predictable using in vitro methods. The movement of drugs across the BBB can be influenced either by efflux or influx transporters or by a combination of transporters acting on one drug. The influence of these transporters can result in brain unbound drug concentrations ranging from less than 1% of the corresponding plasma concentrations up to five times these concentrations [30, 31, 35]. The term used to depict the steady-state ratio of unbound drug in brain ISF to that in plasma is the partition coefficient $K_{p,uu,brain}$ [36, 37].

$K_{p,uu,brain}$ describes the *extent of transport*, or rather the extent of equilibration, across the BBB. It is determined by the balance of transport into and out of the brain tissue. Both processes mainly take place through the BBB, although metabolism within the brain parenchyma and bulk flow of fluid from the ISF to the CSF can also contribute. Bulk flow into the CSF could contribute more significantly to the efflux of drugs which permeate poorly across the BBB [38, 39].

The *rate of transport* of a drug into or out of the brain is described in terms of the permeability of the BBB to the drug in question. The parameter usually used to describe the rate of transport in vivo is the permeability surface area product

(abbreviated to PA or PS; mL min^{-1} g $brain^{-1}$). The influx PA rates at the BBB can span a large range; for example, for opioids, the influx PA ranges from very low at 1.1×10^{-4} mL min^{-1} g $brain^{-1}$ for morphine-3-glucuronide to relatively high at 1.9 mL min^{-1} g $brain^{-1}$ for oxycodone [30, 40–42].

In pharmacokinetic terms, the flow into and out of the brain can be expressed in terms of the influx clearance (CL_{in}) and the efflux clearance (CL_{out}) [37]. These are the *net* clearances in each direction, i.e., the sum of all processes at the BBB or in the brain for the drug in each direction. The influx clearance has also been expressed as the transfer coefficient K_{in} and the overall loss from the brain as K_{out} [43–46]. CL_{in} is thus the same as K_{in}, and CL_{out} is the same as K_{out}. The overall influx and efflux rates for a drug, often expressed as J_{in} and J_{out}, include the concentration of drug in the plasma or ISF and can then be described as

$$J_{in} = K_{in} \times C_{u,plasma} \tag{1}$$

$$J_{out} = K_{out} \times C_{u,brain} \tag{2}$$

where $C_{u,plasma}$ and $C_{u,brain}$ are defined as the unbound-compound concentrations in plasma and in brain ISF, respectively [47]. Further equilibration within the brain from brain ISF to the intracellular compartment and from unbound to bound compound takes place according to Fig. 1. Michaelis–Menten kinetics are used if there is a saturable transport process involved. The rate of change in the amount of a compound in the brain under linear conditions is described by

$$\frac{dQ_{brain}}{dt} = J_{in} - J_{out} = \left(K_{in} \times C_{u,plasma}\right) - \left(K_{out} \times C_{u,brain}\right) \tag{3}$$

where Q_{brain} is the amount of compound present in the brain parenchyma apart from in the brain capillaries. $K_{out} \times C_{u,brain}$ in Eq. (3) can be expressed as $k_{out} \times Q_{brain}$, where k_{out} is the rate constant equal to K_{out}/V_{brain}, and V_{brain} is the effective volume of distribution of the compound in the brain (expressed in mL g $brain^{-1}$) [47], which in turn is equal to the volume of distribution of unbound compound in the brain, $V_{u,brain}$ (see below):

$$\frac{dQ_{brain}}{dt} = J_{in} - J_{out} = \left(K_{in} \times C_{u,plasma}\right) - \left(k_{out} \times Q_{brain}\right) \tag{4}$$

At very early time-points, the influence of this term in Eq. (4) is very small, as there is as yet very little drug in the brain. This fact has been used to look at initial uptake [43, 45]. Equation (4) can then be simplified to

$$\frac{dQ_{brain}}{dt} \approx K_{in} \times C_{u,plasma} \tag{5}$$

By further integration of the equation [44, 47, 48], it is possible to determine K_{in} as

$$K_{in} \approx Q_{brain} / \int C_{u,plasma} dt \tag{6}$$

Although Q_{brain} refers to the amount of drug in the brain minus the brain capillary contents, the measurements are often made on the whole brain concentration including the blood ($Q_{tot,brain}$). Compensation for the amount of solute present in the blood is therefore needed. Further development of the equation (see [47]) results in the Patlak equation [44]

$$Q_{tot,brain} / C_{u,plasma} \approx K_{in} \left[\int C_{u,plasma} dt / C_{u,plasma} \right] + V_i \tag{7}$$

where V_i is the effective vascular space in which the studied compound could be found, including endothelial binding. Another, more practical way of expressing the Patlak equation is

$$K_{in} \approx \left(Q_{tot,brain} - V_{blood} \times C_{blood} \right) / \int C_{u,plasma} dt \tag{8}$$

where V_{blood} is the volume of blood in the brain, often measured using an impermeable vascular marker such as [^{14}C]dextran or [^{3}H]inulin, and C_{blood} is the total concentration of the compound in the blood.

Three parameters influence the clearance of drugs from the capillaries: the rate of blood or plasma flow (F), the capillary surface area (A), and the permeability of the capillaries to the solute (P). Thus, K_{in} is not a permeability coefficient, but an in vivo clearance parameter. The relationship was derived by Renkin [49] and Crone [50] as

$$K_{in} = F \left[1 - \exp^{-PA/F} \right] \tag{9}$$

This equation is called the Crone–Renkin equation. Smith has evaluated the limiting conditions for K_{in} [46]. When F is much larger than PA, K_{in} approaches PA in value, and when F is much smaller than PA, K_{in} approaches F in value. This means that the upper limit of K_{in} is the rate of capillary blood flow and the lower limit is the permeability surface area product. It has been suggested that K_{in} can be used to estimate PA when PA is lower than F by a factor of at least 5. PA can then be estimated by rearranging the Crone–Renkin equation (Eq. (9)) as

$$PA = -F \ln(1 - K_{in}/F) \tag{10}$$

F can be estimated using radioactive iodoantipyrine, microspheres, or diazepam [47].

At equilibrium, the rate of solute transport in each direction across the BBB is similar, i.e., $J_{in} = J_{out}$. Using Eq. (3) with clearance terminology, this gives [37]

$$CL_{in} \times C_{u,plasma} = CL_{out} \times C_{u,brainISF} \tag{11}$$

and, thus, the extent of transport can be described as

$$\frac{C_{u,brainISF}}{C_{u,plasma}} = \frac{CL_{in}}{CL_{out}} = K_{p,uu,brain} \tag{12}$$

Equation (13) describes the intricate collaboration between the different transport processes that results in $K_{p,uu,brain}$, showing that $K_{p,uu,brain}$ describes the balance between all influx and efflux processes:

$$\begin{aligned} K_{p,uu,brain} &= \frac{CL_{in}}{CL_{out}} \\ &= \frac{CL_{passive} + CL_{act\_uptake} - CL_{act\_efflux}}{CL_{passive} - CL_{act\_uptake} + CL_{act\_efflux} + CL_{bulk\_flow} + CL_{metabolism}} \end{aligned} \tag{13}$$

In this equation, $CL_{passive}$ describes the passive movement of the drug across the BBB, assumed to be the same in both directions; CL_{act_uptake} describes the sum of the active uptake transport processes; CL_{act_efflux} describes the sum of the active efflux transport processes; CL_{bulk_flow} describes the bulk flow; and $CL_{metabolism}$ describes the removal of the drug from brain tissue or the BBB by metabolism.

For a drug transported across the BBB mainly by passive transport, $K_{p,uu,brain}$ equals unity (Fig. 2). If the active efflux of a drug is faster than the influx, $K_{p,uu,brain}$ will be lower than unity. The lower the value, the more influential is the active efflux process. If $K_{p,uu,brain}$ is higher than unity, there is a net influx of the drug. To date, $K_{p,uu,brain}$ has been estimated to range from <0.01 for drugs like loperamide, methotrexate, and paclitaxel to 5 for diphenhydramine [31, 34].

The extent of delivery of drugs to the brain can also be measured as the total brain concentration at steady state ($C_{tot,brain,ss}$) divided by the total plasma concentration at steady state ($C_{tot,plasma,ss}$), i.e., $K_{p,brain}$ (also known as logBB). This ratio includes any binding of the drug that occurs in the brain and/or plasma and can be expressed in relation to $K_{p,uu,brain}$ as

$$K_{p,brain} = \frac{C_{tot,brain,ss}}{C_{tot,plasma,ss}} = \frac{C_{u,brainISF}/f_{u,brain}}{C_{u,plasma}/f_{u,plasma}} = K_{p,uu,brain} \times \frac{f_{u,plasma}}{f_{u,brain}} \tag{14}$$

$K_{p,brain}$ is thus influenced by three independent properties [37]: the intra-brain binding, as described here by the fraction of unbound drug in the brain ($f_{u,brain}$), the fraction of unbound drug in plasma ($f_{u,plasma}$), and the BBB transport (described by $K_{p,uu,brain}$). The $f_{u,brain}$ parameter needs to be compensated for pH partitioning into acidic organelles, mainly lysosomes [51]. Alternatively, and preferably, the unbound drug volume of distribution in the brain, which can be expressed as

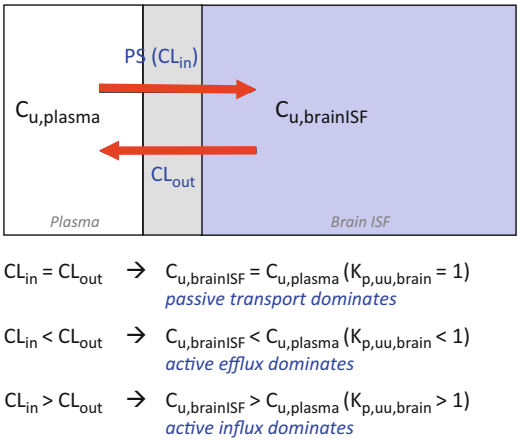

Fig. 2 Interplay between influx and efflux processes at the BBB leading to different unbound drug concentrations in the brain ISF ($C_{u,brainISF}$) from the unbound drug concentrations in plasma ($C_{u,plasma}$). This ratio is the $K_{p,uu,brain}$ (see also Eq. (12)). $K_{p,uu,brain}$ is thus not determined by the absolute values of the influx or efflux clearance, but by the relationship between the two

$1/V_{u,brain}$ in Eq. (14), should be used (see Sect. 3.9). $K_{p,brain}$ is therefore a composite parameter that is not optimal for determining whether a new drug is able to reach the brain in sufficient quantities. The higher the binding in the brain vs that in plasma, the higher the $K_{p,brain}$ value. At the same time, the more efficient the efflux, the lower the $K_{p,brain}$.

3 Methods

The methods described below and in Table 1 are used to estimate either the rate of transport of the drug across the BBB, by measuring PA, or the extent of transport. Some methods are able to measure both properties. The methods that measure the rate of initial unidirectional uptake of drug at the BBB are generally not influenced by elimination from the brain. They are, however, influenced by active processes at the BBB (both influx and efflux) in addition to passive transport. They therefore measure the rate of net uptake or net efflux.

The methods for measuring BBB transport have been described by several authors [11, 23, 27, 47, 52]. The review by Smith et al. is very insightful and offers much additional information on the methods presented here [47]. Additional methodological issues associated with some of these methods have also been discussed by Hammarlund-Udenaes [53].

Table 1 Overview of methods used for studying the rate and/or extent of BBB drug transport

Method	Property
Brain uptake index	In principle a rate method, but does not measure PA
i.v. injection technique	Rate method (K_{in})
In situ brain perfusion	Rate method ($K_{in} \rightarrow$ PA)
Brain efflux index	Rate method ($k_{el} \rightarrow CL_{efflux}$)
Percentage of injected dose	Extent method
Microdialysis	Extent method ($K_{p,uu}$); rate method if data are modeled and $V_{u,brain}$ is measured
Brain-to-plasma ratio of total drug concentrations	Extent method (K_p); a composite parameter using total drug concentrations
Brain-to-plasma ratio of unbound drug concentrations	Extent method ($K_{p,uu}$); maps BBB transport using unbound drug concentrations
CSF sampling	Extent method; but estimates CSF-to-blood partitioning and not necessarily brain-to-blood transport
Positron emission tomography	Measures both the rate and extent of transport; uses total drug concentrations
Brain slice method measuring $V_{u,brain}$	Neither rate nor extent of transport; measures intra-brain distribution
Brain homogenate method measuring $f_{u,brain}$	Neither rate nor extent; measures intra-brain distribution

3.1 *Brain Uptake Index (Carotid Artery Single Injection Technique)*

The brain uptake index (BUI) provides an estimate of the rate of uptake of drug injected into the brain in relation to the rate of uptake of a reference compound. In the original publication, the BUI technique was called the carotid artery single injection technique [54]. A radioactively labeled reference compound that is freely diffusible across the BBB, often ^{3}H-water, ^{3}H-diazepam, or ^{14}C-butanol, is rapidly (0.5 s) injected into the common carotid artery in about 0.2 mL of buffered Ringer's solution. The animal is decapitated 5–15 s after administration. The assumptions are that there is no transport of drug from brain to blood and that there is no metabolism during the time of the experiment. The BUI is calculated according to Eq. (15) as

$$BUI = \frac{\left(C_{brain}/C_{injectate}\right)_{test}}{\left(C_{brain}/C_{injectate}\right)_{reference}} \times 100 \quad (15)$$

where C_{brain} is the concentration of the drug in the brain devoid of blood, and $C_{injectate}$ is the concentration of the drug in the injected buffer. Figure 3 shows the BUI for four opioids, demonstrating one of the disadvantages associated with this technique (see also Table 2). As the transit time through the brain capillaries is very short (1 s), there is too little time for morphine to be transported into the brain. Thus,

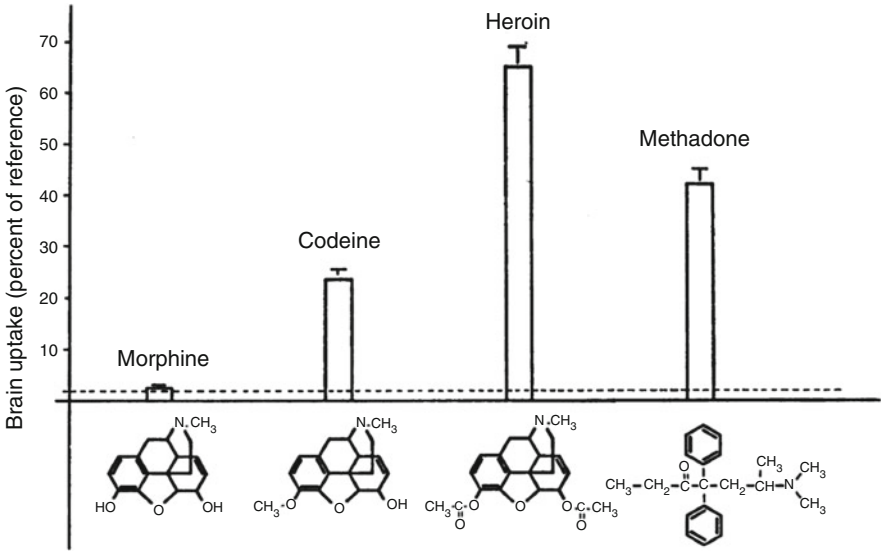

Fig. 3 Classical figure of the brain uptake index of four opioids. A radioactively labeled drug is injected into the carotid artery with ^{3}H-water or ^{14}C-isopropanol as the diffusible reference compound and a sample is taken at 15 s. From Oldendorf et al. [91] with permission from the publisher

Table 2 Advantages and disadvantages of the BUI method for studying BBB drug transport

Advantages	Disadvantages
Technically easy	Only provides relative uptake compared to a reference compound
Rapid (sampling after 5–15 s)	Only 10% of compound reaches the brain, which decreases the detection limit
	Short capillary transit time (1 s) precludes PA $<$ 10 μL min^{-1} g^{-1} being measured

morphine is on the limit of detection while heroin has the highest BUI value. In general, PA values below 10 μL min^{-1} are difficult to measure with this method [52]. As a technical caveat, the administered compounds may be transported to the rest of the body and only 10% of the compound might reach the brain [23]. This lowers the detection limit of the method. The BUI method is now considered less useful than, for example, the i.v. injection technique or the in situ brain perfusion technique presented below.

3.2 The i.v. Injection Technique

The aim of the i.v. injection technique is to measure the rate of unidirectional uptake of a molecule into the brain (K_{in}). The method was first published by Ohno

Table 3 Advantages and disadvantages of the i.v. injection technique for studying BBB drug transport

Advantages	Disadvantages
Low technical difficulty (no access to carotid artery needed)	Compensation for metabolite concentrations in blood (and brain) is needed because of the longer times for sampling (easy if LC-MS/MS is used, but difficult if radioactivity is used)
Relatively sensitive; can measure poorly permeating compounds with PA < 0.5 μL min^{-1} g^{-1}	The assumption that only unidirectional uptake is taking place is probably violated because of the relatively long sampling time
Independent of cerebral blood flow (F) when PA $\ll F$	
Both plasma and brain pharmacokinetics can be obtained	
Studies an intact system	

and coworkers [43]. It is currently considered the gold standard for BBB transport studies [47]. Patlak et al. developed the method to allow graphical representation and multiple time-point measurements [44]. They also discussed aspects of the methodology that required optimizing [48].

Equations (4)–(10) describe this method, which can be used to estimate K_{in}, k_{out}, V_{brain}, F, and PA. There are no assumptions made regarding the intra-brain distribution, but it is assumed that no elimination from the brain takes place during the measurement.

Briefly, an i.v. bolus containing the drug is injected into the femoral or tail vein of the model animal. Samples are then taken serially from the femoral artery. The brain and sometimes also the CSF are sampled at the last time-point. Alternatively, only one arterial blood sample is taken at the same time as the brain sample. It is possible to study the process over a period of less than a minute to hours; however, most studies are no longer than 60 min. This extended time-span violates the assumption of unidirectional uptake, as the compound studied is able to be transported back from the brain to the blood in possibly quantitatively important amounts. Compensation for metabolite formation is also necessary.

The use of the i.v. injection technique to measure the PA is probably not as relevant today as it has been; the available improved cell models can more easily measure the PA without the need for animals. However, cell models are not fully able to describe the in vivo situation to estimate the other parameters, and this is where the i.v. injection technique is of value (Table 3).

3.3 In Situ Brain Perfusion

The in situ brain perfusion method also measures the rate of transport, providing PA and F via measurement of K_{in} (see Eqs. (5–10)). It was developed by Takasato

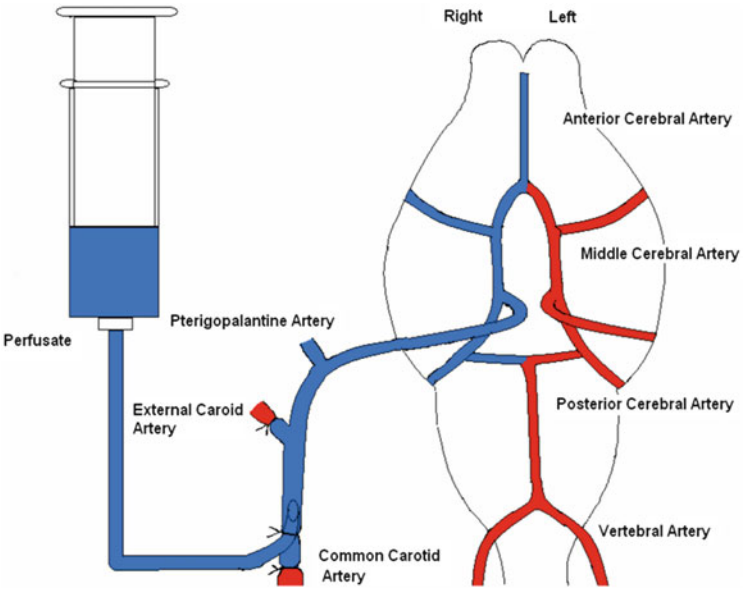

Fig. 4 Surgical procedure and perfusion in the in situ brain perfusion method. From the thesis by H. Mandula, Texas Tech University, 2005, with permission from the author

et al. [45] and was described in detail by Smith and Allen [55]. The method has been further developed by others for studies in mice [56–58].

The procedure is performed in anesthetized rats or mice. The ipsilateral pterygopalatine, superior thyroid, and occipital arteries are ligated and cut. The external carotid artery is ligated and the perfusion catheter is placed either in the external carotid artery [45] or directly in the common carotid artery distal to the bifurcation of the common carotid artery, as in Fig. 4. The ipsilateral common carotid artery is then ligated. The perfusion fluid flows towards the brain at a rate of 3.5–4 mL min^{-1} (some sources say 5–20 mL min^{-1}). This is to produce an arterial pressure equal to the systolic pressure to prevent the perfusate mixing with the circulating rat plasma within the cerebral circulation. D-glucose is added to provide energy. The perfusion can be sustained from 5 s to 10 min [55], but is normally no longer than 120 s.

A reference compound is perfused with the compound(s) of interest, to measure the brain plasma volume. Radiolabeled sucrose or inulin is often used for this purpose [55]. After perfusing the compound of interest and the reference compound, a physiological buffer can be perfused for 10–30 s to separate the bound and transcytosed compounds [55].

The in situ brain perfusion method is more sensitive than the BUI method because the experimental time is longer and the vessels that do not lead to the brain are ligated, thereby resulting in 100% of the perfused solution entering the brain (Table 4).

Table 4 Advantages and disadvantages of the in situ brain perfusion technique for studying BBB drug transport

Advantages	Disadvantages
Rapid	Technically challenging
More sensitive than BUI	Unsuitable for high-throughput use
Lack of systemic exposure of the compounds studied means no influence from peripheral metabolism	
The composition and flow rate of the perfusate fluid can be fully controlled	
Provides mechanistic information	
Competitive processes at the BBB can be studied	
Negligible mixing of perfusion fluid with blood	

Table 5 Advantages and disadvantages of the brain efflux index method for studying BBB drug transport

Advantages	Disadvantages
Can measure carrier-mediated transport of both small and large molecules	Technically challenging
	Very small injection volumes

3.4 Brain Efflux Index

The BEI method is used to characterize the rate of efflux transport from the brain (cerebrum) to the blood across the BBB, and to describe the relative amounts of test and reference compounds effluxed (using k_{el} to estimate CL_{efflux}). The method was developed by Kakee et al. [59, 60]. This and other methods were used by Ohtsuki et al. to study the elimination of the uremic toxin indoxyl sulfate and various neurotransmitters from the brain to the blood via the transporter OAT3 [61]. The advantages and disadvantages of the method are presented in Table 5.

Briefly, anesthetized rats are placed in a stereotactic frame. The skull is exposed and a hole is burred so as to place the cannula in the PAR2 (cortex) region (Fig. 5). A radiolabeled compound is microinjected together with a nonpermeating reference compound (which will remain in the brain parenchyma) in a volume of 0.1–1 μL. It is very important that the injection and retraction of the needle are performed very slowly. The PAR2 region was chosen because it allows minimal diffusion into the rest of the brain. [^{14}C]carboxyinulin can be used as the reference compound for [^{3}H]-labeled compounds, and [^{3}H]inulin, [^{3}H]dextran, or [^{3}H]D-mannitol can be used for [^{14}C]-labeled compounds. Brain and plasma are sampled at various times after the injection to provide an elimination profile (Fig. 6).

The BEI is defined as the relative percentage of drug injected into the cerebrum that is effluxed from the ipsilateral cerebrum to the blood:

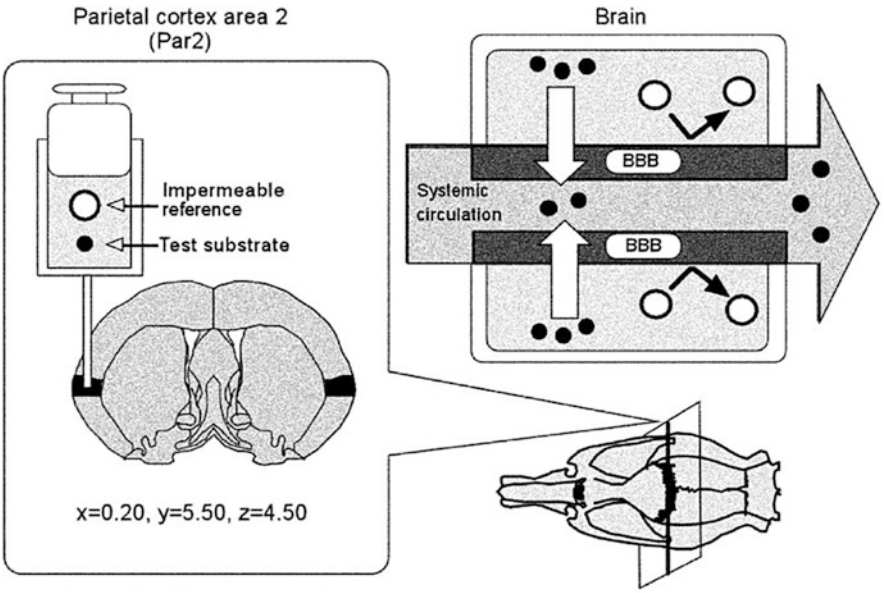

Fig. 5 Depiction of the brain efflux index method showing placement of the co-injection of an impermeable reference compound and the test substrate in the parietal cortex area 2 of the rat. The *top right* picture shows the elimination of the test compound while the reference stays within the tissue. From Hosoya et al. [92] with permission from the publisher

$$\text{BEI } \% = \frac{\text{Compound effluxed at the BBB}}{\text{Compound injected into the brain}} \times 100 \tag{16}$$

The reference compound is used to determine the amount of drug injected. To determine the BBB efflux clearance, 100–BEI% is calculated as

$$100 - \text{BEI}\% = \left(\frac{\frac{\text{Amount of test compound in brain}}{\text{Amount of reference in brain}}}{\frac{\text{Amount of test compound injected}}{\text{Amount of reference injected}}} \right) \times 100 \tag{17}$$

Nonlinear regression analysis of 100 – BEI% against time gives the apparent elimination constant k_{el}. The efflux clearance is obtained by multiplying k_{el} by the distribution volume $V_{u,brain}$, determined using the brain slice method [59] according to

$$\text{CL}_{\text{efflux}} = k_{el} \times V_{u,\text{brain}} \tag{18}$$

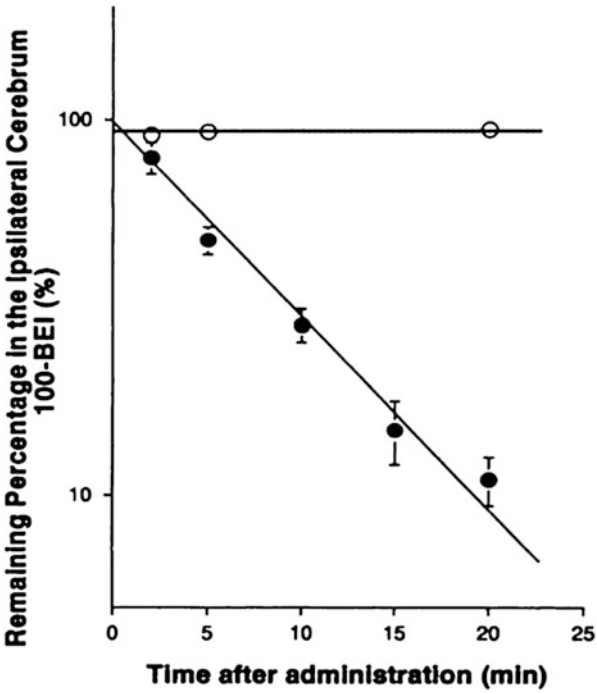

Fig. 6 Time course of BBB efflux clearance using 100–BEI (%) (see Eq. (16)) for [^{3}H]3-O-methyl-D-glucose (*closed circles*) and [^{3}H]L-glucose (*open circles*) in the ipsilateral cerebrum after intracerebral microinjection into the PAR2 region of rats. The reference compound was [^{14}C]inulin. From Kakee et al. [59] with permission from the publisher

3.5 *Percentage of the Injected Dose*

The percentage of a systemically injected dose (ID) that is delivered to the brain provides an estimate of the extent of drug transport into the brain. The percentage of the dose at time t after administration can be determined from the PA and the area under the curve of the plasma concentrations between times 0 and t (AUC) [52]:

$$\%\mathrm{ID/g\_brain}_0^t = \mathrm{PA} \times \mathrm{AUC}_0^t \tag{19}$$

Thus, the amount of the dose that reaches the brain is dependent on the plasma pharmacokinetics and the permeability of the BBB to the drug (influx). However, the amount reaching the brain is also determined by the influx/efflux ratio according to Eqs. (11)–(13), making the estimation of PA in Eq. (19) erroneous if the times studied are not very short. The percentage of the dose reaching the brain is not usually compared with the AUC, but is calculated directly. The value of this method is questionable if other methods are available.

3.6 Microdialysis

Microdialysis has become a well-established technique in the field of neuroscience, mostly for measuring the concentrations of endogenous substances, but also a very important technique for measuring drug concentrations in the brain [25, 26, 62–66]. The specific property of microdialysis that sets it apart is that it maps the concentrations of the unbound compound in the tissue in which the probe is placed, making it possible to correlate concentrations with pharmacological responses and receptor binding.

Microdialysis is mainly used to measure the extent of transport, but can also be used to estimate influx and efflux clearances at the BBB by including $V_{u,brain}$ measurements and modeling the data [67, 68]. The advantages of microdialysis are that it can be used to sample the local concentrations of unbound drug and to sample multiple time-points within the same individual. This reduces the number of animals needed while at the same time improving the amount of detailed information. The major disadvantage of microdialysis for studying drug pharmacokinetics is that tubings and probes can adsorb the compound of interest, resulting in erroneous concentrations and time profiles. An in vitro check of the adsorption behavior is thus required before proceeding to in vivo studies. See Fig. 7 and Table 6.

The microdialysis probe is placed in the selected area of the brain with the help of stereotactic coordinates. For BBB transport studies, a probe can also be placed in the jugular vein or regular blood can be sampled. The probe can be positioned during surgery on the day, or even several days, before the study is performed. Alternatively, a guide cannula can be surgically positioned on the day of surgery and the probe inserted just before the experiment. In some studies, the probe is inserted in anesthetized animals just before the experiment starts; however, this can cause leakage and disturbances in BBB function. On the other hand, waiting for too long (>3–5 days) after insertion of the probe can result in an inflammatory response which may hamper exchange across the probe [69, 70]. Nonetheless, this does not seem to be too influential when studying exogenous compounds.

In vivo recovery estimations are needed for quantitative studies when mapping the extracellular environment and BBB transport of drugs. Measurement of in vitro recovery can never adequately replace in vivo estimations, as the tissue surrounding the probe significantly influences the exchange across the probe membrane. These peri-probe processes include the exchange between extracellular sites and the vasculature, where active efflux transporters like P-gp have a substantial influence on the recovery, as well as the diffusion and metabolism, of the compound within the tissue [62]. In general, all processes that increase the turnover of the compound will increase its recovery.

Alternative methods of estimating the recovery of a drug in these studies include retrodialysis by drug or by calibrator [71], the no-net-flux method, and the dynamic no-net-flux method [72]. A deuterated version of the compound to be studied is the best choice for the calibrator used in retrodialysis, as recovery can then be mapped

Fig. 7 A cartoon of a microdialysis probe on a cast of the capillary network in the brain, showing the semipermeable membrane where exchange between the brain ISF and the dialysis fluid takes place. The picture can be found at http://www.leidenuniv.nl/en/researcharchive/index.php3-c=205.htm

Table 6 Advantages and disadvantages of microdialysis for studying BBB drug transport

Advantages	Disadvantages
Measures unbound drug concentrations	Possible tissue damage during probe implantation
Continuous measurements possible for relatively long periods	In vivo recovery measurements necessary for quantitative measurements (validation crucial)
Any tissue can be studied	The analyte has to be suitable for dialysis (to prevent drug adsorption to probe and tubing material)
Different sites can be measured simultaneously	No access to the intracellular biophase (although no method can accomplish this yet)
Gives detailed, thorough information	Slow
Because crossover studies are possible in small animals, fewer animals are needed	Necessary to adapt the experimental design to methodological aspects, including analytical sensitivity vs collection interval and flow rate
No loss of body fluids (blood, CSF, etc.)	
Possible to administer test compounds locally	
Low variability because fewer animals required	
No vascular contamination of tissue samples	
No sample cleanup needed	
Can correlate drug concentrations with effects in the same tissue	

throughout the study [73]. This requires liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. Alternatively, a compound that is closely related chemically can be used, with the caveat that it may be treated differently by transporters at the BBB, thus resulting in a different recovery than the drug of interest. This is especially important if drug interactions are studied, as the extent of changes in in vivo recovery of the study compound as a result of the interaction may differ from that of the calibrator. The compound of interest could also be studied before it is administered, to check its retrodialysis recovery. A washout period is then needed before the compound can be administered. However, changes during the study itself are then not mapped. This method cannot be used if the studied compound is endogenous.

With the microdialysis method, there is always a trade-off between the sampling duration wanted, the flow rate, and the analytical sensitivity. The lower the flow rate, the higher the recovery, but the smaller the recovered volumes for analysis, given a certain time-interval for sampling. A flow rate of 0.3 μL min^{-1} is used in clinical microdialysis studies, while preclinical studies use 0.5–2 μL min^{-1} or even higher. It may be advantageous to administer drugs as constant-rate infusions to reach steady state in order to counteract these problems. For retrodialysis recovery estimations, a lower recovery is often associated with a greater likelihood of error, as the concentrations of drug in the inflow of perfusate and the outflow of dialysate will be similar, in addition to the inherent variability in the chemical analysis [71].

An in vitro check of adsorption to the tubing should always be made before the in vivo studies [66]. This can be done as a first step, using various types of tubing and sampling every 10 min for 1 h, before adding the probe. Loss to and gain from the tubing are checked by adding the compound of interest to a test tube surrounding the probe and to the perfusate through the probe in sequence, with only blank buffers added between. The percentage loss and gain should be very similar if the data from the study are to be trusted, and there should be a "square-wave" curve when the solutions are changed, indicating that drug recovery does not lag behind as a result of slow release from the tubing and/or the probe. Addition of 0.5% albumin to the perfusate may help prevent the drug from sticking to tubings and the probe membrane, but this will not work if the drug has a high albumin-binding propensity (unpublished observations). Alternatively, the tubing and probe can be coated with a poloxamer such as Pluronic [74].

3.7 CSF Sampling

The CSF offers an accessible sampling site that has been used for many years to estimate the concentration of drugs in the brain. It is important to note that the CSF is a compartment on its own, with the blood–cerebrospinal fluid barrier (BCSFB) at the choroid plexus forming the interface with the blood. About 10% of the CSF is

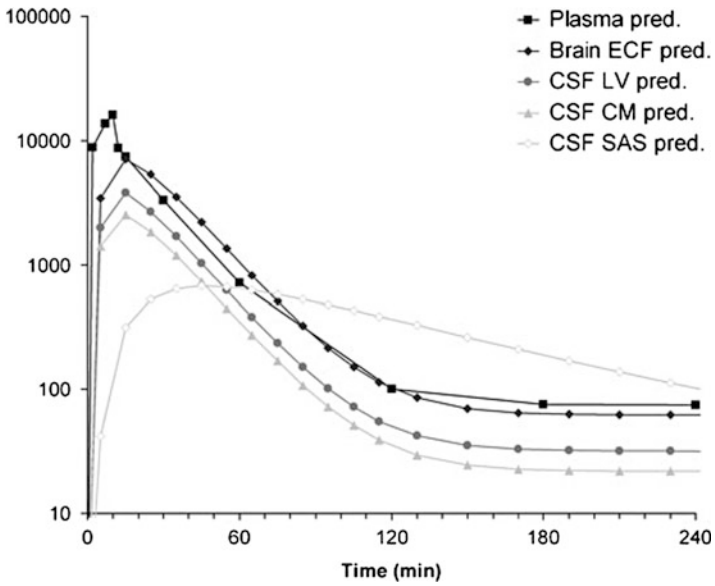

Fig. 8 Predicted concentrations of acetaminophen in plasma and various locations in the brain (*ECF* extracellular fluid) and CSF (*LV* lateral ventricle, *CM* cisterna magna, *SAS* subarachnoidal space), showing the delay in reaching peak concentrations in the CSF vs plasma, with the longest delay in the SAS. From Westerhout et al. [76] with permission from the publisher

made up from the bulk flow of fluid from the brain. The rest is produced at the choroid plexus.

The expression of transporters at the BCSFB may or may not be similar to that at the BBB. It has been shown in rats, for example, that the expression of P-gp is lower at the BCSFB than at the BBB, but that of multidrug resistance protein 1 (Mrp1) is significantly higher [75]. The introduction of microdialysis and other current methods has allowed the comparison of unbound drug concentrations between the brain and the CSF. As suggested by the differing expression of transporters [75], CSF concentrations often overpredict unbound drug concentrations in the brain if the drug is actively effluxed at the BBB and underpredict brain concentrations if the drug is actively taken up at the BBB [35].

Equilibration between the CSF and blood appears to be somewhat slower than that between the brain ISF and blood, as shown in Fig. 8 [76]. The profiles may differ between humans and rats, because the site of sampling differs: the subarachnoidal space (SAS) in humans and the cisterna magna in rats. Westerhout et al. developed an elegant model regarding the correlation of rat and human concentrations [76].

Sampling the CSF from the cisterna magna can be achieved either as a single sample or via a permanent cannula. After sampling, the volume of the CSF is decreased, and this could affect equilibration across the BCSFB. Therefore, the number of serial samples taken should be limited so as not to influence the equilibrium. Microdialysis in the ventricle is an alternative [76].

Table 7 Advantages and disadvantages of CSF sampling for studying BBB drug transport

Advantages	Disadvantages
CSF concentrations of unbound drug are closer to those in the brain than plasma concentrations	Different transporter expression in the BCSFB than at the BBB
Samples can also be obtained from humans	Different sampling sites in rodents and humans make comparisons difficult
	The possible increase in protein content in the CSF in some disease states can lead to erroneous estimations of drug concentrations for drugs that have high protein binding if this is not compensated for

If the differences between the CSF and brain parenchymal concentrations of drugs are taken into consideration, the CSF is an accessible surrogate site that gives an indication of the concentration range that can be expected in the brain (Table 7) [35, 77–80].

3.8 Positron Emission Tomography

Positron emission tomography (PET) can be used to measure both the rate and the extent of transport across the BBB. This method has the great advantage in that it is a noninvasive method that can be used in humans and can be used to study disease states [81]. Measuring BBB transport with PET requires both blood sampling and PET images of brain concentrations; this method is not used as commonly as some of the others [82]. The compound of interest is usually labeled with [^{11}C] and the total radioactivity is measured. The decay half-life ($t½$) of the tracer limits the time frame of the study to about three half-lives, which is about one hour for [^{11}C] ($t½ = 20$ min) and is about 5 h for [^{18} F] ($t½ = 110$ min).

It is necessary to subtract the radioactivity signals from the metabolites when using PET. It is highly likely that the metabolite-to-parent drug ratio in the brain differs from that in the plasma. The different ratios in the brain may be due to differences in nonspecific binding to brain parenchyma or in the extent of BBB transport of the drug and its metabolites (Table 8) [53, 82].

3.9 Combinatorial Mapping of $\mathbf{K}_{p,uu,brain}$

The partition coefficient ($K_{p,brain}$) can be determined by sampling whole brain tissue and plasma (Eq. (14)). Normally, a single dose is administered systemically, and brain and plasma samples are taken at one or several time-points. It is, of course, important that equilibrium has been attained between brain and plasma. The notion of

Table 8 Advantages and disadvantages of PET for studying BBB transport

Advantages	Disadvantages
Noninvasive	Expensive
Can be used in humans	Not all molecules can be labeled with a radioactive atom
Possible to obtain local information from specific brain sites	High technical challenges in equipment and data handling
	Measures total radioactivity

cassette (multiple) dosing has been studied to determine whether the administration of several compounds simultaneously works as well as individual administration, in order to save animals [83]. The results showed that interactions at the BBB between several compounds administered simultaneously are unlikely at the low doses used (1–3 mg kg^{-1}).

A *combinatorial map of* $K_{p,uu,brain}$ can be made using $K_{p,brain}$ determined as above, and $V_{u,brain}$ (or $f_{u,brain}$) and $f_{u,plasma}$ measurements [34]. Equation (14), or a modification that includes $1/V_{u,brain}$ instead of $f_{u,brain}$ (Eq. (20)), can then be used to calculate $K_{p,uu,brain}$. $K_{p,uu,brain}$ can also be determined using microdialysis [36]; however, the time needed for this method and the lack of its success with many lipophilic compounds mean that microdialysis is less feasible in a drug discovery setting.

Two parameters are used to measure the binding of drugs to the brain parenchyma. These are the $f_{u,brain}$, which is determined from equilibrium dialysis of diluted brain homogenate, and the volume of distribution of unbound drug in the brain, $V_{u,brain}$, which is determined from fresh brain slice measurements. These two parameters are related according to

$$f_{u,brain} \approx {}^{1}/_{V_{u,brain}} \tag{20}$$

The parameters describe the intra-brain distribution of the compounds studied, rather than the actual BBB transport of the compounds. Either one of the parameters are then used to obtain $K_{p,uu,brain}$. As discussed below, Eq. (20) should be used with caution as it is not always appropriate.

$V_{u,brain}$, which describes the average nonspecific binding to brain tissue, can be determined using the *brain slice technique* (Eq. (21)) [34, 59, 84, 85]. Fresh rat brain is sectioned into six 300 μm slices and put into a buffer. The proportion of buffer for specific slice weights is crucial for optimal equilibration [86]. The buffer is gently stirred at 37°C for 5 h. One slice is then used to measure viability, while the other five slices are used to determine the total brain concentrations. The buffer is sampled as a measure of the unbound drug ISF concentration:

$$V_{u,brain} = \frac{Q_{slice}}{C_{buffer}} \tag{21}$$

Table 9 Advantages and disadvantages of the combinatory mapping of $K_{p,uu}$ for studying BBB drug transport

Advantages	Disadvantages
Rapid	The combination of three measurements increases uncertainty
Can obtain $K_{p,uu}$ without the need for microdialysis, which is otherwise the only alternative	

where Q_{slice} is the amount of compound per gram of brain slice and C_{buffer} is the concentration of compound in the surrounding buffer, which is assumed to be equal to the brain ISF concentration, using units of mL g brain^{-1}. Values above unity indicate binding to brain parenchymal cells and values below unity indicate restricted distribution of the compound into brain parenchymal cells. If cassette dosing is used, the combined concentration of the compounds should be 1 μM at most [34]. A detailed protocol for the brain slice procedure has been published by Loryan et al. [85].

The *brain homogenate method* is used to determine $f_{u,brain}$ [87, 88]. A homogenate of the brain tissue is mixed with 2–9 volumes of buffer and is dialyzed across a semipermeable membrane against buffer until equilibrium is reached. Frozen brain homogenate can be used. The disadvantage of this method is that the parenchymal cells are destroyed during homogenization, and differences in pH between subcellular structures are subsequently lost. When the brain slice and brain homogenate methods were compared, it was seen that the brain homogenate results required recalculation using the pH partitioning model to better estimate the binding and intracellular partitioning of the drug [51]. Unpublished observations show that results may differ between the two methods even when pH partitioning is taken into account. Di et al. have shown that binding to brain tissue homogenate is very similar between species (Table 9) [89].

4 Conclusions

There are currently several methods available for studying the rate and extent of drug transport across the BBB, both preclinically and clinically. It is important that the question to be answered correlates with the method used. Methods that measure the extent of delivery to the brain are more likely to give clinically relevant estimations of BBB penetration than those measuring the rate of transport.

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ABC Transporters at the Blood–Brain Barrier

David S. Miller

Abstract In the blood–brain barrier several ABC transporters are expressed at the luminal, blood-facing, plasma membrane of the brain capillary endothelial cells. There they function as ATP-driven efflux pumps for xenobiotics and endogenous metabolites, thus providing an important element of the barrier. When these transporters limit neurotoxicant entry into the CNS, they are neuroprotective; when they limit therapeutic drug entry, they become major obstacles to drug delivery to treat CNS diseases. Here I review function and regulation of ABC transporters at the blood–brain barrier, with an emphasis on recently disclosed mechanisms that alter transporter expression and transport activity.

Keywords BCRP, Blood–brain barrier, Brain capillary endothelium, Disease, Drug delivery, MRP, P-glycoprotein, Regulation

Contents

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Abbreviations

ABC	ATP-binding cassette
AD	Alzheimer's disease
AEDs	Antiepileptic drugs
AhR	Arylhydrocarbon receptor
Akt	Protein kinase B
ApoE	Apolipoprotein E
BCRP	Breast cancer resistance protein (ABCG2)
BSEP	Bile salt export pump
CAR	Constitutive androstane receptor
CFTR	Cystic fibrosis transmembrane regulator (ABCC7)
CNS	Central nervous system
COX-2	Cyclo-oxygenase-2
E2	17-β-estradiol
EP-1	Prostaglandin E2 receptor
ER	Estrogen receptor
FXR	Farnesyl-X receptor
GSK-3β	Glycogen synthase kinase 3 beta
hAPP	Human amyloid precursor protein
*i*NOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
LXR	Liver-X receptor
MRP	Multidrug resistance-associated protein
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	*N*-methyl-D-aspartate
PCBs	Polychlorinated biphenyls
PCN	Pregnenolone-16-alpha-carbonitrile
PCR	Polymerase chain reaction
PI3-K	Phosphatidylinositide 3-kinase
PK	Pharmacokinetics
PKCβ1	Protein kinase C isoform β1
PTEN	Phosphatase and tensin homolog
PXR	Pregnane-X receptor
S1P	Sphingosine-1-phosphate
S1PR1	Sphingosine-1-phosphate receptor 1
TNF-α	Tumor necrosis factor-α
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor

1 Introduction

The present review is focused on blood–brain barrier transporters that are members of the ABC family and that largely handle foreign chemicals (xenobiotics). These membrane proteins function as multispecific, ATP-driven efflux pumps and importantly influence the pharmacokinetics of many signaling molecules, waste products of normal metabolism, therapeutic drugs, environmental toxicants, and drug and toxicant metabolites. Those ABC transporters expressed on the luminal, blood-facing plasma membrane of the brain capillary endothelium act to limit brain accumulation of substrates. They do this in two ways: preventing transport from blood into the endothelial cells and mediating efflux from the brain parenchyma through the endothelium into the blood. Conversely, those ABC transporters expressed at the abluminal, CNS-facing plasma membrane of the brain capillary endothelium can facilitate transport into the brain.

To the extent that ABC transporters expressed at the blood–CNS barriers limit exposure to potentially toxic chemicals and endogenous metabolites, they are xenoprotective and neuroprotective. However, ABC transporters distinguish poorly between toxicants and therapeutic drugs. Thus, high ABC transporter expression on the luminal membrane of brain capillary endothelial cells is the major reason why it is such a challenge to deliver small-molecule drugs to the brain for treatment of diseases such as brain cancer, neuroAIDS, and epilepsy. In addition, recent findings implicate the blood–brain barrier and its transporters in CNS disease progression [1, 2], suggesting that the barrier is not just a bystander but rather an active participant and a potential target for therapy. Clearly, a full understanding of ABC transporter function and its regulation is needed to improve the delivery of small-molecule therapeutics to the CNS and to treat CNS disease.

2 ABC Transporters at the Blood–Brain Barrier

In 1976, Juliano and Ling identified an overexpressed gene in multi-drug-resistant cells that conferred resistance to a wide range of chemotherapeutics [3]. It was subsequently shown that the gene coded for a plasma membrane transport protein that coupled ATP splitting to the active, outward transport of many drugs. This transporter was named P-glycoprotein and the gene, MDR1. P-glycoprotein transported such a remarkably wide range of therapeutic drugs and chemical structures that it was designated as a multispecific transporter [4]. Subsequently, it was demonstrated that P-glycoprotein is widely expressed in the body, with highest levels of expression in the cells of barrier and excretory tissues, such as liver, intestine, and blood–brain barrier [5]. This tissue distribution underlies the importance of P-glycoprotein in determining the PK and pharmacodynamics of many drugs. Altered drug PK is best seen in studies in which specific P-glycoprotein inhibitors are used in patients and animals and in organisms with the alterations in the gene itself,

e.g., patients and animals with single nucleotide polymorphisms and mice and rats in which the gene has been deleted [6, 7].

P-glycoprotein was the first member of the ABC family of transporters to be identified. The human genome contains 49 genes encoding ABC transporters [8]. These genes are divided into seven different subfamilies, A–G, based on their evolutionary divergence. Members of the ABC family are classified as such based on the presence of several consensus sequences including two ATP-binding motifs (Walker A and Walker B), as well as the ABC signature C motif (ALSGGQ). ABC family members include proteins that function as ATP-driven transporters on both surface and intracellular membranes, ion channels, and receptors. Mutations in some of the ABC genes result in genetic disorders such as cystic fibrosis (ABCC7, CFTR, a chloride channel), Dubin–Johnson's syndrome (ABCC2, MRP2, a metabolite and drug transporter), progressive familial intrahepatic cholestasis (ABCB11, BSEP, a bile salt efflux pump), and retinal degeneration (ABCA4, a lipid flippase). For vertebrates, three ABC subfamilies, B, C, and G, contain transporters that largely handle foreign chemicals (xenobiotics) and these importantly influence the pharmacokinetics of many signaling molecules, waste products of normal metabolism, therapeutic drugs, environmental toxicants, and drug and toxicant metabolites.

Multiple ABC transporters that handle therapeutic drugs are expressed in the brain capillary endothelium that makes up the blood–brain barrier [9] (Fig. 1). Certainly for an efflux transporter to be effective in limiting blood to brain movement of drugs it should be pointed towards the vascular compartment, i.e., localized to the luminal plasma membrane. Several transporters appear to fall into this category, P-glycoprotein, MRP2, and BCRP (Fig. 1). Others, e.g., MRP4 and MRP1, may be expressed on both sides of the endothelium, but the fraction of transport protein on luminal membrane still counts as an obstacle to blood to brain transport. In this regard, there is lingering controversy over where some ABC transporters are located. For example, available evidence indicates that MRP1 could be abluminal, luminal, or both [10]. The same can be said for MRP4. There is no definitive published localization of MRP5 and MRP7. In addition, some studies have found evidence for transporter localization in other membrane structures. For example, immuno-electron microscopy and biochemical methods indicate that 30–50% of P-glycoprotein within brain capillary endothelial cells is not on the luminal membrane [11, 12]. These results raise interesting possibilities with regard to regulation of transport activity through insertion and retrieval of preformed protein at surface membranes (see below). Indeed, this is one way by which transport activity can be altered in the absence of change in transporter protein expression.

It should be noted that other elements of the neurovascular unit express ABC transporters and these are known to respond to stressors, e.g., inflammation, by altering expression and transport activity. Astrocyte and microglial P-glycoprotein, MRPs, and BCRP likely contribute to multidrug resistance in situations where drug targets are within the glia themselves and CNS inflammation has upregulated transporter expression, e.g., neuroAIDS [10, 13, 14]. Similarly, neurons express some ABC transporters and it is not clear to what extent neuronal transporter expression changes

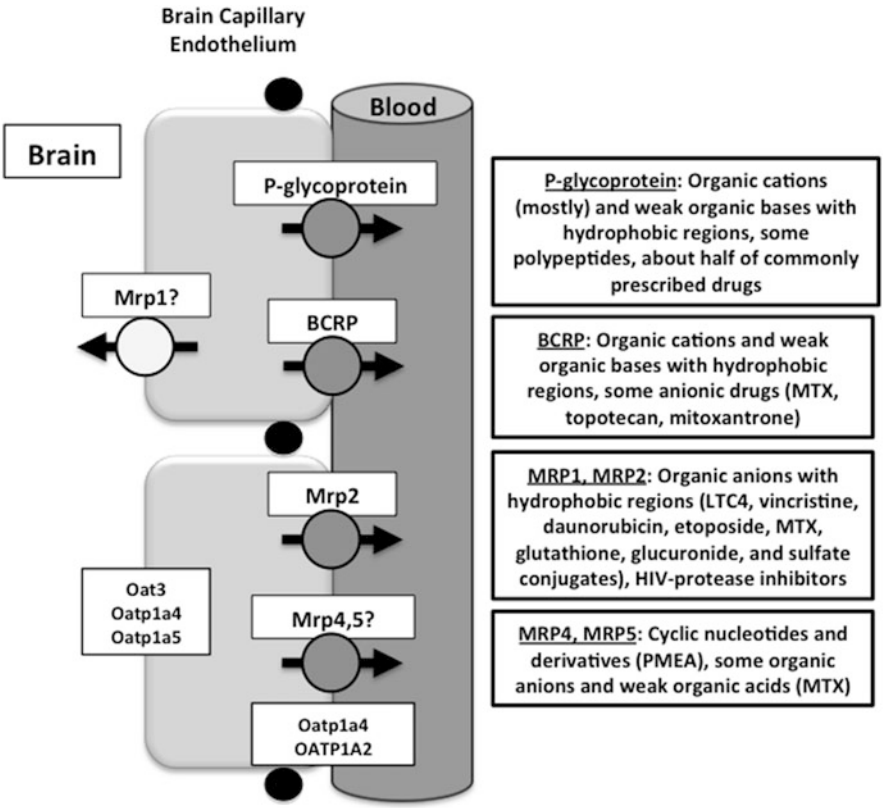

Fig. 1 The distribution of xenobiotic transporters at the blood–brain barrier. Shown in *boxes* are specificity characteristics of the luminal ABC transporters

in response to stressors [10, 15]. Finally, the extent to which pericytes express drug efflux transporters is unknown. This multilayer arrangement of transporters suggests that drugs must overcome more than one transporter-derived barrier to access targets within specific cells in the neurovascular unit and the CNS as a whole.

2.1 Assessing ABC Transporter Activity/Expression

There are multiple experimental strategies for studying the expression, function, and regulation of ABC transporters at the blood–brain barrier (see other chapters in the present publication). One can measure expression at the mRNA level using specific molecular probes designed for PCR and microarray analysis and using deep sequencing combined with statistical/bioinformatic methods. Specific antibodies for many ABC transporters are commercially available; these provide measures of

protein expression level and, when used for immunostaining, of subcellular transporter localization. Few of these ABC transporter antibodies recognize external epitopes, so cells and tissues must be permeabilized prior to antibody exposure. Finally, improved, sensitive mass spectroscopy-based techniques now provide absolute quantitation of protein levels.

Measuring ABC transporter function is another matter. Function has been measured in isolated brain endothelial cells (primary cells in culture and cell lines), endothelial cell monolayers, isolated brain capillaries, intact animals (brain to plasma concentration ratio, brain perfusion, brain efflux index, and brain uptake index), and in human subjects. Indeed, in human subjects, positron emission spectroscopy and single photon emission computed tomography with labeled transporter substrates provide a measure not only of drug uptake but also of drug distribution within the brain, albeit at low spatial resolution [16]. One must be aware that each approach has inherent strengths and weaknesses, requiring one to balance tradeoffs. In general, moving away from the in vivo situation increases the potential to bring powerful molecular tools to bear on underlying mechanisms of transport and their regulation. However, in doing that, one has to be concerned about altered expression of key proteins and altered signaling and loss of critical cell–cell interactions within the endothelium and the larger neurovascular unit and thus physiological relevance. For these reasons, it is important to validate critical in vitro findings with in vivo measurements.

Since the ABC transporters function as unidirectional, drug efflux pumps, direct measurements of substrate efflux rates are difficult to make. Endpoints measured include exclusion and efflux of fluorescent or radiolabeled substrates by cells, net transport of fluorescent or radiolabeled substrates across monolayers of cells, and secretion of fluorescent substrates from bath into capillary lumens. In vivo measurements of ABC transporter activity at the blood–brain barrier require careful selection of the substrate, specific inhibitors, and analytical techniques. Changes in transport activity can be seen as altered uptake from the vascular compartment or as altered efflux from the brain.

In many cases, transport activity is defined by measurements of steady-state drug distribution, so actual rates of transport are not computed. Moreover, at least one of these transporters, P-glycoprotein, seems to extract substrates from the plasma membrane's lipid bilayer. As a result, estimates of transport affinities are dependent on substrate partitioning into the membrane and thus on the lipophilicity of the substrate and composition of that membrane. Thus, medium substrate concentration may not accurately reflect what is seen by the transporter embedded in the plasma membrane.

3 Modulation of ABC Transporter Expression and Activity

Over the past 10 years it has become evident that ABC transporter expression and transport activity at the blood–brain barrier are altered by multiple factors, including disease, stress, diet, therapy, and toxicant exposure [17, 18]. Certainly, it is clear

that ABC transporter expression at the blood–brain barrier can be upregulated through the action of a number of ligand-activated receptors, leading to selective tightening of the barrier to both neurotoxicants and therapeutic drugs [17]. The consequences of efflux transporter upregulation are increased neuroprotection but reduced drug delivery. In certain situations, e.g., chemotherapy to the periphery, one might want to take advantage of these mechanisms to upregulate ABC transporter expression and augment neuroprotection. Conversely, recent studies show that targeting of blood–brain barrier signaling to manipulate transporter activity has the potential to selectively improve drug delivery to the CNS [18]. In addition, this barrier is not just a bystander in CNS disease but rather an active participant and thus a potential target for therapy [1, 2]. The mechanisms that underlie changes in ABC transporter expression in disease are not well understood. A detailed understanding of the mechanisms underlying changes in transporter expression/activity is essential for devising strategies to improve CNS pharmacotherapy and for an appreciation of how changes in barrier properties contribute to neuroprotection, neurotoxicity, and CNS disease.

3.1 Altered Transporter Expression

Being at the interface between the CNS and the periphery, the blood–brain barrier has the potential to sense conditions on both sides and to respond by altering its own function. Through signaling, it is capable of passing information between the CNS and the periphery and to other elements of the neurovascular unit. One way by which brain capillary endothelial cells sense their environment is through multiple receptors and transcription factors. When activated, they translocate to the nucleus, bind to specific promoter regions of target genes, turn on transcription, and alter gene expression. These receptors/transcription factors activated through specific interactions with hormones, metabolites, or xenobiotics or through upstream intracellular signaling. Recent studies show that a number of receptors and transcription factors largely increase expression of ABC transporters at the blood–brain barrier. Figure 2 summarizes the results of several of these studies.

3.1.1 Response to Xenobiotics

In peripheral barrier and excretory tissues, xenobiotic-activated intracellular receptors signal increased expression of Phase 1 (cytochrome p450 enzymes) and Phase 2 (transferases) xenobiotic metabolizing enzymes and xenobiotic excretory transporters (Phase 3). This coordinated response to xenobiotic exposure can lead to complicated effects, since some receptor ligands are substrates for the affected enzymes and transporters. Thus, through receptor binding and increased transcription, these xenobiotics upregulate their own metabolism and excretory transport. In addition, because of the wide specificity limits of both the nuclear receptors and

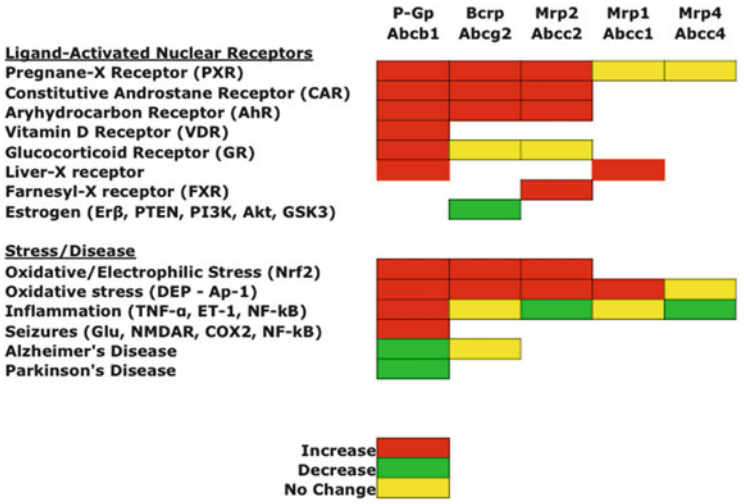

Fig. 2 Heat map showing how xenobiotics and stressors alter ABC protein expression at the blood–brain barrier. The figure summarizes published and unpublished data from this laboratory and others. See text for discussion and specific references

several ABC transporters, members of one class of drug can alter the metabolism and transport of other classes without having to interact at the level of the enzyme or transporter. This has led to documented adverse drug interactions in the clinic. Indeed, exposure to a drug that activates a specific receptor can increase expression of multiple target genes that ultimately influence the pharmacokinetics of multiple drugs, generally reducing plasma levels (reduced absorption in the gut and increased metabolism and excretion in liver and kidney) as well as brain drug levels (increased efflux transport at the blood–brain barrier and likely increased metabolism within the endothelial cells).

The best-studied xenobiotic receptors are the PXR, CAR, and AhR, which function as a major part of our first line of defense against potentially toxic endogenous metabolites and xenobiotics [19, 20]. PXR and CAR share activating ligands and target sequences in gene promoter regions and receptor-responsive genes; activation of one receptor can alter the expression of the other; once in the nucleus, both partner with the retinoid-X receptor before binding to DNA. PXR and CAR are activated by endogenous ligands, e.g., bile acids, and by numerous therapeutic drugs, many of which are handled by the enzymes and transporters that the receptors regulate. AhR is activated by a number of planar aromatic chemicals, many of which are widespread and persistent environmental pollutants, e.g., PCBs and dioxins [21].

PXR, CAR, and AhR are expressed in brain capillaries or brain capillary endothelial cells from mouse, rat, pig, and human [22–25]. Ligand activation of any of the three in vitro increases protein expression and transport activity of multiple ABC transporters, including P-glycoprotein, MRP2, and BCRP [22, 26–28]. Importantly, dosing animals with PXR, CAR, and AhR ligands increases protein expression for

P-glycoprotein, MRP2, and BCRP in brain capillaries and decreases brain accumulation of drugs that are P-glycoprotein substrates [28–30].

With regard to altered ABC transporter function at the blood–brain barrier, PXR, CAR, and AhR are the xenobiotic-activated nuclear receptors for which we have the most complete picture (Fig. 2). Other ligand-activated receptors are expressed in the tissue and available evidence indicates that some ABC transporters are their targets (Fig. 2). Studies have shown increased expression of MRP2 with FXR ligands [26], increased expression of P-glycoprotein and MRP1 with LXR ligands in ischemia [31], increased P-glycoprotein expression with VDR ligands [32, 33], and increased expression of P-glycoprotein with GR ligands [34]. The latter is particularly relevant to the clinic, since synthetic glucocorticoids, e.g., dexamethasone, are among the most highly prescribed drugs. Indeed, our recent experiments show reduced brain access of drugs that are P-glycoprotein substrates in rats dosed with dexamethasone (Miller et al., unpublished data).

3.1.2 Response to Disease

Blood–brain barrier properties are clearly altered in CNS disease. For each disease, one wonders whether changes in barrier properties are a consequence of disease progression or active driving forces. In most diseases, the hallmark of barrier involvement has been taken as increased junctional permeability. However, profound changes in ABC transporter expression have also been documented in patient samples and studies with animal models (Fig. 2). Altered expression of ABC transporters at the blood–brain barrier accompanies several neuropathologies. Reduced expression or transport function for blood–brain barrier P-glycoprotein is associated with AD [35], Jakob-Creutzfeldt disease [36], Parkinson's disease [37], HIV infection [38], and normal aging [39]. Increased expression of P-glycoprotein, MRP1, MRP2, and BCRP is associated with epileptic seizures [40]; increased expression of P-glycoprotein and MRP1 is associated with ischemic stroke [31, 41]. Certainly, along with changes in tight junction permeability, these findings for ABC transporters have immediate and obvious implications for the delivery of therapeutic drugs to the CNS. For HIV infection, Alzheimer's disease, and epilepsy, recent reports are beginning to disclose mechanisms underlying changes in ABC transporter expression. In addition, both inflammation [42] and oxidative stress [43] (Miller et al., unpublished data) can alter ABC transporter expression at the blood–brain barrier. Below is summarized recent progress in understanding those mechanisms for epileptic seizures, Alzheimer' disease, and ischemic stroke.

Limited drug delivery to the brain is a common cause of therapeutic failure in epilepsy. One suggested basis for pharmacoresistance is the overexpression of ATP-driven drug efflux pumps at the blood–brain barrier, including P-glycoprotein, MRP1, MRP2, and BCRP [40]. Evidence connecting transporter overexpression with pharmacoresistance to AEDs is strongest for P-glycoprotein. Bauer et al. used brain

capillaries isolated from rat and mouse to demonstrate that the neurotransmitter glutamate signals through an NMDA receptor, COX-2, prostaglandin E2, and NF-κB to increase expression of P-glycoprotein [44]. They found that microinjection of glutamate into the hippocampus in rats locally increased brain capillary P-glycoprotein expression and that indomethacin, a nonselective COX inhibitor abolished seizure-induced increases in capillary P-glycoprotein expression following pilocarpine-induced status epilepticus. Subsequent in vivo studies with rodent seizure models [45, 46] have shown similar effects with a specific COX-2 inhibitor, an NMDA receptor antagonist, and a prostaglandin E2 receptor antagonist, thus validating the major elements of the blood–brain barrier signaling system.

In AD, ABC transporters may play more than a spectator role. Zlokovic has proposed that a cascade of neurovascular events alters BBB function and fuels disease progression in AD [1]. One element of this hypothesis is that reduced amyloid-β efflux from the brain increases brain accumulation of that pathological protein in AD [1]. P-glycoprotein and BCRP have been implicated as possible efflux pumps for amyloid-β [47–49] and studies show that Alzheimer's patients exhibit reduced expression of P-glycoprotein and increased expression of BCRP in brain capillaries [35, 49]. Hartz et al. found that P-glycoprotein specifically mediates efflux transport of amyloid-β from mouse brain capillaries into the vascular space, thus identifying a critical component of the amyloid-β brain efflux mechanism [50]. Using a transgenic mouse model of AD (hAPP-overexpressing mice; Tg2576 strain) they also found that brain capillary P-glycoprotein expression and transport activity are substantially reduced compared with wild-type control mice [50]. Note that reduced P-glycoprotein expression is seen in Alzheimer's patients [35]. Hartz et al. hypothesized that upregulating expression of P-glycoprotein, perhaps through diet, would slow amyloid-β deposition and possibly disease progression. Testing that hypothesis, they found that dosing 12-week-old (asymptomatic) hAPP mice over 7 days with PCN to activate PXR restores P-glycoprotein expression and transport activity in brain capillaries and significantly reduces brain amyloid-β levels compared with untreated hAPP mice [50]. Thus, targeting signals that upregulate blood–brain barrier P-glycoprotein in the early stages of AD has the potential to increase amyloid-β clearance from the brain and reduce amyloid-β brain accumulation.

In an animal model of ischemic stroke (middle carotid artery occlusion in mice), the expression of P-glycoprotein increases and expression of MRP1 decreases [41, 51]. In recent experiments, ElAli and Hermann investigated the mechanistic basis for such changes. They found that ApoE, possibly released from astrocytic end feet during ischemia, controls P-glycoprotein and MRP1 expression and abundance [41]. Thus, ApoE accumulating on the abluminal surface of brain capillaries binds to ApoER2, deactivating JNK1/2 by dephosphorylation. The resulting decrease in c-Jun activation increases P-glycoprotein transcription and protein expression at the luminal plasma membrane. At the same time, MRP1 transcription decreases, as does transporter protein expression.

3.2 *Altered Transporter Activity Through Signaling*

Recent studies focused on understanding the signals that regulate basal transport activity (independent of expression) of P-glycoprotein and BCRP suggest novel ways to improve drug delivery to the CNS. Both transporters have been identified as critical gatekeepers for many CNS-acting drugs and drug candidates [52]. Surprisingly, recent studies from a number of laboratories show much greater than additive effects of knocking out both transporters or inhibiting both transporters when measuring drug delivery to the brain [52, 53]. Thus, for drugs that are modest substrates for both P-glycoprotein and Bcrp, e.g., the tyrosine kinase inhibitor lapatinib, a large benefit for drug delivery to the CNS may be obtained by reducing transport on both transporters in concert. Given the important roles of the two transporters, singly and in combination, in limiting drug entry into the brain, a detailed understanding of the cellular signals that determine their basal transport activity could provide new options for improving small-molecule drug delivery to the CNS. To date, two signaling pathways have identified that reduce basal P-glycoprotein activity and one pathway has been identified that reduces basal BCRP activity. These experiments provide a proof of principle that targeting signaling has the potential to enhance delivery of therapeutic drugs to the brain.

3.2.1 Regulation of P-Glycoprotein Activity Through Sphingolipid Signaling

Over 10 years ago, Fellner et al. used nude mice to demonstrate that inhibiting P-glycoprotein at the blood–brain barrier would increase brain accumulation of a chemotherapeutic (Taxol) tenfold and reduce the mass of an implanted human glioblastoma by 90% [54]. This suggested a simple way to circumvent the barrier to drug entry into the CNS. Unfortunately, the use of ABC transporter-specific inhibitors to improve drug delivery to tumors has not translated well to the clinic [55–58]. As an alternative strategy, we sought to identify intracellular signals that rapidly modulate basal P-glycoprotein activity without altering transporter expression. At the time there was no evidence that such signals existed. Over several years, we found that basal transport activity of P-glycoprotein in rat brain capillaries is rapidly and reversibly reduced through a signaling pathway that is part of an extensive and complex pro-inflammatory response. It involves signaling through a TNF-α receptor, an endothelin receptor *i*NOS, and PKCβ1 [59, 60]. The pathway is shown in Fig. 3.

Although these experiments demonstrated that basal transporter activity could be rapidly and reversibly modulated, none of the signaling elements identified provided a way to safely target the pathway in the clinic. Exploring events downstream of PKCβ1 in rat and mouse brain capillaries, we recently identified multiple sphingolipid-based steps, involving sphingosine kinase, S1P, and S1PR1 (Fig. 4). S1P and S1PR1 agonists rapidly reduced P-glycoprotein transport activity in brain capillaries; these effects were blocked by S1PR1 antagonists [61]. Importantly, the

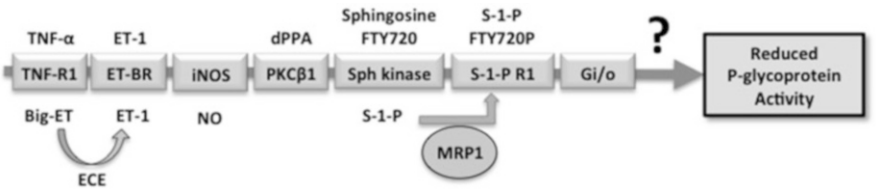

Fig. 3 Extended signaling pathway that regulates basal P-glycoprotein activity at the blood–brain barrier. Activation of the pathway in vitro (isolated brain capillaries) causes rapid and reversible loss of transport activity. Activation of the pathway in intact rats increases drug delivery to the brain [61, 62]

Fig. 4 VEGF signaling pathway that regulates basal P-glycoprotein activity at the blood–brain barrier. Activation of the pathway in vitro (isolated brain capillaries) causes rapid and reversible loss of transport activity. Activation of the pathway in intact rats increases drug delivery to the brain [12]

receptor could be targeted by fingolimod (Gilenya, FTY720), a prodrug that is approved for use in patients with relapsing-remitting multiple sclerosis. FTY720 generates an S1P analog (S1P720P) that is an S1PR agonist; both the prodrug and its phosphorylated metabolite rapidly reduced P-glycoprotein transport activity in brain capillaries (Fig. 3).

Recent experiments showed that another ABC transporter was an essential component of this signaling system (Fig. 3). S1P must exit the cells to bind to S1PR1. Since S1P is sufficiently polar to limit membrane permeability, access to external S1PR binding sites requires carrier-mediated efflux. Several studies in other cells have implicated ABC transporters in S1P efflux from cells [63, 64]. ABCA1 and MRP1 were leading candidates. Recently, a novel S1P transporter (Spns2), not a member of the ABC transporter family, was discovered in zebrafish [65, 66]. An ortholog of this transporter is expressed in mammals [67]. Using brain capillaries from MRP1 knockout mice, Cartwright et al. identified MRP1 as the ABC transporter that mediates S1P efflux from brain capillary endothelial cells [62]. In those capillaries, signaling upstream of sphingosine kinase, e.g., initiated by TNF-α, sphingosine, or FTY720, no longer reduced P-glycoprotein activity, but S1P and FTY720P were as effective as in wild-type mice [62].

Importantly, the involvement of the signaling pathway shown in Fig. 3 in regulating P-glycoprotein activity in vivo was validated using in situ brain perfusion in rats [59–61]. Treating rats with a specific PKCβ1 agonist, S1P, FTY720, or FTY220P rapidly and specifically increased brain uptake of several drugs that are radiolabeled P-glycoprotein substrates (in situ brain perfusion), indicating loss of P-glycoprotein activity in vivo antagonists. For Taxol, the chemotherapeutic used

in the initial mouse study with the implanted human glioblastoma [54], brain accumulation increased fivefold. In these experiments, brain uptake of ^{14}C-sucrose, a sensitive measure of changes in tight junction permeability, was not altered by the specific PKCβ1 agonist, S1P, FTY720, or FTY220P [59–61].

Note that this activation of signaling pathway (Fig. 3) also reduces P-glycoprotein activity after a xenobiotic-induced increase blood–brain barrier transporter expression. AhR activation by a dioxin increases blood–brain barrier P-glycoprotein expression in vitro and in vivo [30]. Activating PKCβ1 reversed the effect of P-glycoprotein induction on transporter activity in rat brain capillaries exposed to dioxin in vitro, in brain capillaries from TCDD-dosed rats, and in intact TCDD-dosed animals (increased brain accumulation of ^{3}H-verapamil with a PKCβ1 activator) [27]. Thus, signaling to P-glycoprotein can be used to increase access of drugs to the CNS, even in a drug-resistant population, one in which blood–brain barrier transporter expression has been induced.

3.2.2 Regulation of P-Glycoprotein Activity Through VEGF Signaling

The second distinct pathway that signals rapid, reversible loss of P-glycoprotein activity in brain capillaries is signaled by VEGF binding to a membrane-bound receptor, Flk-1, and activating Src kinase [12] (Fig. 4). Increased brain expression of VEGF is associated with neurological disease, brain injury, and blood–brain barrier dysfunction [68]. VEGF release and action are critical signals in angiogenesis. Hawkins et al. found that exposing isolated rat brain capillaries to VEGF acutely and reversibly decreases P-glycoprotein transport activity. This occurs without changes in transporter expression or in tight junction permeability [12]. VEGF increases Tyr-14 phosphorylation of caveolin-1 in an Src kinase-dependent manner. Thus caveolin-1 phosphorylation is downstream of Flk-1 and Src kinase signaling, but it is not clear whether this event leads to reduced P-glycoprotein transport activity [12]. Previous studies using brain capillary endothelial cells had suggested a role for caveolin-1 in regulation of P-glycoprotein activity [69, 70]. However, glycoprotein/caveolin-1 association, as measured by co-immunoprecipitation, is not altered in VEGF-exposed brain capillaries exhibiting reduced P-glycoprotein transport activity [12].

In intact rats, intracerebroventricular injection of VEGF increases brain accumulation of the P-glycoprotein substrates, ^{3}H-morphine and ^{3}H-verapamil, but not the tight junction marker, ^{14}C-sucrose. These VEGF effects on P-glycoprotein-mediated transport are blocked by systemic administration of an Src kinase inhibitor [12]. Taken together, these findings imply that P-glycoprotein activity is acutely diminished in pathological conditions associated with increased brain VEGF expression. They also imply that once the more downstream elements of VEGF signaling to P-glycoprotein are identified, there could be additional that modulate P-glycoprotein activity acutely and thus improve drug delivery to the brain.

3.2.3 Regulation of BCRP Activity Through Estrogen Signaling

Through a combination of transporter trafficking, transporter protein degradation, and reduced transporter mRNA levels, estradiol exposure reduces BCRP transport activity in mouse and rat brain capillaries [71, 72]. Multiple signaling pathways are involved (Fig. 5). Rodent brain capillaries express both ERα and ERβ, with expression of the latter dominating at both the mRNA and proteins levels [71, 73]. Exposing rat and mouse brain capillaries to subnanomolar to nanomolar concentrations of E2 rapidly and reversibly reduces BCRP-mediated transport activity without altering protein expression. The reduction in activity is not altered by inhibitors of transcription and translation, but could be blocked by brefeldin A, an inhibitor of intracellular vesicle trafficking. Both ER receptor subtypes, ERα and ERβ, are involved since capillaries isolated from ERα-null mice or ERβ-null mice do not show reduced BCRP transport activity in response to E2. The rapid response to E2 and the lack of effect of inhibitors of transcription and translation point to a nonclassical mechanism of E2 action, perhaps through intracellular signaling.

Extending the time of exposure to E2 reduces BCRP mRNA and protein expression. These effects are mediated by ERβ signaling though PI3-K, PTEN, Akt, and GSK-3β (Fig. 5). ERα is not involved. Such signaling increases ubiquitination of Bcrp protein, which leads to transporter protein degradation at the proteasome [71]. E2 also reduces Bcrp mRNA after 90 min of exposure, although it is not clear whether this is a result of reduced transcription, increased mRNA degradation, or both [72].

Dosing mice with E2 (0.1 mg/kg by i.p. injection) recapitulates the complex time course of changes in BCRP activity and expression seen when brain capillaries are exposed directly to E2 [71, 72]. That is, capillaries isolated from mice 1 h after E2 dosing show reduced BCRP transport activity with no change in protein expression, but capillaries isolated from mice 6 h and 24 h after E2 dosing show both reduced transport activity and transporter protein expression. Pharmacokinetic studies showed that plasma E2 levels rose rapidly after E2 dosing, but then fell. Six hours after dosing, levels had returned to those seen in controls, indicating long-term effects of a transient E2 exposure on blood–brain barrier physiology [71]. These studies suggest two estrogen-based strategies for reducing basal BCRP activity at the blood–brain barrier, with ERα-specific agonists rapidly and reversibly reducing transport activity and ERβ-specific agonists initially reducing transport activity followed by loss of transporter protein.

3.2.4 Mechanisms Underlying Decreased Transporter Activity

By what mechanisms do intracellular signals rapidly reduce ABC transporter activity when transporter protein expression is not altered? To date, two general types of mechanism have been proposed to underlie reductions in the activity of plasma

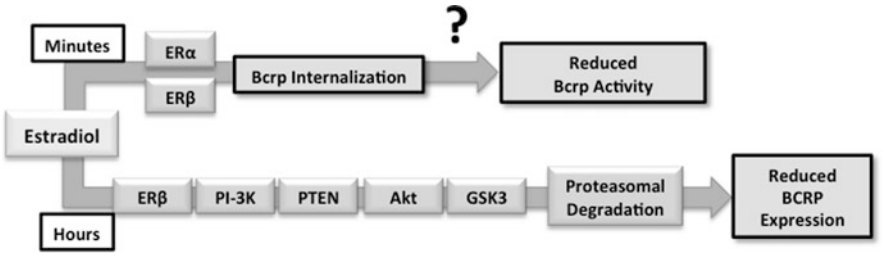

Fig. 5 Signals underlying the loss of BCRP transport activity and protein expression following E2 exposure [73]

membrane ABC transporters at the blood–brain barrier: (a) trafficking between the plasma membrane and intracellular compartments, i.e., transporter internalization, and (b) altered microenvironment within the plasma membrane. With regard to the first mechanism, for many proteins that function primarily at the cell surface, a fraction of total cellular protein is stored away from the surface in intracellular vesicular compartments. In hepatocytes, P-glycoprotein and other ABC transporters move rapidly in both directions between intracellular, membrane-bound compartments and the canalicular membrane [74]. In brain capillary endothelial cells, both immuno-electron microscopy and our biochemical measurements indicate that a significant fraction of total P-glycoprotein protein is not present in the luminal plasma membrane [11, 12]. These stored proteins are potentially available for rapid insertion and retrieval, resulting in changes in transport activity independent of transcription and translation. Recent experiments in rat using an in vivo protease K protection assay in which the protease was infused into the brain's vasculature examined this possibility for signaling initiated by VEGF and by PKCβ1 [12]. In control experiments, protease K infusion reduced levels of luminal plasma membrane proteins (western blots of P-glycoprotein, MRP2), but did not alter levels of intracellular proteins (β-actin) or levels of proteins localized to the abluminal plasma membrane (Na, K-ATPase) [12]. VEGF caused reduced proteolysis of P-glycoprotein, but not of MRP2 (transport activity of MRP2 is not affected by VEGF) [12]. This result indicates that VEGF signaling drives the transporter away from the luminal membrane surface, perhaps to a vesicular compartment where it cannot contribute to efflux transport at the luminal plasma membrane.

In contrast, PKCβ1 activation resulted in no detectable protection of P-glycoprotein from luminal protease, indicating no movement of the transporter away from the luminal membrane surface. How transport activity is lost as a consequence of TNF-α PKCβ1/S1PR1 signaling remains unknown. Loss of activity could be the result of covalent modification of the transport protein, perhaps through phosphorylation–dephosphorylation, redox reactions or cross-linking at cysteines, or changes in membrane microenvironment, e.g., non-covalent associations with other proteins or membrane phospholipids and altered local ion activities. Both caveolae and lipid rafts have been implicated in regulation of P-glycoprotein in brain endothelial cells [69, 70, 75] and of BCRP in tumor cells [76]. Recent experiments

using an animal model of peripheral inflammatory pain show complex changes in membrane protein biochemistry that accompanies altered P-glycoprotein activity [77]. McCaffrey et al. showed that the blood–brain barrier responds to localized, peripheral inflammatory pain (λ-carrageenan model in rats) by increasing P-glycoprotein transport activity likely through protein–protein interactions, i.e., a concerted redistribution of P-glycoprotein and caveolin-1, involving disassembly of high-molecular-weight P-glycoprotein-containing structures [77].

4 Perspectives

Delivery of small-molecule drugs designed to access CNS targets remains a problem in the clinic. Blood–brain barrier ABC transporters contribute substantially to the problem. Recent progress in understanding the regulation of these transporters provides good news and bad news. The good news is that the basal activities of P-glycoprotein and BCRP appear to be regulated and the signaling pathways responsible contain multiple elements that could be manipulated with drugs already in use in the clinic [18]. If that could be done [56], efflux transport through those transporters could be rapidly and reversibly reduced. This would provide a window in time when drugs that are transporter substrates could enter the CNS unimpeded. Note that for certain drugs that are handled by P-glycoprotein and BCRP, the benefit of reducing the activity of both transporters would be even more substantial [52].

The bad news is threefold. First, studies that identified pathways that signal reduced transporter activity have not yet provided a strategy which is immediately translatable to the clinic [56]. Second, little is known about the extent to which drug-metabolizing enzymes in the blood–brain barrier to present an additional obstacle to the delivery of biologically active drugs to the CNS. It is clear that the capillary endothelium expresses a number of Phase 1 and Phase 2 enzymes and that enzyme expression can be induced through xenobiotic-activated nuclear receptors, e.g., PXR and CAR. However, the effect of these enzymes on drug PK and how well they are coupled to efflux transporters remain to be determined. Third, the list of stressors that upregulate ABC transporter expression at the blood–brain barrier is growing. Based on available data from animal models, it includes inflammation, therapeutic drugs, dietary constituents, environmental pollutants, oxidative/electrophilic stress, and seizures (Fig. 2). Given the breadth of the list, it is hard to believe that a substantial portion of the human population is not already induced and thus drug resistant. Whether transporter expression can be reduced, for example, through a modified diet, remains to be seen.

Finally, another CNS barrier, the blood–spinal cord barrier, resides within the spinal cord capillary endothelium. Like the blood–brain barrier, the blood–spinal cord barrier is a very tight endothelium and thus an obstacle to the diffusive movement of solutes between blood and spinal cord. Recent studies with rats and mice show that this tissue expresses P-glycoprotein, MRP2, and BCRP and that the

regulation of transporter expression and activity closely parallels the patterns found for the blood–brain barrier [62, 78]. That is, transporter expression increases when spinal cord capillaries are exposed to PXR, CAR, and AhR ligands and P-glycoprotein activity decreases in response to sphingolipid signaling. Moreover, expression of P-glycoprotein and BCRP in spinal cord capillaries is increased in a mouse model of ALS [79]. Increased P-glycoprotein expression is also seen in spinal cord samples from ALS patients, suggesting reduced ability to deliver drugs to the spinal cord. The similarities between the blood–brain and blood–spinal cord barriers are striking. Whether a full characterization of the later will reveal any major differences remains to be seen.

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Nanoparticles as Blood–Brain Barrier Permeable CNS Targeted Drug Delivery Systems

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Abstract Research in the field of nano-neuroscience is becoming a promising future direction given the advantages presented by nanosystems for central nervous system (CNS) drug delivery. Since the blood–brain barrier (BBB) represents an invincible obstacle for the majority of drugs such as antineoplastic agents and a variety of psychoactive drugs such as neuropeptides, "smart" CNS drug delivery systems with high ability to deliver substances across the BBB are highly desired and will not only enable drugs to reach the CNS but also target specific areas of the CNS. Thus, injectable biodegradable nanoparticles have an important potential application in the treatment of a variety of neurological and psychiatric disorders. Therefore, in the following, we will highlight the requirement and importance of CNS drug delivery systems with particular emphasis on nano-scale systems. It is the objective of this article to offer a perspective on the complexity and challenges in fabrication of nanostructures, *in vivo* nano–bio interactions and also to highlight some of the most used nanosystems for drug delivery into the CNS.

Keywords Blood–brain barrier, Central nervous system, Nanomedicine, Nanoparticles

Contents

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1 Introduction

The human brain is a plastic organ constantly shaped by developmental processes and life's experiences resulting in changes of the biochemical structure at the molecular and cellular level, thereby affecting information processing and flow. However, the brain is susceptible to a multitude of disorders that may manifest at every stage of life. Developmental brain disorders such as autism spectrum disorders are apparent from birth on. In contrast, psychiatric diseases that also may have underlying genetic defects, such as schizophrenia, depression, and obsessive-compulsive disorder, typically have their onset in early adulthood. Neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, or amyotrophic lateral sclerosis manifest with advancing age. Despite being among the most serious health problems in our society causing a high economic burden, the aforementioned pathologic conditions affecting the brain are amongst the most prevalent untreatable syndromes.

However, the last few decades have witnessed an unprecedented advance in the development of pharmaceuticals with excellent potential for the treatment of brain disorders. Unfortunately, a great number of pharmacologically active molecules are not able to exert their activity *in vivo* owing to the CNS barriers [1] namely, blood–brain barrier (BBB), blood-cerebrospinal fluid barrier (BCB), CSF–brain barrier, and some specialized barriers such as blood–tumor barrier (BTB) (in case of brain tumor).

1.1 Blood–Brain Barrier

The BBB separates blood from the brain extracellular fluid (Fig. 1). The transmissivity of the BBB is limited through the presence of tight junctions (*zonula occludens*) between epithelial cells of the blood capillaries in vertebrate brain and spinal cord. This so-called "tight epithelium" restricts the passage of substances from blood to the brain. Additionally, pericytes and glial cells encapsulate the

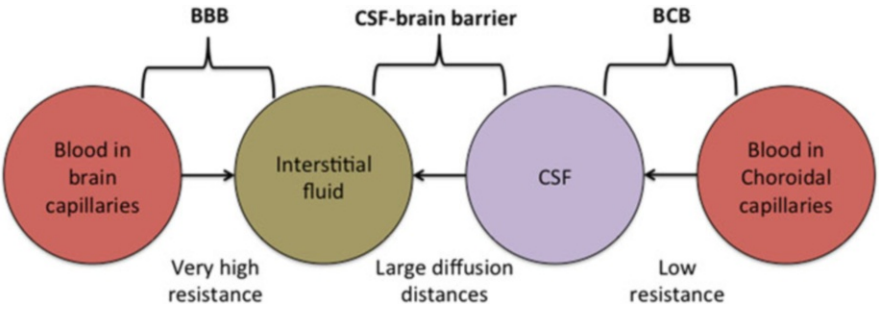

Fig. 1 Illustration of barriers between different compartments of the brain

surface of the capillaries, thereby producing an electrical resistance of 1,500–2,000 Ωcm^2 much higher than that of the other systemic endothelia (3–33 Ωcm^2) [2]. Brain capillaries do not possess an intercellular cleft, fenestrae and mechanisms of pinocytosis. Therefore, the diffusion of lipid insoluble and large hydrophilic molecules into the brain is hindered and metabolic products need to be exchanged trans-cellularly by active transport across the BBB with specific proteins [3]. Only lipid-soluble molecules that can freely diffuse through the capillary endothelial membrane may passively cross the BBB, which is practically inaccessible for lipid-insoluble compounds such as polar molecules and small ions. However, lipophilicity alone does not determine the membrane permeability of a molecule. For instance, lipophilic tranquilizers such as benzodiazepines rapidly cross the blood–brain barrier [4] in contrast to other lipophilic molecules such as cyclosporin A (immunosuppressant) [5] and vinca alkaloids (anticancer) [6]. Brain uptake of a substance depends on various factors such as affinity of a substrate for specific transport system and molecular weight (discussed in Sect. 1.3). The absolute cutoff for significant BBB passage regardless of lipophilicity for molecules is 400 Da [3]. Moreover, solutes crossing the cell membrane are exposed to degrading enzymes present in large numbers inside the endothelial cells. These enzymes recognize and rapidly degrade most peptides, including naturally occurring neuropeptides [3]. In addition, the brain capillary endothelial cells possess a high concentration of drug efflux transporter proteins such as P-glycoprotein (Pgp) [7], multidrug resistance-associated proteins (MRPs) [8], and breast cancer resistance protein (BCRP) [9], which limits penetration of a variety of therapeutic agents (including compounds that are relatively lipophilic) into the brain parenchyma.

1.2 Blood–Cerebrospinal Fluid Barrier (BCB) and CSF–Brain Barrier

Some regions of the CNS located adjacent to the ventricles of the brain, the so-called circumventricular organs (CVOs), do not show a BBB. These regions

consist of choroid plexus, the median eminence, pineal gland, neurohypophysis, organum vasculosum of the lamina terminalis, subfornical organs, the area postrema, and the subcommisural organ. Unlike the capillaries that form the BBB, the blood capillaries in CVOs are fenestrated and lack tight junctions. Although the capillary endothelium is permeable to solutes, the epithelial cells of the choroid plexus (and tanycytes of other CVOs) have tight junctions between them to restrict permeability of solutes from blood to CSF, thus forming the BCB (Fig. 1). However, the choroidal epithelial cells offer low resistance (150–200 Ωcm^2) in comparison with capillary endothelial cells that form the BBB [10]. As a result, various substances are able to move from the blood into the CSF in a molecular weight-dependent manner and irrespective of their movement across the BBB. For example, azidothymidine (AZT), an antiretroviral drug used for the treatment of HIV/AIDS, rapidly enters CSF across the choroid plexus epithelium but cannot easily cross the BBB [11, 12].

For the development of effective drug delivery systems, it is crucial to understand that the presence of a drug in the CSF compartment does not guarantee its penetration into the brain parenchyma. Until quite recently there was a prevailing misconception that the trans-cranial drug delivery to the CSF can overcome the obstacle caused by the BBB in delivering drugs to the brain. Unlike at the BBB, where a solute once having crossed the capillary barrier undergoes a rapid distribution throughout the brain parenchyma, penetration of solutes from CSF to brain parenchyma is achieved through diffusion, a process that decreases exponentially with distance. For example, the maximum penetration of brain-derived neurotrophic factor (BDNF) is just 0.3 mm from the ependymal surface of the brain [13]. This barrier caused by the large diffusion distances is referred to as the CSF–brain barrier (Fig. 1). Additionally, the CSF flow rate influences uptake. The estimated volume of CSF is 140 mL in the human brain [14] and 90 μL in a rat brain [15]. In a healthy adult, the CSF is replaced completely 4–5 times a day. CSF produced by the choroid plexus passes from the lateral ventricles to the third and subsequently into the fourth ventricle. From there, the CSF passes from the foramina of Luschka and Magendie to the cisterna magna and then into the cranial and spinal subarachnoid spaces. Finally it is absorbed into the bloodstream across the arachnoid villi. Thus, drugs injected into the CSF are rapidly removed via bulk flow through the CSF flow track owing to the high turnover rate of CSF.

1.3 CNS Drug Delivery Strategies

To circumvent the CNS barriers that hamper penetration of potentially efficacious drug molecules into the CNS, various strategies have been developed (Fig. 2). These include the disruption of blood–brain barrier by injectable solvents and metals, by inducing conditions such as hypertension and ischemia, by administration of convulsive drugs, e.g., metrazol [16] with simultaneous delivery of anticonvulsant agents, and by using neoplastic agents such as cisplatin [3]. The osmotic

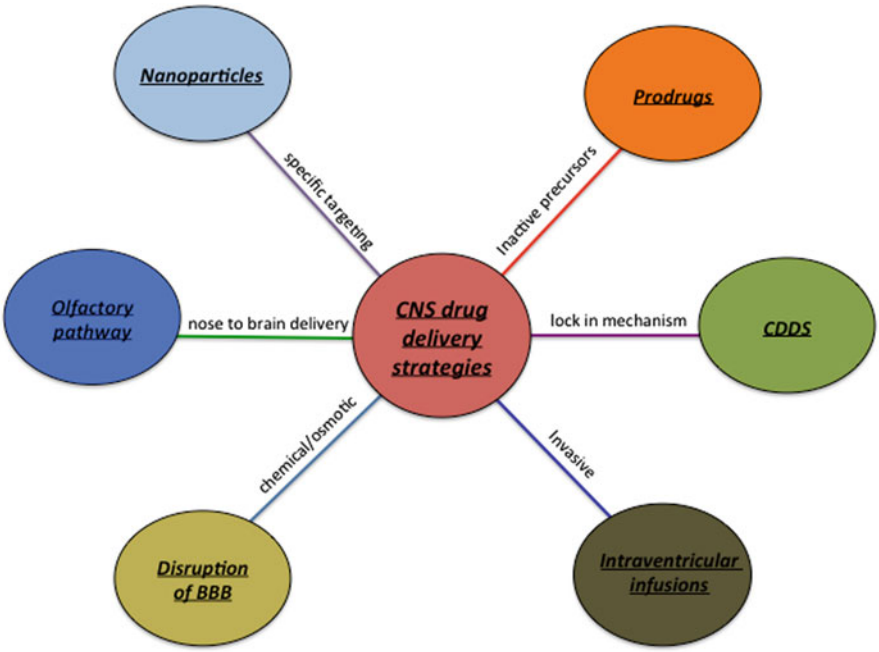

Fig. 2 Various strategies for drug delivery to the CNS

disruption of BBB has been used in the treatment of gliomas [17]. In this technique, endothelial cells of the blood capillaries are forced to shrink leading to an opening of the tight junctions for few hours. Another approach used to treat brain tumors is the selective opening of BTB by intracarotid administration of leukotriene C4 [18]. However, all the aforementioned strategies have detrimental effects on CNS homeostasis due to the destruction of the brain's protective mechanisms and thus predisposing it to life-threatening infections.

An alternative approach for delivery of substances across the BBB is to chemically modify the drugs to facilitate membrane permeability. One of the strategies exploits the lipophilic characteristic of a substance that influences its brain penetration. Brain permeability of otherwise non-permeable drugs can be increased by the use of lipophilic precursors of the drug or by coupling drugs to a sphere of lipids (discussed in Sect. 2.3). However, these techniques have not lived up to their theoretical potential as lipophilicity increases not only the brain influx of a substance but also it's efflux from the brain parenchyma (for details see Sect. 1.4). Another drug modifying strategy makes use of prodrugs and chemical drug delivery systems (CDDS). These are inactive molecules of the parent drug, which can relatively easily cross the BBB, and inside the brain parenchyma become metabolized to the active parent drug. The prime difference between prodrugs and CDDS is that CDDS undergo multi-step transformation to get into the active

form whereas prodrugs are converted to the active form in just one metabolic step. Moreover, CDDS make use of the "lock-in" mechanism to sustain the drug within the brain. For the lock-in mechanism a drug is attached to a moiety serving particularly as a lipophilizer, which in turn increases its brain uptake and within the brain gets converted into a lipid insoluble compound. Thus, it is "locked-in" behind the BBB [19, 20].

Drugs can also be manipulated to exploit endogenous pathways for their delivery across the BBB, such as carrier-mediated drug delivery and receptor-mediated drug delivery (discussed in Sect. 1.5). Other alternative routes bypass systemic circulation altogether. For example, using olfactory pathways, drugs can be delivered directly from nose to brain [21]. However, this approach is limited by factors such as low pH of the nasal epithelium and inflammation of nasal mucosal lining. Another strategy is the intraventricular infusion of substances achieved through implantation of pumps such as Ommaya reservoir but it has grave limitations [22]. For instance, in addition to being invasive, it lacks efficiency as shown by low intracranial concentrations of the administered drugs. Delivery of drugs by this method is limited by the blood–CSF barrier and is useful only in conditions where the drug is needed in the nearby regions of the site of injection, e.g., carcinomatous meningitis [23]. Thus one of the major challenges of today's pharmaceutical research is to discover attractive strategies for an effective delivery of drugs to the desired site of action. To achieve this, an emerging promising approach is the use of nanosized carriers as drug delivery platforms.

1.4 Nanomedicine Exploitation

Nanocarriers such as liposomes and polymeric nanoparticles are able to protect loaded drugs from being metabolized and assure a timed and quantitatively controlled release of the embedded substances.

In general, nanoparticles (polymer based, lipid based or with solid–lipid interface) display several advantages with regard to

1. the feasibility of the formulation
2. the possibility to encapsulate different types of molecules (ranging from high to low molecular weight, hydrophilic, lipophilic and genetic material)
3. the possibility to modify biodistribution and metabolism profiles of the loaded molecules
4. the preferential targeting of otherwise inaccessible organs such as brain
5. the selective targeting of diseased cells within the intended site of action and the targeting of subcellular organelles for treatment of organelle-specific diseases
6. the broad spectrum of applications ranging from the treatment of life-threatening diseases such as cancer, cardiovascular diseases, and neurological disorders to improved vaccination, gene therapies, and development of more effective imaging agents (Fig. 3).

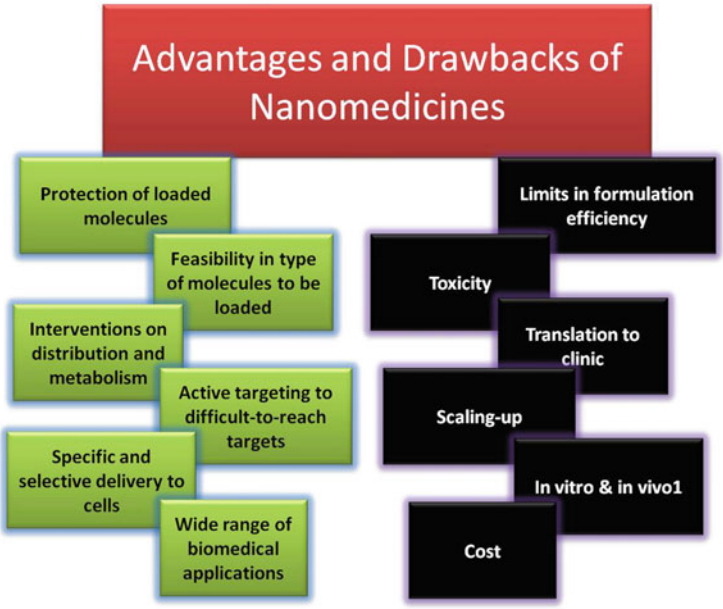

Fig. 3 Advantages and drawback in nanomedicines

On the other hand, nanomedical strategies for drug delivery have several drawbacks that must be acknowledged considering the future development of nanomedicines (Fig. 3).

In this view, some limitations and aspects that need to be investigated are evident. For example,

1. the formulation efficiency (the encapsulation efficiency rate which could vary depending on the chemico-physical properties of the loaded drug)
2. the detailed understanding of toxicity profiles of the formulated nanomedicine (as the nanocarrier could behave like a new entity when coupled with the loaded drug)
3. the possible difficulty in translational process from preclinical to clinical applications (primarily related to toxicity/safety profiles)
4. the possible limitations in scaling up the formulation processes, from small academic research laboratory to large-scale production, mainly due to the use of organic solvents needed for the production of nanocarriers
5. the drawbacks in translating *in vitro* results to *in vivo* results, which is an obvious criticism for all pharmaceuticals but in some cases, this could be exacerbated, for example, in the use of nanomedicines for treating neurodegenerative disorders
6. the high cost of production and scaling up (Fig. 4)

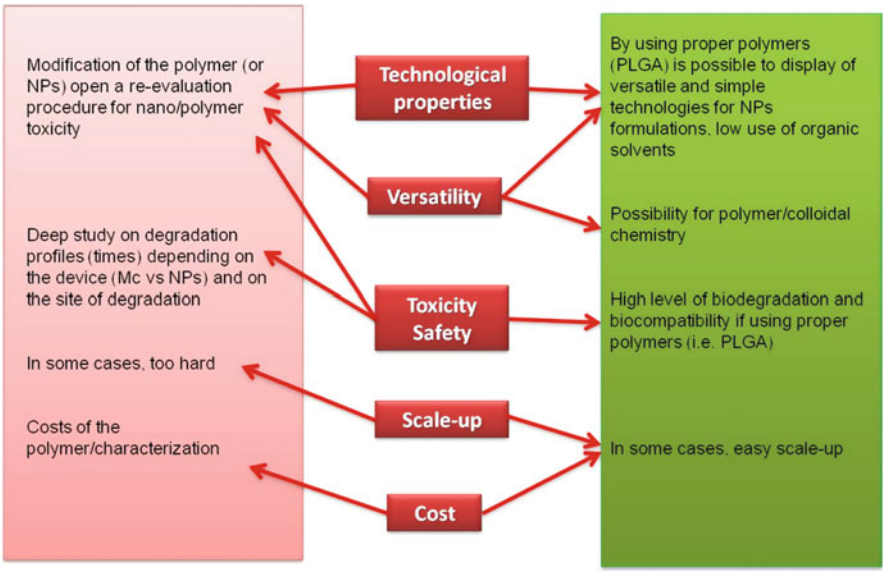

Fig. 4 Balancing positive and negative aspects in nanocarriers production and development

To design efficient drug delivery system with expected therapeutic effects, deep understanding of the physicochemical properties of the drug and carrier (separately and when coupled) is imperative. Various modifications used to increase the brain uptake of a drug might not yield the expected pharmaceutical effect due to the impact of modification on some other neglected parameters. For instance, lipid solubility of a substance is positively correlated with brain uptake. However, increased lipophilicity can also result in a number of negative effects such as decreased solubility in plasma and relatively more binding with plasma proteins, ultimately resulting in low bioavailability of the administered drug [3]. To minimize impediments in clinical trials, physiological determinants that might affect drug carrier performance *in vivo* should be taken into consideration from the initial developmental stages of the drug. These include nano–bio interactions, nonspecific toxicities and physiological differences between human beings and commonly used rodent models.

Drug carriers can be administered into the body through various routes such as pulmonary, oral, and most often by intravenous injections. From the site of injection, drug carriers enter the systemic circulation. Blood, being the fluid connective tissue, acts as the transporter of drug carriers to different organs. It should be taken into account that the human body perceives most of the carriers as foreign particles and will generate immune response towards them and will also try to rapidly clear them from the system. Within the blood, drug carriers can interact with leukocytes and neutrophil extracellular traps (NET) have been shown to clear extracellular nanoparticles *in vitro* [24]. In blood, carriers can also interact with blood plasma proteins such as albumin. Interaction of drug carriers with albumin reduces their

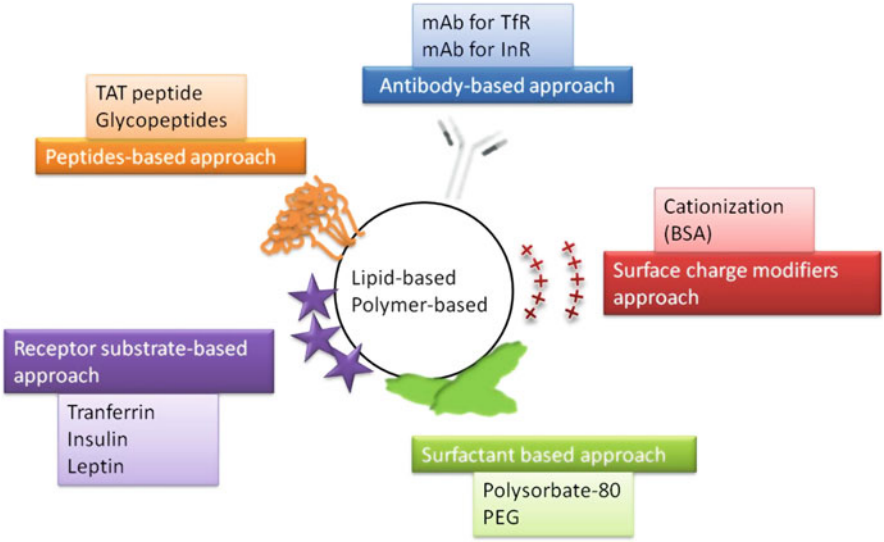

Fig. 5 Nanocarriers for brain targeting: schematic representation of the strategies for blood–brain barrier crossing

renal clearance [25]. In contrast, interaction with proteins of the complement system results in phagocytosis (depending on surface charge of the carriers) [26]. Various physical properties of the carrier can also influence its fate *in vivo*, for example; size and shape of the carrier impacts the glomerular filtration, renal clearance, and speed of internalization [27]. In addition, a plethora of other factors determines the drug profile in a biological system. These include rate of diffusion from blood to the brain, interaction between drugs and receptors in the brain, rate of transport from brain to blood, amount of blood flow to the targeted organ and potential of the drug to form hydrogen bonds [3].

1.5 Nanomedicines for BBB Crossing: General Considerations

Research in the field of nanotechnology offers a promising future for targeted drug delivery by introducing tools such as nanoparticles (NPs), capable of directed delivery of drugs into the brain. However, nanocarriers need to be modified on their surface with suitable ligands to ensure targeting to a specific tissue or a specific organ, such as the CNS (Fig. 5). Indeed, several studies show that these nanocarriers with proper ligand are able to cross the BBB without apparent damage [28] and can be used to deliver drugs or genetic material into the brain [29]. The mode of transport of NPs across the BBB has been hypothesized to be mediated by passive diffusion and/or receptor-mediated endocytosis [30], fluid phase endocytosis or phagocytosis, carrier-mediated transport or by absorptive-mediated transcytosis.

Passive diffusion can be facilitated through the enhancement of a drug's plasma concentration, resulting in a larger gradient at the BBB and thus an increase in the amount of drug entering the CNS. Moreover, degradation products of NPs could have pro-adsorption properties [31], thereby adding to an increased passive diffusion.

Receptor-mediated endocytosis is a common strategy for NP targeting to the brain, which relies on the interaction of the NP surface ligand with a specific receptor in the BBB. Examples for suitable ligands include transferrin, transferrin receptor binding antibody, lactoferrin, melanotransferrin, folic acid, and a-mannose for NPs undergoing receptor-mediated transcytosis [32–39]. The hypothesized steps in BBB crossing pathways consist of interaction of engineered NPs with the selected receptor, creation of endocytotic vesicles, transcytosis across the BBB endothelial cells, and subsequent exocytosis of NPs. Thus, possible limitations in this kind of receptor-based approaches are based on extremely high and strong linkage between the receptor and the ligand attached onto the NP surface, creating tight bonding resulting in low exocytosis rate. This limit is confirmed by a number of publications [40] highlighting a much higher percentage of NPs inside capillary endothelial cells compared to NPs inside the CNS parenchyma. Another possible limitation of receptor-mediated endocytosis is a possible saturation mechanism due to the binding of the endogenous ligand to the receptor, hampering the efficiency of receptor-mediated endocytosis. Moreover, besides playing a role in NP uptake, surface engineering can target different cell compartments [41–44]. Given that the vascular density in the brain is very high, once NPs have crossed the BBB, they will spread rapidly throughout the brain. Various techniques can be used to engineer the surface of nanocarriers, such as covalent linkage of molecules (ligands) to nanosystems (polymers or lipids) [45]. However, the ligand-based approach faces the difficulty that a molecule able to exploit endogenous targeting mechanisms, i.e. endocytosis-mediated pathways present at the BBB level [4, 46] has to be identified as ligand beforehand. Thus, under a multitude of possibilities (peptides, proteins, specific antibodies, etc.), choosing the most suitable ligand is one of the most important steps in designing efficient nanocarriers.

Besides this, some challenges in the development of nanoparticulate systems have arisen. Targeting of NPs to the brain is hampered by the failure of NPs to reach the CNS in sufficient quantity due to their uptake by the reticulo-endothelial system (RES), also known as the mononuclear phagocytic system. The RES is comprised of a group of mononuclear cells with increased localization in the liver, spleen and bone marrow, responsible for a rapid clearance of small foreign particles from blood circulation [47]. However, stabilizers such as ionic and nonionic molecules like polyethylene glycol polymers, lecithins, polysorbates, poloxamers, derivatized fatty acids, and their combinations can be employed [48]. That way, NPs have been deployed successfully in the past in several studies showing their high value for targeted CNS drug delivery [49]. For example, NP-mediated delivery of doxorubicin in a rodent model of glioblastoma revealed significant remission with minimal toxicity [50, 51]. Furthermore, 3H-Dalargin conjugated to NPs and injected systemically into mice showed accumulation in the CNS [52, 53]. Moreover,

a tyrosine hydroxylase (TH) expression plasmid was delivered to the striatum of adult rats using NPs in a model of Parkinson's disease and the expression of TH was verified [54]. Chelators carrying nanoparticles have been shown to cross the BBB in fixed AD brains by preferential adsorption of apolipoprotein E [55].

However, these examples open the discussion about the physiological state of the BBB with respect to BBB crossing and targeting. It is important to remember that the BBB is strongly influenced by a healthy or diseased brain. In the case of a healthy and intact BBB (found in animal models and in patients with Parkinson's disease or epilepsy), the permeability of the BBB remains the same as in the healthy state, so the BBB crossing is considered as a real challenge. On the contrary, in some kind of pathologies, e.g. Alzheimer's disease, multiple sclerosis, various infectious diseases, the BBB demonstrated an increased permeability due to an increase in BBB crossing pathways such as overexpression of receptors or increased pinocytic processes. In such cases, BBB crossing by means of nanocarriers should be fine-tuned in order to take advantage of these modifications in membrane permeability [56].

For example, in the case of glioblastoma, the BBB state is strongly dependent on the grade of tumor formation. Unfortunately, the evaluation and the diagnosis of glioblastoma normally take place at very high grades (3–4th) with an almost succumbed BBB integrity. In this case, NPs can easily cross the BBB and engineering of the NP surface should be aimed to directly target the tumor rather than to facilitate BBB crossing.

2 BBB Crossing Nanocarriers

NPs that can act as drug carriers are defined as submicroscopic colloidal systems such as nanospheres (matrix system in which the drug is dispersed) or nanocapsules (reservoirs in which the drug is confined surrounded by a single polymeric membrane) [57–60]. Polymeric BBB crossing nanocarriers are ideally composed of a natural or synthetic polymer, which is inexpensive, biodegradable, biocompatible and thus nontoxic. Moreover, NPs have to be nonthrombogenic, nonimmunogenic, noninflammatory, and stable in blood to ensure a prolonged circulation time. To date, there are a number of NPs that meet these criteria, however sometimes with limitations.

*2.1 Poly(*n*-butylcyanoacrylate) (PBCA) NPs*

PBCA polymers have been often combined with the nonionic surfactant polysorbate-80 coating and have been proven useful for the delivery of a variety of small polar drugs into the CNS in multiple studies [61–66]. For example, doxorubicin, loperamide, tubocurarine, and dalargin were adsorbed onto PBCA

NPs and successfully targeted to the CNS, where they induced a pharmacological effect [67].

PBCA NPs do not induce a nonspecific disruption of the BBB. Instead they are taken up by endocytotic mechanisms triggered by apolipoprotein E, reported to adsorb on polysorbate-20,-40, -60, or -80-coated NPs thus being subject to similar endocytotic processes that low-density lipoproteins undergo [42]. However, an alternative to the brain uptake of PBCA NPs was proposed, where NPs induce a nonspecific BBB permeabilization [68]. Interestingly, polysorbate-80 has been shown to be effective in minimizing uptake by the RES [69], resulting in increased systemic circulation of the drug.

PBCA NPs have also been reported to be able to deliver BBB-impermeable fluorophores of a wide range of sizes: from 500-Da targeted polar molecules to 150,000-Da tagged immunoglobulins into the brain of living mice [70]. However, PBCA NPs have a limited potential for clinical applications. High doses of PBCA NPs with polysorbate-80 may lead to a damage of the BBB. Additionally, only short pharmacological effects were observed after administration of drugs delivered by PBCA NPs and the use of these carries in clinical applications would need frequent administrations [71].

2.2 *Methoxypoly(ethylene glycol)-polylactide or Poly(lactide-co-glycolide) (mPEG-PLA/PLGA) NPs*

Polymeric NPs made of natural or synthetic polymers such as polylactide-co-glycolide (PLGA) or polylactide (PLA) polymers are nanosized carriers (1–1,000 nm), with the capability of drug encapsulation. Given that PLGA and PLA polymers have good CNS biocompatibility [72, 73], are FDA-approved and are widely used in the manufacturing of sutures, fixation nails and screws, the use of polymeric NPs is one of the most promising approaches for CNS drug delivery [4, 74, 75]. Polymeric NPs possess various advantages over other drug delivery systems, such as high drug-loading capacity [75] and protection of the embedded drugs against chemical or enzymatic degradation, thus increasing chances for the active molecule to reach the CNS. Release of drugs occurs through degradation of PLA or PLGA by autocatalytic cleavage of the ester bonds through spontaneous hydrolysis into oligomers and D,L-lactic and glycolic acid monomers [76]. PLGA-NPs conjugated to five short synthetic peptides, thus mimicking the synthetic opioid peptide MMP-2200 [77] and, Lectin–PEG–PLA NPs were reported to be equally effective in CNS targeting.

The surface properties of polymeric NPs can be modified to improve RES escape, to actively target a tissue, or to increase their ability to cross BBB by means of specific mechanisms, such as adsorptive-mediated transcytosis or receptor-mediated transcytosis. Targeting receptors at the luminal side of the BBB, such as transferrin receptors or insulin receptors can be suitable for the delivery of drugs across the BBB. Unfortunately, the amount of nanocarriers that

can be transported into the brain using uptake mechanisms mediated by these receptors is very limited [40], which makes this approach useful only for drugs which require a very low therapeutic dose. However, recently, a novel approach to BBB crossing using a simil-opioid peptide as ligand was reported [78–81]. In particular, PLGA NPs modified with a simil-opioid peptide (g7) were found to be able to cross the BBB and above all, act as drug carriers [81]. Computational analysis showed a Biousian conformation of the g7 peptide, suggesting its pivotal role in the mechanism of BBB crossing [45]. The biodistribution of these modified NPs that are able to cross the BBB via multiple-pathways such as membrane–-membrane interaction and macropinocytosis-like mechanisms shows localization into the CNS that is about two orders of magnitude greater than that found with the other known NP-drug carriers [79, 80]. Alternatively, the modification of PLGA NPs using the sequence 12–32 (g21) of leptin results in NPs being able to cross the BBB and to enter the brain parenchyma after intravenous administration [82].

2.3 Liposomes

Given that hydrophilic substances are unable to cross the BBB, one strategy for increasing the lipophilicity is to surround the hydrophilic drug with a sphere of lipids to generate a liposome.

Liposomes have been successfully used for CNS targeting. However, the delivery of liposomes across the BBB is only possible after engineering the liposomal surface so that the liposomes can bypass the RES. Thus, liposomes, even small unilamellar vesicles, do not undergo significant transport through the BBB without further modifications [83]. One solution to this problem is presented by incorporation of gangliosides, i.e. monosialoganglioside, polyethylene glycol (PEG) that prolong the half-life of liposomes in the blood, or chimeric peptide technology. In particular, a bi-functional PEG2000 derivative that contains a maleimide at one end (for attachment to a thiolated antibody) and a distearoylphosphatidylethanolamine moiety at the other end was used. These pegylated immunoliposomes can access the CNS via receptor-based transcytosis, mediated by an antibody such as OX26 that binds to the transferrin receptor, and deliver their content into the brain without damaging the BBB [3, 29, 83–86]. Antibody-directed liposomes have been previously used for delivery of the antineoplastic agent daunomycin to rat brain [83]. Alternatively, modification of liposomes with the RGD peptide (Arg-Gly-Asp) shows a three-fold increase in drug concentration within the CNS compared to uncoated liposomes. The RGD peptide combines with integrin receptors and the liposomes are taken into the CNS in response to an inflammatory recruitment [87].

Recently, solid lipid nanoparticles (SLNPs) have been shown to enable CNS drug delivery. SLNPs can be found in spherical conformation but also platelet-like arrangement with few lipid layers (two or three) forming a 10–18 nm thick structure. Brain targeting is achieved upon surface modification of SLNPs, i.e. using

PEG-derivatives or PEG-containing surfactants. Alternatively, surface charged SLNPs were proposed to achieve brain targeting. Positively (+5 mV) charged SLNPs showed a higher brain accumulation compared to both negatively charged SLNPs and the free labeled drug [88, 89].

2.4 Inorganic Nanosystems

Besides the active transport of liposomal and polymeric NPs across the BBB, inorganic nanoparticles have been demonstrated to possess the potential to deliver multiple agents across the BBB [90]. For example, quantum rods can be co-incorporated with molecules. By linking the iron-transporting protein transferrin with quantum rods it was found that the transferrin mediates BBB crossing since transferrin freely crosses the BBB as part of its function as carrier of essential nutrients into the CNS. Alternatively, NPs consisting of a magnetic metal ferrite core with surface coating of cross-linked serum albumin (SA) were reported to be delivered into the CNS via adsorptive-transcytosis of SA [91]. Furthermore, silica-based NPs (organically modified silica, ORMOSIL) conjugated with transferrin receptor conjugation were shown to penetrate into living brains, neuronal cell bodies, and axonal projections in *Drosophila* [92]. Moreover, mesoporous silica nanoparticles such as silica/titanium hollow nanoparticles can be loaded with drugs. For example, iron oxide nanoparticles embedded in magnetic mesoporous silica nanoparticles were shown to release anticancer drugs through the application of an external magnetic field to induce agitation of the drug loaded materials [93].

Similar to some inorganic nanosystems, incorporation of Fe_3O_4 into NPs might enable magnetic guidance of NPs using an external magnet. However, while the efficacy of drug delivery has been shown by this technique [94, 95], it may be impractical for use in clinical applications on human subjects.

Although the drug delivery capacities of inorganic NPs are very limited, they might act as drug themselves. Nanosized zinc oxide (nanoZnO) may have some interesting properties for CNS application, since nanoZnO was suggested to be beneficial on a behavioral level for mice and to act as a neuroprotectant in some ways [96]. Given that changes in zinc-ion levels are associated with a variety of brain disorders, such as depression [97], other possible mechanisms involved in the therapeutic effects of nanoZnO might lead to a beneficial outcome.

3 Conclusions

In Europe alone, about 35% of the total burden of all diseases is caused by brain disorders and approximately 2 billion people worldwide suffer from CNS disorders. This number is rapidly increasing because of demographically aging population. Therefore, the development of novel therapeutics is the prime focus of pharmacological research. However, more than 98% of the potential therapeutics

are unable to cross the BBB [1]. Thus, the major challenge for the treatment of CNS disorders is not only the production of a pharmaceutically active compound but also a way to deliver it to the brain.

Nano-neuroscience is a promising field of research for CNS drug delivery considering the advantages presented by these novel nanosystems, confirmed by *in vitro* and *in vivo* experiments. The progress in our understanding of the BBB has paved the way for several interesting novel approaches to improve CNS drug delivery. In particular, carrier or transport systems, enzymes and/or receptors that control the uptake of substances have been identified and used in drug delivery systems. With the help of these findings, injectable nanoparticulate drug carriers have been used to successfully cross the BBB and are shown to have important potential applications for the treatment of neurological disorders. By encapsulating drugs into novel modified nanocarriers, an improvement in therapeutic index of the drug could be achieved.

Although many examples of successful CNS drug delivery by the use of nanoparticles can be witnessed, this approach is still in its developing phase and further research needs to be done for successful clinical implementation of nanomedicines. This includes continued research for safe and effective targeting, resolving issues related to toxicity and bioavailability of the encapsulated drug, head-to-head comparison of available and emerging nanomaterials and elucidating the biological response to nanomaterials. Many of the future treatments of neurological disorders will require innovative strategies for the delivery of newly developed therapeutics and to improve the efficacy of already existing drugs. Thus, upcoming years will show unprecedented developments, in "smart" CNS drug delivery systems, with higher ability to deliver drugs across the BBB, not only to the CNS, but also to specific areas of the CNS and to subcellular organelles for the treatment of organelle-specific diseases.

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Blood–Brain Barrier and Stroke

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Abstract Stroke disintegrates communications within a highly dynamic and regulated ensemble of cells that constitutes the blood–brain barrier (BBB), endothelial cells, astrocytic end feet that surround blood vessels, the basement membrane (BM)/extracellular matrix (ECM), and pericytes, inducing and propagating injury. We discuss the effects of experimental stroke on individual cell constituents of the BBB and how these changes affect structural and functional integrity of the BBB in relation to acute injury and repair. The age at the time of stroke, from the newborn period to adulthood and older, can markedly affect the particulars of deregulation, processes that we also discuss in this chapter.

Keywords Extracellular matrix, Inflammation, Microglia, Middle cerebral artery occlusion, Neonatal stroke

Contents

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1 Introduction

The blood–brain barrier (BBB) protects the CNS microenvironment. It serves as a physical barrier, restricts paracellular transport of cells, proteins, and hydrophilic molecules, thus limiting random or uncontrolled entrance of molecules and cells from the blood, and confers high electrical resistance. The BBB also serves as a transport and nutritional barrier by regulating nutrient supply and removal of unwanted molecules through multiple specific transport systems, including glucose transporter 1 (GLUT1) and extruder transporters, such as *P*-glycoprotein. However, the BBB is not static and its integrity is ensured by a tightly controlled cell–cell communication between endothelial cells, astrocytic end feet that surround blood vessels, the basement membrane (BM)/extracellular matrix (ECM), and pericytes embedded in the BM between endothelial cells and astrocytes, a highly dynamic and regulated ensemble of cells often referred to as the "neurovascular unit." Stroke can disintegrate this highly regulated structure in many ways, enhancing injury and affecting the recovery. Reperfusion and reoxygenation of previously ischemic brain regions can further affect BBB function, restoring or disrupting, depending on a number of parameters, such as the length of initial CBF disruption (e.g., severity), and the presence of other confounding factors, such as infection. In this chapter we will discuss the effects of stroke on individual cell constituents of the BBB and how these changes affect structural and functional integrity of the BBB and injury progression. The age at the time of stroke, from the newborn period to adulthood and older, can markedly affect the particulars of deregulation, processes that we will also discuss in this chapter.

2 Injury to BBB Cell Components After Stroke

2.1 Endothelial Cells

Some of the characteristic features of brain endothelial cells that serve to restrict BBB permeability include the presence of structurally integrant adherens junctions (AJs) and tight junctions (TJs), which support high electrical resistance. A low rate of transcytosis and low expression of leukocyte adhesion molecules, which exist under physiological conditions, are drastically altered following ischemia and, further, after reperfusion. TJs are the major structural component that limits paracellular

diffusion between brain endothelial cells. The proper location and assembly of TJ proteins relies on multiple factors, including the balance between TJ protein synthesis and degradation, phosphorylation, and intracellular docking [1], all of which can be potentially altered by brain ischemia. Degradation of the transmembrane proteins occludin and claudin-5 and of an intracellular anchor protein, zonula occludens-1 (ZO-1), is increased after stroke in part due to activation and/or de novo synthesis of matrix metalloproteinases (MMP-9, MMP-2, and others) [2–4]. Integral TJ proteins can also internalize into the cytosol or redistribute to other membrane domains after cerebral ischemia. The latter scenario has been shown for claudin-5, which is internalized by caveolin-1-mediated endocytosis as soon as 2 h after middle cerebral artery occlusion (MCAO) in adult rats, coinciding with the early post-ischemic BBB opening phase. AJs support BBB properties [5], and changes in protein composition of AJs induced by stroke affect BBB permeability, either directly or indirectly, by altering TJ stability [6–8]. As an example, the degradation or phosphorylation of VE-cadherin following Src activation leads to removal of this protein from the plasma membrane.

Endothelial cells are sensitive to oxidative stress [9]. Reperfusion of ischemic brain tissue leads to reoxygenation of affected brain regions, which can spare endothelial cells and parenchymal cells if an ischemic episode is short, but can injure endothelial cells via a substantial oxidative burst when ischemia is prolonged. Excessive accumulation of reactive oxygen species (ROS) occurs during brain ischemia/reperfusion, which, together with the inability of the overwhelmed endogenous antioxidant mechanisms to metabolize ROS, contribute to damage TJs and other endothelial cell components and promote activation of cell death pathways [10].

The spatial–temporal scope of the response of endothelial cells to stroke is far from being fully understood. There is extensive literature on activation of various individual signaling pathways in these cells in response to cerebral ischemia. Although less sensitive to cerebral ischemia than neurons, endothelial cells can also undergo cell death, which occurs via several mechanisms, including apoptosis, necrosis, and necroptosis [10]. We recently compared gene expression in endothelial cells from injured and uninjured (contralateral) cortex after transient MCAO using endothelial transcriptome. With more than 31,000 probe sets used to determine endothelial gene expression and the chosen significance threshold of $>$2-fold change, the endothelial transcriptome data sets revealed significant upregulation of 877 probes and downregulation of 389 probe sets in injured regions 24 h after reperfusion (Fig. 1) [11]. The expression of several groups of genes directly related to the BBB function, including TJ components, adhesion molecules, extracellular matrix components, angiogenesis regulators, molecular transporters, and mediators of Wnt signaling, was significantly altered. Interestingly, gene expression of different collagens, laminins, and other structural barrier components, as well as MMP-9, was significantly upregulated. Several leukocyte adhesion molecules, including *P*-selectin, *E*-selectin, and ICAM-1, were also upregulated. Ischemia–reperfusion triggered a more than fivefold increase in VEGFR-2 and Angpt2 [11].

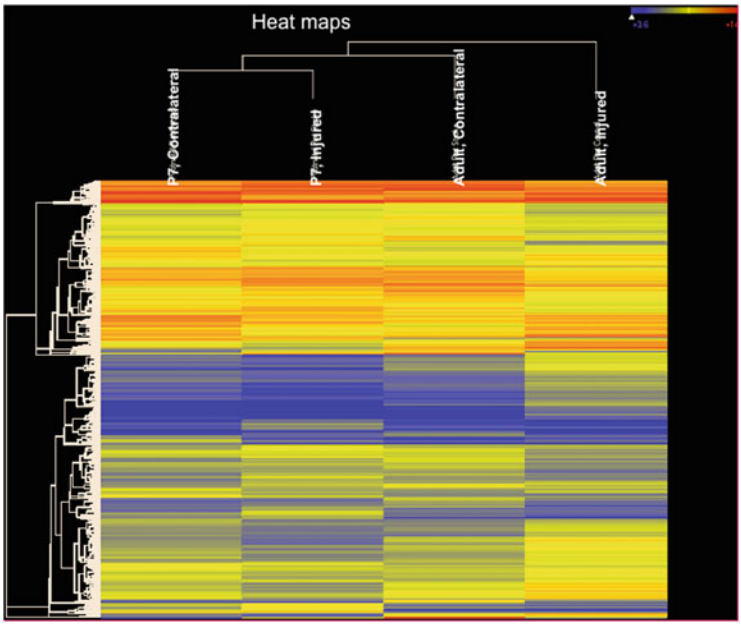

Fig. 1 Stroke induces rapid changes in gene expression in endothelial cells within injured regions. Heatmaps obtained in endothelial cells isolated from injured adult and neonatal brains 24 h after transient 3 h MCAO. Heatmap visualization demonstrates that the expression levels of endothelial genes are markedly changed in injured regions in each age group and that the pattern of changes is distinct following adult and neonatal stroke. The expression levels of genes are indexed by color

2.2 Pericytes

Pericytes have been shown to be important for the formation of the BBB [12, 13] and adequate maintenance of its function [12]. The responses of these cells to cerebral ischemia and their effects on the structure and permeability of the BBB are receiving increasing attention. Under normal conditions, pericytes are in close physical contact with endothelial cells, surrounding them with their cell bodies and processes. An intimate connection between pericytes and endothelial cells occurs in characteristic membrane domains known as *peg-and-socket junctions*, in which *N*-cadherin and connexin-43 are the two major components [14]. Pericytes may also signal to astrocytes, neurons, and possibly other pericytes. These communications are fundamental for the regulation of numerous endothelial BBB properties [15]. It is known that polarization of several BBB components (e.g., astrocyte end feet and TJs) during development relies on vessel coverage by pericytes and the presence of adequate endothelial–pericyte contacts [13]. Similarly, incomplete pericyte coverage of brain vasculature after birth causes BBB abnormalities and malfunction [12].

Ischemia-induced changes in the function of these cells lead to marked abnormalities in BBB structure and function after stroke [16]. Electron microscopy

studies have revealed that after stroke pericytes undergo detachment from the BM and migrate away from the vascular endothelium [17, 18], coinciding with increased endothelial transcytosis [18]. This phenomenon was also observed in human stroke [19]. Pericyte detachment from endothelial cells may be a consequence of disintegration of the ECM by MMPs (and other proteases) and changes in the distribution of integrins in the vascular abluminal surface [20]. Pericytes are themselves an important source of MMPs after brain ischemia [21] and can contribute to their own detachment. Contractile capacity has been described in a subset of pericytes containing high levels of alpha smooth muscle actin [22]. Pericyte contractility depends on the patterns of increases in cytosolic calcium, in part induced by ROS production following stroke, because the reduction of ROS concentration counteracts pericyte contractility and preserves CBF after reperfusion [23]. The concept of pericytes as regulators of CBF relies on their capacity to respond to both vasoconstrictors (e.g., catecholamines) and vasodilators (e.g., prostacyclin, NO, adenosine), which supports their potential role in the control of capillary diameter and brain perfusion [24], but the contribution of pericytes to modulation of CBF under both normal and ischemic conditions remains controversial. A loss of pericytes has recently been suggested to lead to a massive proliferation of resident platelet-derived growth factor receptor beta ($PDGFR\beta^{+}$) stromal cells and consequent effects on scar formation [19]. The role of pericytes in post-stroke angiogenesis has also been demonstrated [25]. Pericyte coverage of vessels formed de novo in response to stroke seems a necessary step for vascular maturation, blood flow restoration, and barriergenesis.

2.3 Astrocytes

Astrocyte end feet comprise the most external layer of the BBB from the circulation lumina. These specialized structures account for a particular polarization pattern of several proteins. Aquaporin-4 (AQP-4) and the potassium transporter Kir1.4 are selectively docked in astrocyte end feet and are organized in orthogonal arrays of particles (OAPs) [26]. Their localization depends on their association with cytoskeleton-associated proteins including α-syntrophin and β-dystroglycan [27, 28]. AQP-4 is a water channel that regulates water passage from and to the circulation across astrocyte membranes. Not surprisingly, AQP-4 is involved in the formation of brain edema in a number of injury types, including stroke [29]. Early after brain ischemia, water accumulates in the cytoplasm of brain cells due to energy failure of ATP-dependent ion osmotic pumps. AQP-4 mediates the trafficking of water into astrocytes, leading to astrocyte swelling and contributing to the generation of cytotoxic edema [30]. Furthermore, cell swelling leads to retraction of astrocyte end feet from the abluminal endothelial surface. The loss of contact of end feet with the parenchymal BM that surrounds capillaries causes further alterations in polarization and structure of the OAPs, leading to the relocation of AQP-4 in membrane domains more proximal to the astrocyte cell body [28]. The latter results in further deregulation of water trafficking

across the BBB and possibly contributes to the formation of vasogenic edema (or leakage of water into the brain parenchyma via disrupted BBB) later after stroke. Stroke studies in mice lacking AQP-4 or AQP-4 polarization to the end feet (mice lacking alpha syntrophin) [28, 31, 32] showed that, interestingly, both types of AQP-4 changes reduce cytotoxic edema during the initial stages of brain injury, but the absence of AQP-4 worsened vasogenic edema later on, suggesting that in spite of the altered localization of AQP-4 in astrocytes after stroke, this protein can still partially counteract water efflux from the blood into the brain.

Astrocytes are attached to the ECM via multiple matrix adhesion receptors. In the primate stroke model, the expression of integrins α1β1 and α6β4 and αβ-dystroglycan was shown to rapidly decrease in the microvasculature following focal ischemia [33]. Mice deficient in the expression of the ECM protein agrin in the brain recapitulate the effects of the lack of AQP-4 polarization on cytotoxic edema formation [34], suggesting that binding of β-dystroglycan to agrin defines AQP-4 localization in astrocyte end feet. Deletion of connexins 30 and 43 from astrocytes is also associated with decreased expression of αβ-dystroglycan in these cells and increased microvascular permeability in response to high vascular pressure [35].

Astrocytes also modulate BBB permeability by mechanisms independent of AQP-4. They are important sources of inflammatory mediators and VEGF, which are known to promote vascular permeability by different mechanisms. Cytokines and chemokines produced in astrocytes can damage astrocytes themselves and other BBB cells, while VEGF binds to receptors in endothelial cells and promotes proliferation, migration, and vascular permeability.

3 Basement Membrane, Extracellular Matrix, and Stroke

The BBB "phenotype" is induced by interactions of the endothelium with the surrounding cells, pericytes, and astrocytes. The proteins of the ECM and their corresponding receptors on endothelial cells and astrocytes provide both physical and biochemical "scaffolding" of the glial–vascular interface. The BM components hold endothelial cells and astrocytes in close proximity and contribute to the regulation of both the permeability and stability of the BBB. Furthermore, BM components laminin, collagen IV (Col-IV), fibronectin, and perlecan serve as signaling platforms within the BBB via providing proper cell–cell interaction, which occur via binding to and signal through integrin and dystroglycan receptors. As we discuss throughout this chapter, integrins play a central role in connecting/disconnecting various components of the neurovascular unit and in clustering and activation of growth factor receptors. Endothelial cells express several receptors for ECM/BM ligands, including a vitronectin receptor αvβ3, Col-IV receptor α1β1, and laminin receptors

α3β1 and α6β1 [36]. Proliferation and survival of endothelial cells also depend on the fibronectin-binding integrins α5β1 and αvβ3 [37]. Proper positioning of astrocyte end feet to the abluminal endothelial surface also occurs via the cross-linked network of the ECM components [38]. Endothelial–ECM interaction via β1 integrins regulates the expression of the TJ protein claudin-5 and interaction with β1 integrin-mediated adhesion modulates permeability. Other molecules of the ECM, like galectin-3, mediate integrin-induced stabilization of focal adhesions and regulate cell motility [39]. Galectin-3 can also directly associate with cytokine receptors to enhance actions of growth factors, including VEGF, mediate VEGF-dependent αvβ3 clustering, and activate integrin-dependent intracellular effectors, including FAK. Integrin αvβ3 is weakly expressed on resting endothelial cells but is strongly expressed on activated endothelial cells. It modulates membrane localization and activation of the pericyte protein NG2 chondroitin-sulfate proteoglycan, Col-IV, laminin, and the growth factor receptors EGFR, TGFβR, and IGFR1.

Col-IV is the major structural BM protein. Primary defects of vasculature assembly due to homozygous mutations in Col-IV are lethal mid-gestation due to blockage of capillary bed development. Mutations in the α1(Col-IV) gene cause intracerebral hemorrhage (ICH) in both mouse and human [40, 41]. Electron microscopy analysis of cerebral structure in mutant α1(Col-IV) mice showed that ICH is caused by weakened vascular BM and structural defects in the cerebral vasculature, including variable and uneven thickness and focal disruptions [40]. While the causative role of α1(Col-IV) mutation in ICH was established and inhibition of secretion of the mutant Col-IV in ICH was demonstrated [40], it remains unclear whether accumulation of unfolded proteins and impaired secretion of Col-IV heterotrimers or the thinning of the collagenous structure resultant from misfolding of α1/α1/α2 heterotrimers in the extracellular space accounts for vessel fragility and pathology. Mutations affecting triple-helix formation and thus the inability of Col-IV heterotrimers to polymerize into flexible sheets interfere with stability and disrupt dynamic biological processes through interactions with laminin, growth factors, and receptors on microglial cells and astrocytes, including integrins. Mutations of α2(Col-IV) and mutations of other BM proteins are generally associated with milder phenotypes [42].

There are multiple isoforms of laminin that make up the BM. In the BBB, endothelial cells generate laminin-411 and -511, whereas astrocytes produce laminin-111 and -211 isoforms. By binding to integrin and αβ-dystroglycan receptors, laminin is involved in cell survival, migration, differentiation, and attachment [43]. The lack of laminin in glial cells causes detachment of astrocytic end feet from the BBB unit, leading to disrupted BBB integrity and ICH, but exogenous laminin-111 can be incorporated into the BM and rescue the defects [44, 45]. Laminin is degraded following focal experimental stroke [20]. Its degradation can contribute to acute and chronic injury not only by weakening and distorting BM structure but also by disrupting signaling between individual components of the neurovascular unit [46].

The expression of MMPs in the adult brain is low under physiological conditions, but MMPs are upregulated after stroke. Several members of the MMP

family have been implicated in the pathophysiology of acute brain damage after stroke [47–50]. Individual MMPs have both distinct and common substrates and can disrupt the BBB by degrading the TJ and BM proteins, including collagen, laminin, and fibronectin, thereby leading to brain edema, BBB leakage, and leukocyte infiltration. MMP-2 and MMP-9, the two most studied MMPs in stroke, play different roles in BBB disruption. For example, genetic deletion of MMP-9 provides significant protection, whereas genetic deletion of MMP-2 does not provide protection in transient or permanent MCAO [51]. However, deletion of MMP-2, MMP-9, or both reduces hemorrhagic transformation after stroke [52]. Local activated microglial cells/macrophages and infiltrating leukocytes, neutrophils in particular, are the major sources of MMPs, especially early after injury [53, 54]. However, the types of cells producing MMPs change over time, with activated astrocytes and neurons producing MMPs at later injury stages [55]. As we discuss later in this chapter, MMP-9 is critical for brain repair; thus, its inhibition for lengthily periods of time would harm brain recovery. MMP-3 (stromelysin-1) does not cleave type I collagen but targets other ECM components, such as laminin and proteoglycans, and mediates BBB opening by inflammatory mediators [56]. Thus, MMP-3 is considered an important component in ICH [56].

Another ECM/cytokine/growth factor, TGF-β, also contributes to the BBB function after stroke. Administration of TGF-β into the brain reduces infarct size in experimental animal models of ischemia, while injection of a soluble TGF-β type II receptor to antagonize the endogenous actions of TGF-β significantly increases infarct area and reduces hemorrhagic transformation.

4 Systemic and Local Inflammation and BBB Permeability After Stroke

Neuroinflammation is a characteristic feature of stroke progression and is a major contributor to brain injury [57]. Parenchymal, perivascular, and peripheral circulating cells independently and in concert contribute to stroke-induced production of inflammatory mediators and neuroinflammation [57] and activate endothelial cells [58, 59]. Perivascular macrophages, microglial cells, and mast cells, which are strategically positioned around brain vessels, further contribute to BBB disruption by induction and release of signaling molecules and proteases that promote vascular permeability [60]. Finally, peripheral leukocytes that adhere to the endothelium also possess highly effective enzymatic machinery aimed to open infiltration routes across the BBB.

4.1 Chemokines, Adhesion Molecules, and Leukocyte Trafficking

Leukocyte entry into the CNS is restricted due to the BBB and the few leukocytes that are present in the CNS enter mostly through the CSF and subarachnoid space [61]. Leukocyte migration and homing is a multistep process dependent on the stepwise coordinated presence of a number of receptors and ligands on both circulating cells and the endothelium. Local inflammation leads to the upregulation and translocation of adhesion molecules in the luminal membrane of endothelial cells. Some of these molecules (e.g., *P*-selectin and Von Willebrand factor) accumulate in endothelial Weibel–Palade bodies under normal conditions and can be rapidly translocated and expressed in the plasma membrane before induction of gene expression [62]. Several cytokines induce *E*-selectin and *P*-selectin expression [63]. Selectins promote rolling and loose adhesion of leukocytes to endothelial cells, the first step necessary for the infiltration of these cells. The tight ligation and crawling of leukocytes to the endothelium are mediated by leukocyte integrins (β1 and β2), which bind to endothelial ICAM-1 and VCAM-1. Completion of transmigration depends on multiple processes, including PECAM-1, CD99, and JAM-A interactions [64], and on the presence of chemokine gradients in the parenchyma [64]. The multifaceted roles for β(CC), α(CXC), and δ(CXC3) classes of chemokines were shown in animal models of stroke [65] and other diseases with an inflammatory component [66, 67]. Substantial redundancy in ligand–receptor interaction often results in temporal and spatial patterns that intervene with ligand–receptor specificity, complicating the understanding of multicomponent pathophysiological responses.

Evidence of the role of neutrophils in ischemic damage is based on data showing that neutrophils are present in ischemic tissue early, prior to, or at the time of substantial neuronal death, that neutropenia is associated with reduced ischemic damage, and that treatments that prevent leukocyte vascular adhesion and extravasation into the brain parenchyma are neuroprotective [68–73]. Chemokine-induced neutrophil chemoattractant-1 (CINC-1, also known as KC in mouse and GROα in human) acts predominantly via a single receptor, CXCR2 in the rat [74] and CXCR1 in human [75, 76], and plays important and nonredundant roles in inflammation [77–79]. As a CXC chemokine, CINC-1 is a potent neutrophil chemoattractant in vitro and in vivo. CXCR2 is predominantly expressed in immune cells and, to a lesser extent, by various other cells, including glial cells [80], endothelial cells, and cortical neurons [81, 82]. Increased expression in the tissue initiates neutrophil recruitment, whereas high circulating CINC-1 can abort neutrophil recruitment due to CXCR2 desensitization [83]. CNS-specific overexpression of CINC-1/KC produces major BBB disruption [84], likely via the recruitment of neutrophils. Transient MCAO in adult rats triggers a transient increase of CINC-1 in the blood and the brain (3–6 h and 3–48 h, respectively) after reperfusion [65], at a time when the BBB is disrupted [54, 85]. Administration of a neutralizing CINC-1 antibody following transient MCAO in an adult stroke model aborts neutrophil transmigration,

reduces brain edema and myeloperoxidase activity, and is neuroprotective [73]. As we discuss later in this chapter, CINC-1 has different and even opposing role in focal stroke when it occurs in a newborn.

Treatment with antibodies against CD11b/CD18 or ICAM-1 or with a neutrophil inhibitory factor UK-279,276 (rNIF) reduces parenchymal neutrophil accumulation and infarct size in focal transient ischemic models [72, 86], but inhibition of neutrophil adhesion and migration does not reduce infarct volume in permanent ischemia [87]. Cumulatively, these data suggest that neutrophils exacerbate reperfusion injury after ischemic insult. The effect also depends on the severity (duration) of an initial ischemic episode [72, 73, 88]. Data from several species, including rodents, rabbits, and baboons, show that, early after ischemia, neutrophils can prime endothelium and contribute to reducing CBF and that administration of the anti-CD18 monoclonal antibody or neutropenia reduces the "no-reflow" phenomenon [69, 89]. The relative roles of transmigrated and intravascular neutrophils in mediating damage are not entirely clear, but infiltrated neutrophils are believed to injure via the production of free radicals, release of proteolytic enzymes, and stimulation of cytokine release from neighboring cells. Inhibition or genetic depletion or proteolytic enzymes in leukocytes such as elastase, cathepsin G, or MMP-9 [54, 90, 91] reduces ischemic injury.

In humans, neutrophil accumulation progressively increases within 24 h after stroke, and neurological outcome in patients correlates with more severe neutrophil accumulation [92]. Based on genomics studies in the blood of patients, the majority of the genes induced during the first 24 h after stroke are expressed by neutrophils [93]. However, clinical trials of anti-adhesion therapies and UK-279,276 did not show improvement in recovery in acute ischemic stroke patients [88, 94, 95]. Yet, it is unclear whether negative clinical data are due to the limited role of neutrophil-dependent injury, due to the "disconnect" between preclinical and clinical trials, or due to shortcomings of the design of previously conducted clinical trials.

T and B cells are detected in the brain days after injury in rodent models of focal ischemia [96], making their participation on BBB disintegration after acutely after stroke less likely.

The role of monocytes as regulators of BBB is increasingly recognized. While earlier studies pointed to toxic features of activated monocytes due to increased production of cytokines, proteases, and ROS, recent studies showed an important role of monocytes in maintaining integrity of the neurovascular unit following brain ischemia [97]. Using several strategies, including pharmacological monocyte depletion, CCR2 receptor knockout, and bone marrow chimeric approach, it has recently been demonstrated in two different murine models of ischemic stroke that depletion of circulating monocytes or selective targeting of CCR2 in bone marrow-derived cells alters ischemic injury and hemorrhagic transformation. The stabilizing effects of monocytes are TGF-β1 dependent as injection of rTGF-β1 into the lesion border zone greatly reduces infarct and bleeding in mice with depleted monocytes [97]. These data are consistent with the notion that monocytes have multiple roles in "bridging" and stabilizing vessels during brain development [98].

4.2 Parenchymal Brain Cells

Parenchymal cells (neurons, astrocytes, and microglia) increase the production of inflammatory mediators post-ischemia [99–102], which can affect BBB integrity. For example, endothelial interactions with the BM are substantially influenced by TNFα and IL-1β and affect integrin β1 expression [103, 104]. TNFα and IL-1β also promote the expression of endothelial adhesion molecules [59, 105] and induce MMP-9 production in endothelial cells, surrounding perivascular cells, as well as in peripheral cells [53, 106, 107]. However, the cellular source of cytokine production may play distinct roles in BBB integrity, as TNFα produced in leukocytes was demonstrated to disrupt the BBB after stroke, whereas TNFα produced in microglia had no effect on this injury component [108]. The upregulation of MCP-1 has been directly linked to increased monocyte and neutrophil infiltration and exacerbation of brain injury [109], in part by inducing TJ protein redistribution and increased endothelial permeability [110, 111]. Altered communication between MCP-1 and its receptor CCR2 alters TJ integrity and increases BBB permeability [110, 112].

Although microglia are not considered a structural cell component of the BBB, recent evidence suggests that these cells exert an important modulatory effect on BBB function after stroke. Microglial cells have been considered toxic after cerebral ischemia production of inflammatory mediators, but these mediators are also produced in high amounts by reactive astrocytes, degenerating neurons, and endothelial cells [99–101, 116]. One of main roles of microglia – surveillance the brain and rapid response to changes in the microenvironment – position these cells in the front line to modulate BBB permeability. Microglia are rapidly activated by plasma components (such as plasminogen, fibrinogen) that appear in the brain parenchyma when BBB is leaky [113, 114]. They also respond to small and locally induced BBB lesions by extending their processes towards the sites of vascular leakage, shielding a lesion, and preventing further leakage [115]. In animal models of multiple sclerosis, reactive microglia cluster around large leaky vessels [114]. Data on direct role of microglia in BBB damage after adult stroke are scarce. We recently reported that in a focal ischemia–reperfusion model in neonatal rats, depletion of microglia worsens parenchymal injury and increases levels of several inflammatory mediators in acutely injured regions [116] and that the absence of microglia adversely affects vascular integrity during sub-chronic injury phase [117]. It remains largely unknown whether these results extrapolate to adult stroke and what are the stabilizing effects of microglia on the BBB.

4.3 Perivascular Inflammatory Cells

Compared to other inflammatory cell populations, the role of perivascular macrophages (PVMs) after brain ischemia has been much less explored, mainly due to the absence of reliable markers for the distinguishing this particular macrophage

subpopulation from parenchymal microglia and blood-borne and meningeal macrophages. PVMs are known as important contributors to inflammation in models of experimental autoimmune encephalitis (EAE). The ability to produce cytokines [118–120], induce MHC II expression and become antigen-presenting cells and activate lymphocytes [118, 121, 122], together with the perivascular location of these cells, suggests that these cells are important "gatekeepers" controlling the trafficking of leukocytes across the BBB.

Mast cells are strategically located in the abluminal surface of brain vessels (mostly penetrating arterioles) and are part of an early inflammatory response element after stroke [123]. The presence of cytoplasmic granules enables mast cells to exert a rapid initial response by releasing their contents to the surroundings. Some of the molecules released during degranulation, including TNFα, histamine, chemokines, and proteolytic enzymes, such as tryptase and chymase, can damage the endothelium and degrade components of the BM and endothelial junctions [123]. Chymase is a known activator of MMP-9, and mast cells are also a source of MMP-9 themselves [123]. Mast cells can also contribute to a more delayed response by producing inflammatory cytokines and chemokines de novo and promoting endothelial activation and leukocyte recruitment and infiltration [123]. The important injurious role of mast cells after brain ischemia has been demonstrated in several studies, showing that mast cell stabilization or their absence considerably reduces BBB leakage, neutrophil accumulation, brain swelling, and injury [124, 125]. These data have encouraged the exploration of modulators of mast cell activation and degranulation as potential agents for the early prevention of BBB leakage following stroke.

5 Post-ischemic Vascular and Brain Repair

Repair after stroke is a very complex process [126, 127]. The formation of new blood vessels, angiogenesis, is a limiting factor in post-ischemic repair [128]. Angiogenesis is a multistep process, which is under strict control by numerous soluble factors. Angiogenic molecules such as VEGFs, FGFs, angiopoietins, and CXC chemokines with the ELR motif, integrins, and VE-cadherin, as well as angiostatic molecules such as angiostatin and CXC ELR$^-$ chemokines, are some of the well-described effectors of angiogenesis [129]. For example, VEGF-A is central to angiogenesis in the brain. Angiogenesis is increased following stroke in rodents and humans. The upregulation of VEGF-A and/or VEGF receptors and angiogenesis are spatially correlated following MCAO in rats [130, 131], and intraventricular or intravenous administration of VEGF-A further enhances post-ischemic angiogenesis and neurogenesis [132]. In addition, circulating endothelial progenitor cells (EPCs) play an important role in repairing blood vessels after stroke, in part signaling through both VEGFR-1 and VEGFR-2 receptors [133]. Permissiveness of the BBB plays a central role in migration of progenitors.

MMPs are critically involved in the remodeling of the ECM, and a balance between MMPs and their inhibitors, TIMPs, is believed to be important for the evolution of brain injury in ischemia [56]. In contrast to the protection achieved via MMP-9 inhibition after acute stroke, the effect of MMPs in general is complex; for example, treatment with the broad-spectrum MMP inhibitor GM6001 and with more specific MMP-9 inhibitors significantly decreased the migration of immature neurons from the SVZ into the striatum [134]. In a stroke model, inhibition of MMP-9 worsens outcome, specifically by reducing angiogenesis via reduced availability of biologically active VEGF [55]. MMP-9 also supports migration of pluripotent stem cells from the subventricular zone after stroke [134]. Thus, during tissue repair, MMP-9 is beneficial rather than harmful.

In naïve brain neurogenesis is restricted to selected regions, the dentate gyrus and the SVZ. Neurogenesis and functional incorporation of newly produced neurons, the ultimate goals of repair, occur in the adult, as has been demonstrated in a variety of rodent models [128, 135–138]. Focal stroke increases SVZ neurogenesis and directs neuroblast migration to sites of damage, but the survival of newly born neurons is often low [136].

An increasing number of studies demonstrate that post-ischemic angiogenesis, enhanced pharmacologically [139, 140] or by cell-based therapy [141, 142], promotes functional recovery, while suppression of angiogenesis by anti-inflammatory strategies, like MMP inhibition [55], or by disruption of SDF1 or Ang1/Tie2 signaling [138], worsens functional recovery. Neurogenesis itself is regulated by a changed microenvironment [143], including a shift between pro- and anti-inflammatory factors and production of growth factors [144–146] and the microglial phenotypes. M1-type microglia can inhibit angiogenesis by initiating a death program in endothelial cells [147] and through release of angiostatic factors, whereas M2-type microglia promote angiogenesis and neurogenesis by releasing growth factors, including VEGF [148], and production of proline, a precursor for collagen biosynthesis, from ornithine generated by arginase 1 [149]. Microglia can also produce mediators, such as MMP-9, that can harm initially but enhance the repair through remodeling of the ECM [55]. Cell-based therapies, including MSC, improve functional outcomes after stroke [150–155]. Exogenously administered MSC reduce apoptosis, promote endogenous cell proliferation [150], significantly reduce the expression of inhibitory factors in astrocytes, including a broad array of glycoproteins [156], and, at the same time, increase the production of growth factors, including VEGF and BDNF [151]. Angiopoietin1/Tie2 and VEGF/Flk1 induced by MSC amplify angiogenesis [151]. There are a number of unresolved questions related to the underlying mechanisms of repair by cell therapies. The literature is still conflicting on whether engrafted cells themselves enhance the repair or they act to change the microenvironment, including the neurovascular interface. Some studies suggest that engrafted cells survive for prolonged periods while other studies show a rapid decline in survival but enhanced repair [157].

6 BBB Responses to Stroke in the Perinatal Period

Emerging evidence suggests that the early postnatal BBB is functional and not as permeable as once thought. Although some barrier mechanisms are different in a fetal brain compared to an adult brain [158, 159], endothelial TJs are already present during early embryonic development [13, 160], specific BBB transporters are present in the brain endothelium during mid-gestation [158], and no fenestrations are observed at birth [161]. While the expression of endothelial BBB proteins undergoes major changes from the embryonic period to adulthood [13], susceptibility of the BBB to injury does not decrease linearly with age. An example of this is the higher BBB disruption observed in P21 rats subjected to a local brain inflammatory challenge (intrastriatal IL-1β-injection) compared to the response in 2-hour-old newborn rats [162].

A recent comparative study provided direct evidence for the intrinsically different functional BBB responses between experimental stroke in adult and neonate rats [11]. Following transient MCAO in P7 and adult rats, functional integrity of the BBB was evaluated by intravenous injection of tracers of different sizes (ranging from 650 Da to 70 kDa). While leakage of large tracers was observed in the adult brain 24 h after stroke, the extravascular distribution of tracers of all utilized sizes remained significantly lower in the neonatal brain (Fig. 2) [11]. The preservation of BBB function occurs in spite of increased vascular degeneration in the ischemic core, observed as early as 24 h after neonatal stroke [117], suggesting that degenerating vessels may not be adequately perfused within the injury regions.

Intrinsic age-related differences in the expression of several proteins involved in BBB function could contribute to higher resistance of the neonatal BBB to stroke. Comparative analysis of the endothelial transcriptome in adults and neonates 24 h after transient MCAO showed a markedly different response (Fig. 1). Among many differences, two major components of the vascular BM, Col-IV and laminin, are more abundant in uninjured neonates than in uninjured adults, while the expression of several TJ proteins is better preserved in neonates [11]. Gene expression of MMP-9 and E-selectin is lower in endothelial cells from neonates in response to stroke, suggesting possible age-related differences in the interaction of the brain endothelium with circulating leukocytes. Consistent with the latter notion, infiltration of neutrophils into injured neonatal brain is minimal within 1–72 h after stroke [11]. Only limited or transient neutrophil infiltration was observed after neonatal hypoxia–ischemia [163, 164]. Alterations in the blood–brain gradient of the cytokine-induced neutrophil chemoattractant (CINC-1) led to increased presence of neutrophils in the brain parenchyma that was correlated with locally increased BBB permeability [11], suggesting that neutrophils mediate BBB damage in association with transmigration. Compared to adult stroke, infiltration of circulating monocytes across the BBB is also low during the acute phase after stroke in the neonate [165].

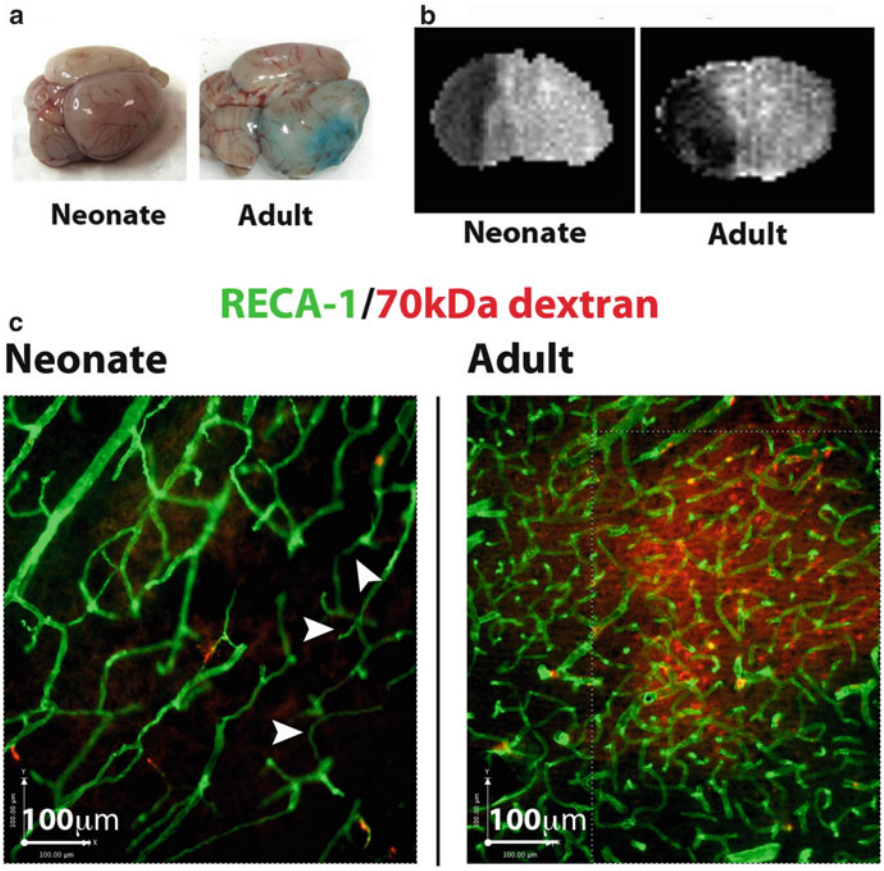

Fig. 2 Age at the time of the insult affects BBB permeability after stroke. (**a**) Accumulation of Evans Blue injected at 2 h after stroke and let circulate for 22 h was observed in the ischemic regions of adult brains, but was minimal in neonate brains. (**b**) Apparent diffusion coefficient (ADC) maps showing a similar initial extent of injury (measured as brain edema during MCAO) in adults and neonates. (**c**) Immunofluorescence for the rat endothelial cell marker RECA-1 (*green*) showing the vascular coverage in neonate and adult brains 24 h after stroke. Although abnormally looking vessels are present in injured regions in the neonate brain (*white arrowheads*), leakage of 70 kDa dextran (*red*) injected intravenously is negligible at 24 h after injury, as opposed to adult brains

The exact mechanisms that restrict leukocyte infiltration in the ischemic neonatal brain are not completely understood, and it remains unclear whether the higher resistance of the neonatal BBB to stroke is a cause or a consequence of reduced leukocyte transmigration.

7 BBB Integrity, Angiogenesis, and Brain Repair After Stroke During the Perinatal Period

The presence of leaky angiogenic brain vessels has been commonly assumed, but recent evidence has shown that during embryonic and postnatal angiogenesis the BBB is integrant and functional [11, 13, 161]. Vascular outgrowth continues during the first two postnatal weeks in the rat brain, including endothelial cell proliferation and abundant present endothelial tip cells with extended filopodia [11, 166–168]. Following stroke in P7 rats physiological angiogenesis is arrested in injured brain regions up to 14 days after injury, and angiogenic response is subtle in the ischemic boundaries in the cortex [117]. Thus, the response of neonatal brain to stroke differs to that in the adult in this aspect, since in adults endothelial cell proliferation and vascular outgrowth have been reported as soon as 24 h after stroke [127, 169, 170]. Consistently, endothelial transcriptome in neonatal and adult rats 24 h after stroke revealed reduced gene expression of proteins involved in angiogenesis (angiopoietin 2, VEGF receptors) in neonate rats but not in adults [11]. Brain vessels with active endothelial proliferation in the ischemic boundaries of the injured regions 14 days after neonatal stroke showed abnormal expression of the endothelial barrier antigen (EBA) [117], a protein necessary for proper BBB function in adolescent and adult rats [171–173] which expression is maturation dependent [174, 175]. Therefore, the relatively preserved BBB after neonatal stroke may also negatively impact angiogenesis and account for a delay in vascular remodeling [117] and ultimately endogenous neurogenesis, although the relationships between the two processes are still poorly understood.

8 Concluding Remarks

The fine-tuned selective permeability of the intact BBB lies on a delicate structural and functional interplay between several cell types and ECM components. Multiple cell components of the BBB are affected by cerebral ischemia and reperfusion, with the extent of injury, the size and anatomical location, as well as genetic background and gender play a role. Recent studies have improved our understanding of the events at the neurovascular interface after stroke. For example, the relative roles of local and systemic inflammation as well as the need to be careful about when and for how long it is safe to give particular therapies are better understood from studies of effects of ROS, inflammatory cytokines/chemokines, and MMPs. More attention has been given to the role of local parenchymal cells, microglia and astrocytes, and perivascular cells as modulators of neurovascular integrity. Another important aspect under intense investigation in the stroke field is the relationship between BBB integrity and angiogenesis. Interaction of the BBB components with neuroprogenitors, endogenous or engrafted, which is needed for adequate trophic support and local homeostasis, migration, and differentiation of neural progenitors during stroke-induced neurogenesis, is another important area of investigation in

the stroke field. Technological advances in noninvasive imaging that has enabled visualization of dynamic interactions within the BBB and with cells surrounding the BBB in living injured brains will further help us understand how to control BBB permeability and design therapeutics to improve stroke outcome.

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Inflammation at the Blood–Brain Barrier in Multiple Sclerosis

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Abstract The blood–brain barrier is specialized to function as a barrier to protect the central nervous system (CNS) by restricting entry of unwanted molecules and immune cells into the brain and inversely, to prevent CNS-born agents from reaching the systemic circulation. The blood–brain barrier endothelium, together with the cells involved in its regulation, forms the neurovascular unit. Blood–brain barrier dysfunction is an important hallmark of early multiple sclerosis pathophysiology, leading to a consequent loss of the imperative brain homeostasis. The unrestrained access of immune cells and blood-borne compounds into the CNS play a central role in demyelination and axonal damage, two major hallmarks of multiple sclerosis pathology underlying the clinical symptoms of patients. The neuroinflammatory changes at the blood–brain barrier are numerous and include the loss of barrier function, altered communication with surrounding cells, and activation of both inflammation promoting and dampening mechanisms. A better understanding of the blood–brain barrier alterations in neuroinflammation might lead to new ways to promote blood–brain barrier function in neurological diseases like multiple sclerosis.

Keywords Astrocytes, Blood–brain barrier, Endothelial cells, Multiple sclerosis, Neuroinflammation

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Contents

1 Multiple Sclerosis and the Blood–Brain Barrier

The vasculature of the brain functions as a specialized barrier to protect the central nervous system (CNS) from the systemic circulation by restricting entry of unwanted molecules and immune cells into the brain, by active removal of cytotoxic compounds, and by supplying the brain with essential nutrients and oxygen through specific transport mechanisms. The blood–brain barrier (BBB) is not a rigid barrier but a dynamic structure that receives continuous input from the CNS cells it protects. This allows for a thorough response to the local demands for oxygen, nutrients, and buffering which is crucial for the maintenance of a CNS homeostasis that favors optimal neuronal function.

Several neuroinflammatory and neurodegenerative diseases like multiple sclerosis (MS), capillary cerebral amyloid angiopathy (capCAA), Alzheimer's disease (AD), epilepsy, and Parkinson's disease (PD) are associated with an impaired function of the BBB. Especially in MS, disruption of BBB function is paramount and an early marker for MS pathophysiology. The following chapter will cover various aspects of the known involvement of BBB dysfunction in MS pathology, therapeutic aspects, and future implications of BBB research in MS.

1.1 Clinical Features and Diagnosis of Multiple Sclerosis

MS is a chronic inflammatory disorder of the CNS. MS pathology is characterized by the presence of focal inflammatory lesions scattered throughout the brain. Depending on temporal stage, lesions are hallmarked by inflammation, demyelination, gliosis, axonal injury, and diffuse axonal degeneration [51, 109]. The global median estimated prevalence is 30 per 100,000, resulting in over two million people affected with MS worldwide. With an average age of onset between 25 and 32 years of age, MS is one of the most common neurological disorders and causes of disability in young adults [160].

Presentation and symptoms of MS are characterized by great variability and diversity. In general, the initial symptoms and signs are sensory impairment, optic neuritis, motor deficits, limb ataxia, and difficulty with balance [155]. The majority of MS patients are subject to a relapse with onset of MS, referred to as clinically isolated syndrome (CIS), which may eventually convert to MS [97]. The clinical manifestation of MS varies and can be described by three clinical course definitions: relapsing-remitting (RR) MS, accounting for the onset of disease in about 85% of MS patients, is described by clearly defined disease relapses with full or partial recovery. Secondary-progressive (SP) MS is described by initial RR disease course, followed by progression with or without occasional relapses, minor remissions, and plateaus [93]. Primary-progressive (PP) MS, accounting for the onset of disease in about 10% of MS patients, is described by rapid disease progression from onset with occasional plateaus and temporary minor remissions.

Diagnosis of MS is primarily based on clinical grounds, comprising neurological exams and clinical history. If a diagnosis based on clinical presentation is not possible, radiological and laboratory assessments such as magnetic resonance imaging (MRI) and cerebrospinal fluid (CSF) analysis may be essential for diagnosing MS. MRI analysis detects MS lesions in brain and in spinal cord and can therefore provide evidence of dissemination of MS lesions in both time and space, two potential criteria for diagnosis of MS. CSF analysis may provide supportive evidence by the presence of CNS-derived antibodies (oligoclonal bands).

1.2 Etiology

The precise etiology of MS remains unknown. Epidemiological studies indicate that environmental factors may contribute to the development of MS [36], but that development of MS will probably arise in the genetically susceptible population, upon exposure to environmental factors [118]. Family studies have revealed that first degree relatives of MS patients are more likely to develop MS compared to non-related individuals [36, 121]. Further support for a genetic risk factor for MS susceptibility derives from twin studies, which show a higher concordance rate of MS in monozygotic twins compared to dizygotic twins [37, 75, 96]. Certain human leukocyte antigen (HLA) alleles are associated with susceptibility to MS. The allele with the strongest association with MS is HLA-DRB1*15 (HLA-DR2) showing consistency of effect across several Western European and Scandinavian countries and the USA. In addition, various genetic mutations or polymorphisms in genes coding for cytokines (IL7, IL12A, IL12B), cytokine receptors (CXCR5, IL2RA, IL7R, TNFRSF1A, IL12R), adhesion molecules (CD6, VCAM-1), and co-stimulatory molecules (CD37, CD40, CD80, CD86) are associated with pathogenesis of MS [123].

Environmental risk factors described for MS are diverse of character. Several infectious pathogens such as varicella zoster virus, herpes viruses, and chlamydia are described as environmental risk factors; however, current scientific interest is

oriented toward the Epstein–Barr virus (EBV) [8, 40, 49, 84, 85, 94, 101, 102, 110, 135]. Involvement of EBV in MS pathology may be explained by its aptitude to elicit a persistent infection in the CNS inducing an immune response that contributes to pathology directly or through autoimmunity. Although literature about involvement of EBV in MS pathology is expanding, consensus about its complicity is not reached due to major controversies concerning sensitivity and specificity of detection methods of the virus in the CNS [84]. Two important risk factors amongst the non-infectious environmental risk factors for MS are latitude and vitamin D. Populations living at higher latitude show an increased prevalence of MS compared to populations living near the equator, a finding most likely associated with vitamin D serum levels. Interestingly, studies show that populations living at high latitude but with rich vitamin D food intake also show reduced MS prevalence [72, 118]. Pinpointing MS etiology has thus far proved elusive. Therefore, understanding the mechanisms of disease in MS might result in an enhancement of the current therapeutic strategies to combat the progression of MS.

1.3 Pathogenesis

A distinct feature of MS pathology is the formation of demyelinated lesions, or plaques, in the CNS. Four patterns of demyelination were identified by systematic analysis of MS plaques: T-cell and macrophage-mediated demyelination, antibody and complement-mediated demyelination, oligodendrocyte dystrophy, and primary oligodendrocyte degeneration. To improve and standardize appropriate diagnosis and to support uniformity in research material, several different staging attempts have emerged in the last 20 years. These were named according to the pathologists involved in these staging systems: The Bö/Trapp system, The De Groot/van der Valk modification, The Luchinetti/Lassmann/Brück system, and the Vienna consensus [143].

According to De Groot/van der Valk staging, MS lesions can be classified as pre-active, active demyelinating, active but not demyelinating, chronic active, and chronic inactive lesions [143]. Pre-active lesions may be located near existing demyelinated plaques and in “healthy” white matter areas. The lesions do not show demyelination but are characterized by modest white matter abnormalities including clusters of activated microglial cells and few perivascular leukocytes. In contrast to pre-active lesions, active demyelinating lesions are characterized by loss of myelin and presence of abundant macrophages containing myelin degradation products. In addition, parenchymal and perivascular infiltrates of macrophages and lymphocytes are observed as well as abundantly present reactive astrocytes. A chronic active MS lesion is a demyelinated lesion containing a hypocellular center and a hypercellular rim of hypertrophic astrocytes, microglia, and macrophages [165]. Finally, chronic inactive lesions are demyelinated and hypocellular with only moderate expression of major histocompatability complex class II (MHCII) and few lipid-phagocytosing macrophages present [129].

Complementary to demyelination, axonal damage is known to be of great importance in MS pathology. Early axonal damage is found at areas of acute demyelination and inflammation [45, 140]. Axonal loss has been shown to be a major cause of irreversible neurological disability in MS [18]. The irreversible nature of axonal damage and its association with inflammation suggest that anti-inflammatory treatment should be utilized early and that future therapies could benefit from the inclusion of a neuroprotective component to prevent neurological deterioration.

Despite many advances in both molecular and clinical MS research, MS still remains incurable. Nevertheless, various therapies for treatment of MS are available and more therapies will most likely become available in the following years. Current MS therapies are limited to reduction of relapse rates, slowing down disease progression, accelerating recovery of relapses, and palliative treatment.

2 The BBB in MS

In MS pathology, numerous changes in BBB structure and function have been described. These observations derived from in vitro systems, animal models, and patient tissue studies show a high involvement of the disruption of BBB integrity and function in MS pathophysiology. The combined outcome of these studies has led to the notion that BBB disruption represents an early event in MS lesion formation, preceding both the massive infiltration of leukocytes (mainly T lymphocytes and monocyte-derived macrophages) and nervous tissue destruction [99]. Even before clinical symptoms arise, MRI scans of animals with experimental allergic encephalomyelitis (EAE), a well-established and validated animal model for the inflammatory phase of MS, show leakage of the BBB before leukocyte infiltration [48]. However, before leukocytes adhere and transmigrate through the BBB, the cerebral endothelium must be activated by inflammatory mediators which induce expression of cell adhesion molecules (CAM) on BEC, with which leukocytes interact. A better understanding of the molecular changes occurring at the BBB during MS pathophysiology could shed light on the crucial steps needed for the breach of the barrier in MS, eventually leading to a better understanding of the mechanism that can be utilized to halt the inflammatory component of MS. A schematic overview of the inflammatory changes at the BBB described in this chapter is depicted in Fig. 1.

2.1 Inflammation at the BBB in MS

The BBB is composed of highly specialized brain endothelial cells (BECs) and limits both transcellular and paracellular passage of cells and molecules from the systemic circulation into the CNS and vice versa. Transcellular passage of

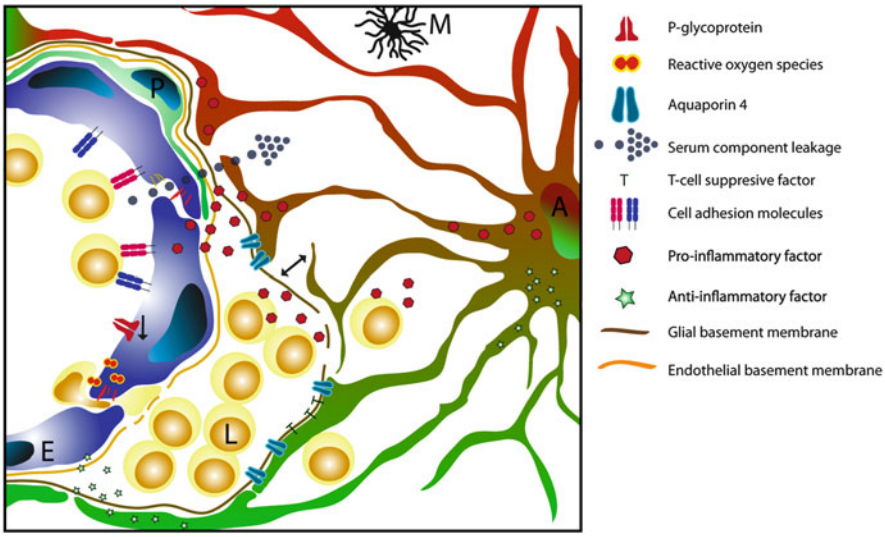

Fig. 1 Neuroinflammatory changes at the blood–brain barrier. During neuroinflammation in MS, the inflamed BBB shows loss of barrier integrity, resulting in leakage of serum components into the CNS. Endothelial cells (**E**) express cell adhesion molecules leading to adhesion and migration of activated leukocytes (**L**) into the CNS. Leukocytes locally release reactive oxygen species to disrupt TJ complexes. Efflux transporter P-glycoprotein expression is decreased on endothelial cells. Reactive astrocytes (**A**) and activated microglia (**M**) contribute to the neuroinflammatory process by releasing pro-inflammatory chemokines and cytokines. Aberrant astrocyte endfeet aquaporin 4 expression is thought to aggravate BBB disruption. The protective role of reactive astrocytes is illustrated by expression of T-cell suppressive factors and the release of anti-inflammatory factors like sonic hedgehog. The role of pericytes (**P**) in inflammatory BBB disruption is not known, although the loss of pericytes is associated with the damaged BBB

hydrophilic molecules is limited due to a low rate of transcytotic vesicles, low pinocytotic activity, expression of active efflux membrane pumps of the ATP-binding cassette (ABC) family such as P-glycoprotein, and high metabolic activity (cytosolic enzymes and transporters). To buffer excess amounts of neurotransmitters like glutamate from the CNS, BECs possess excitatory amino acid transporters (EAAT) 1–3 to limit neurotoxicity. In order to closely regulate the influx of only those components that are necessary in the CNS, BECs harbor specific transporters that actively transport nutrients like glucose into the CNS by glucose transporters (Glut1-3).

Paracellular diffusion of hydrophilic molecules and trafficking of immune cells is restricted by a network of TJ complexes which allow firm adhesion of BECs to each other and sealing of the inter-endothelial space [58, 92, 124, 159]. Adjacent BECs express continuous rows of transmembrane proteins that make homophilic contact in the intercellular space and form TJs [147]. Claudins and occludin are the most important membranous components of TJs, but the participation of junctional adhesion molecules (JAMs) and adherens junctions (Cadherins) are important as well [159]. One of the specific characteristics of the brain endothelium is the

absence of pan-endothelial marker plasmalemmal vesicle-associated protein-1 (PLVAP). PLVAP is a transmembrane protein associated with transendothelial transport and the caveolae of the fenestrated microvasculature, and is developmentally silenced during BBB differentiation [57]. The mechanism of PLVAP downregulation in endothelial cells during CNS development is not fully understood, and conflicting reports exist on the role of CNS pericytes in PLVAP regulation [6, 29]. The endothelium of the CNS microvasculature shows a high degree of specialization to form the BBB, and the regulatory process behind this specialization is still largely unknown.

Tumor necrosis factor-α (TNF-α) and chemokine (C–C motif) ligand 2 (CCL2) are two examples of numerous pro-inflammatory molecules which cause an upregulation of endothelial CAMs such as E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) [90], activated leukocyte cell adhesion molecule (ALCAM) [24], and melanoma cell adhesion molecule (MCAM) [82]. While it remains unclear what triggers initial vascular activation in MS, reactive astrocytes and perivascular microglia are potent contributors to endothelial inflammation since they secrete pro-inflammatory cytokines and chemokines such as TNF-α, interleukin (IL)1β, IL6, IL12, and CCL2 during the disease process [1, 64, 136]. Through secretion of pro-inflammatory molecules, astrocytes and microglia not only contribute to direct disruption of the BBB but also facilitate upregulation of CAMs and form a chemo-attractive gradient, thereby promoting recruitment and adhesion of more leukocytes to BECs.

Inflammation-related tissue damage in the CNS of MS patients is driven by both autoreactive, antigenic CD4 T cells and CD8 T cells [9, 13, 16, 17, 32, 56, 114, 166]. In addition, IL17 producing memory CD4 T cells (Th17 cells) are found within active MS lesions [74]. Of the antigen-presenting cells (APCs), infiltrated monocyte-derived macrophages are thought to possess a crucial role in orchestrating processes such as demyelination and axonal damage [3, 21, 27, 43, 62]. Before entering the CNS, leukocytes have to transmigrate through the ECs of the BBB. Monocytes, the effector cells within MS lesions, are attracted to the perivascular space in high numbers. Within the process of monocyte trafficking across the BBB, it has been demonstrated that reactive oxygen species (ROS) play a dominant role. ROS are produced by monocytes upon firm adhesion to ECs and subsequently enhance migration and adhesion of monocytes [142]. Treatment of EAE animals with antioxidants such as flavonoids and lipoic acid suppressed the development of EAE by lowering the entry of inflammatory cells into the CNS. Histological examination demonstrated a reduced number of infiltrated T cells and macrophages, suggesting a role for ROS in BBB permeability [69, 125]. Moreover, it was shown that super oxide is the predominant ROS treatment which induces BBB disruption by inducing TJ rearrangements and cytoskeletal changes, allowing cell migration [142].

The exact mechanism leading to BBB integrity loss during neuroinflammation is still not fully understood. Interestingly, the lack of various pro-inflammatory mediators like TNF-α or IFNγ in EAE animals alters the composition and amount of infiltrating immune cells in spinal cord lesions, but not the increase in BBB permeability [44]. Furthermore, this study shows that EAE disease severity is directly

correlated with BBB permeability. This suggests that the loss of BBB integrity is a requisite for EAE development and can occur independently of classical pro-inflammatory mediators, possibly by the direct interaction with activated leukocytes. Immune-activated brain endothelium can furthermore promote barrier disruption by the expression of matrix metalloproteinase (MMP)-9 [59], an extracellular matrix (ECM)-degrading enzyme that has been associated with the specific breakdown of the glia limitans during leukocyte infiltration in EAE [4]. Although MMPs expression during neuroinflammation is usually attributed to immune cells, BECs highly increase the expression of both MMP2 and MMP9 upon activation [153]. Blocking MMP activity with fluoxetine after spinal cord injury resulted in the prevention of BBB disruption, as well as reduced infiltration of immune cells in vivo [87]. In the same study fluoxetine was also shown to decrease MMP9 expression in BECs. Protecting BBB integrity during neuroinflammation by targeting BEC activation might therefore reflect an interesting therapeutic possibility.

2.2 Immune Cell Trafficking Across the Brain Endothelium

The transmigration of leukocytes across the vascular wall requires the sequential activation and interaction of numerous molecular effectors expressed by BECs and immune cells, including selectins, chemokines, adhesion molecules of the immunoglobulin superfamily, and their integrin counter ligands. The importance of leukocyte migration in MS is highlighted by the fact that the healthy CNS is devoid of immune cells and has been further demonstrated by the clinical efficacy of pharmacological blockers of migration in human MS patients. Interfering with leukocyte extravasation and diapedesis by blocking the adhesion cascade has indeed proven to be beneficial in reducing clinical disease activity and pathological indices in MS. Natalizumab, which blocks VLA-4, the ligand of VCAM-1, is reported to reduce migration of most leukocyte subtypes into the brain. Therefore, validation of the biological importance and of the clinical relevance of immune cell trafficking in MS is provided by the important clinical benefit of anti-VLA-4 blocking therapies. These VLA-4 blocking strategies prevent immune cell recruitment to the CNS, reduce myelin and axonal damage, and alleviate clinical symptoms and disease progression in both animal models of MS [141] and MS patients [98].

Although the presence of leukocytes within demyelinating lesions is indisputable in MS and EAE, the route and adhesion molecules by which these cells access the CNS are still not fully understood. As immune cell transmigration across BECs represents a critical step for initiation of CNS-directed immune reactions, a better understanding of the molecular mechanisms involved in leukocyte diapedesis could identify novel therapeutic targets to modulate CNS immune responses. In this sense, VCAM-1, ICAM-1, ALCAM, JAM-L, CD90, and CD137 have all previously been shown to influence leukocyte transmigration in a nonrestrictive manner, affecting the recruitment of antigen-presenting cells, but also of T and B lymphocytes. Furthermore, ICAM-1 and VCAM-1 blockade only partially restrict migration of immune cells

across BECs, and it was suggested that additional CAMs are involved in the leukocyte transmigration process. These new CAMs need still to be identified.

MCAM, also known as CD146, is a new molecule of particular interest. MCAM is a member of the immunoglobulin superfamily, such as ALCAM, ICAM, and VCAM. The only ligand reported to bind MCAM is MCAM itself (homotypic interaction), although a recent report shows the binding of MCAM to the matrix protein laminin 411 [47]. MCAM is expressed by endothelial and smooth muscle cells. MCAM associates with the actin cytoskeleton and could contribute to the stabilization of inter-endothelial junctions. MCAM is also reported to mediate rolling of immortalized immune cells on BECs, although such data have not been confirmed using primary cells. Recently, MCAM was shown to be expressed by subsets of human peripheral blood memory CD4 and CD8 T lymphocytes. Interestingly, MCAM-expressing T lymphocytes are $CCR7^{neg}$ and thus bear the phenotypic properties of immune cells that have the capacity to migrate to inflamed organs. Blocking MCAM in vivo delayed disease and reduced the severity of EAE, using MOG-injected C57/BL6 animals [82]. Taken together, these observations suggest that MCAM is an adhesion molecule expressed by activated T_H17 lymphocytes and used to enter the CNS by binding either to MCAM expressed by the BBB or to the matrix protein laminin 411.

Recent evidences also suggest that encephalitogenic T_H17 lymphocytes can migrate to the CNS via capillary structures of the choroid plexus, and not through the BBB. This seems to be uniquely dependent on the chemokine CCL20 and the chemokine receptor CCR6 [119]. However, entry of encephalitogenic lymphocytes via choroid plexi remains a matter of debate, as other groups have not been able to confirm these data or have provided some contradictory findings [39, 149].

In addition to the family of CAMs members of another class of cell surface molecules are involved in the transendothelial migration process. The transmembrane 4 superfamily (TM4SF), or tetraspanins, are small membrane proteins differentially expressed by all mammalian cells. The size of tetraspanins ranges from 204 to 355 amino acids and they contain four transmembrane domains, the first of the two resulting extracellular loops is short while the second loop is long [127]. This long, second loop in combination with the four transmembrane domains are important in promoting associations of the tetraspanin with additional proteins such as other tetraspanins, integrins, CAMs, and intracellular signaling molecules [89]. Resulting structures are referred to as tetraspanin-enriched microdomains (TEMs) and they operate as molecular organizers for other transmembrane proteins [68]. The biological function of tetraspanins depends on the ability of the tetraspanin to organize TEMs. Biological functions associated with tetraspannins include adhesion, proliferation, differentiation, and motility of many different cell types [65–67]. Of the more than 30 mammalian tetraspanins, three are associated with intercellular junctions in endothelial cells [162]. Moreover, these tetraspanins, CD9, CD81, and CD151, also localize to docking structures on endothelial cells which are formed at sites of leukocyte adhesion [10]. More specific, presence of microdomains containing tetraspanins and adhesion receptors were present on activated endothelial cells even before leukocytes adhered and studies demonstrated that CD81 and CD9 play a role in the transendothelial migration of immune cells [11, 120].

2.3 Astrocyte–Endothelial Interactions in MS

Astrocytes are strongly represented within the neurovascular unit, ensheathing over 95% of the abluminal microvascular surface. It was this observation that gave rise to the idea that astrocytic processes formed the BBB, until electron microscopic studies showed that BECs were responsible for barrier function in brain microvasculature [20].

Astrocytes are able to influence a number of features of the BECs, leading to increased integrity of the BBB. TJ expression and TJ complex formation and maturation, expression and localization of BEC transporters, and specialized enzyme systems have been shown to be upregulated under astrocyte influence [2]. The notion that astrocytes can induce and maintain BBB properties in BECs through physical interaction and secreted agents has been widely accepted [60]. Astrocyte processes extending toward CNS microvessels terminate in specialized (perivascular) endfeet structures onto the basal lamina surrounding the BECs. Astrocyte endfeet associated with BECs show a high density of orthogonal arrays of particles (OAPs), organized arrays of ion- and volume-regulating membrane particles identified by freeze fracture [33], containing channels like the water channel aquaporin-4 (AQP4) and the potassium ion channel Kir 4.1 [104]. Membrane proteins in OAPs represent a strong polarization of perivascular astrocyte function and correlate with the expression of the basement membrane molecule agrin, an important proteoglycan for BBB integrity [108], responsible for the correct localization of AQP4. The distribution of these channels in OAPs is most likely important in the regulation of BBB homeostasis, as disruption of this distribution is associated with microvascular damage in, among other pathologies, AD [14].

The observation of astrocyte-conditioned medium inducing junction formation in BECs in vitro [7] gave rise to the idea that astrocyte-derived secreted factors were able to influence the BBB properties of BECs. Numerous astrocyte-derived agents have since then been described, mainly by in vitro studies, as modulators of BEC barrier function. Among these soluble BBB-promoting factors are transforming growth factor-β (TGF-β) [139], glial-derived neurotrophic factor (GDNF) [71], fibroblast growth factor (FGF) [38], and angiopoietin-1 (ANG1) [88]. Recently, sonic hedgehog (Shh), a member of the Hh pathway, was shown to be produced and secreted by perivascular astrocytes in the human and mouse adult brain and that microvascular BECs expressed the receptors and the intracellular machinery to respond to Hh ligands [5]. These observations confirm the important role of perivascular astrocytes in the regulation of the BBB in the adult CNS, and therefore represent an important topic in BBB dysfunction research in MS.

During MS pathogenesis, reactive astrocytes participate in various mechanisms that contribute to neuroinflammation. Reactive astrocytes aggravate inflammation by increasing vascular activation and leukocyte accumulation in the CNS, and are involved in loss of BBB integrity, mediated by local release of pro-inflammatory molecules like IL-1β, IL6, and CCL2 [34, 117, 137]. In addition, once inflammation has abated, astrocytes are the major cell type involved in glial scar formation and

are thereby directly associated with inhibition of axonal regeneration [30]. In contrast, during pathophysiology, astrocytes may also exert protective properties and promote cellular regeneration. Astrocytes are able to produce antioxidant enzymes and glutamate metabolizing enzymes and transporters suggesting an important role in scavenging ROS and extracellular glutamate [107, 146]. Furthermore, reactive astrocytes maintain the capacity to secrete T-cell suppressive factors [78], anti-inflammatory cytokines, and neurotrophic factors [22]. Finally, astrocytes in active MS lesions produce semaphorins, which are known to form chemotactic gradients for developing oligodendroglial cells, thereby possibly promoting remyelinization [156]. This accentuates the important and dual role of astrocytes in CNS damage, which is not limited to BBB damage, but encompasses all neuroinflammatory changes in the CNS. Inflammatory changes affecting the interaction between astrocytes and the BBB in MS are described below.

2.3.1 The Hedgehog Pathway

Neuroinflammatory conditions such as MS are associated with a breakdown of the BBB. A recent study showed that human astrocytes treated with TNF-α and IFN-γ increased Shh expression and that BECs grown in astrocyte-conditioned media (ACM) and treated with TNF and IFN-γ increased their expression of Hh receptors Ptch-1 and Smo [5]. Addition of Shh to BEC cultures induced a reduction in both CAM expression and chemokine secretion. Within control brain tissue and normal-appearing white matter (NAWM) obtained from MS brains, astrocyte processes and endfeet surrounding parenchymal vessels displayed Shh immunoreactivity. However, Shh immunoreactivity was strikingly enhanced in hypertrophic astrocytes and processes throughout active demyelinating MS lesions, and the Hh transcription factor Gli-1 was increased in BBB-ECs [5, 152]. Upon inflammatory stimulation, astrocyte-secreted Shh therefore induces expression of Hh receptors in BECs, which leads to the translocation of the Hh transcription factor Gli-1 into the nucleus of BECs. The hedgehog pathway, where Hh ligands are secreted by astrocytes and Hh receptors, are expressed by BECs, and therefore acts as a molecular repressor of CNS inflammation and promotes BBB repair.

2.3.2 Aquaporin-4 and Kir4.1 in Astrocyte Endfeet

Astrocytes with endfeet terminating in the neurovascular unit perform specific functions in the maintenance of perivascular ion and water homeostasis [132]. Extracellular potassium ions released by neurons require spatial buffering by astrocytes to maintain homeostasis. The inwardly rectifying Kir4.1 potassium channels which are highly expressed in the polarized astrocyte endfeet meet this need for potassium buffering. Potassium ion buffering by astrocytes is accompanied by osmotic changes and slight cell swelling. The AQP4 water channels present at high densities in the OAPs of astrocytic endfeet regulate these osmotic changes by redistribution of excess

water. The tight regulation of expression and distribution of the ion and water channels on astrocytic endfeet is necessary for homeostasis, and disruption of this compensatory system has been shown for BBB disruption in Alzheimer's disease [14] and glioblastomas [154], both involving aberrant agrin expression. The increase of AQP4 expression observed in brain edema, probably serving as an adaptive mechanism, tends to aggravate the BBB disruption [164]. AQP4 upregulation has also been shown in reactive (hypertrophic) astrocytes in response to injury, correlating with BBB disruption [150]. Reactive astrocytes in MS lesions were shown to have increased levels of AQP4 expression [134], which could possibly contribute to further edema-induced BBB damage after initial disruption.

The observation that the astrocytes with the highest AQP4 expression are located at the outer rim of active MS lesions, resembling ischemic foci [111], suggests that altered AQP4 expression, localization, or regulation by agrin could be contributing to aggravation of MS pathology.

2.3.3 Connexin 43

Astrocytes in the neurovascular unit are coupled together via gap junctions (GJ), mainly formed by connexin43 (Cx43) [105]. The coupling through GJ provides the network of astrocytes with a cytoplasmic continuity which allows the free and fast passage of (signaling) ions and metabolites between astrocytes. This syncytium of cells provides the BBB with a network of continuously communicating astrocytes, where fast responsiveness can be crucial in maintaining homeostasis.

In EAE a decrease in astrocytic Cx43 expression was observed in the inflammatory regions of EAE pathology, suggesting a decreased astrocytic connectivity in these areas [19]. Whether reduced astrocyte–astrocyte communication during inflammation is detrimental or beneficial remains to be determined, although the possible involvement of Cx43 in maintaining BBB integrity through co-localization with TJ-proteins in porcine BEC has recently been reported [103]. In contrast to findings in EAE, the enhanced expression of Cx43 in MS lesions was recently reported [95]. Besides the formation of gap junctions between astrocytes, Cx43 forms hemichannels resulting in enhanced exchange to the extracellular space [50]. Increased Cx43 hemichannel formation in this study was associated with promoting neuronal degeneration during NMDA-induced cytotoxicity. The effects of the loss of GJ-contact between astrocytes on astrocyte activation, BBB integrity, and inflammatory response should be investigated further to address the impact on MS pathology.

2.3.4 P-glycoprotein

The drug-efflux transporter P-gp is an ATP-dependent efflux pump highly expressed on the luminal side of BEC, responsible for the active removal of a broad range of hydrophobic molecules from the BEC cytoplasm [12]. P-gp function leads to the prevention of potentially neurotoxic molecules entering the CNS tissue,

also leading to the low penetration of CNS-therapeutical drugs [52]. The expression of P-gp is not confined to BECs, but expression was also shown to localize in astrocytic endfeet structures [112]. In a recent study, P-gp expression in the inferior colliculus was shown to be heavily reduced in BECs, following a chemically induced focal loss of astrocyte contact. Interestingly, P-gp expression returned to normal when astrocytes were seen to repopulate the affected area [157]. This observation indicates a role for astrocytes in the induction and maintenance of P-gp expression by BECs.

Recent data by our group showed a significant reduction of microvessel P-gp expression in various MS lesions in patients, compared to normal-appearing white matter [77]. These results suggest that a loss of P-gp expression might be involved in lesion formation or aggravation. A follow-up study showed that P-gp expression increased in astrocytes in MS lesions, suggesting a possible role for astrocytes as a complementary drug resistance barrier in areas of BBB disruption. However, P-gp was found to mediate the release of CCL2 and the proinflammatory lipid platelet activating factor [76] which may actively contribute to the neuroinflammatory process by attracting more immune cells into the lesion.

2.3.5 Sphingolipid Metabolites

In recent years, it has become increasingly clear that sphingomyelin metabolism plays a key role in biological processes in the CNS. Sphingomyelin is the major sphingolipid present in cell membranes, where it serves as a building block for biological membranes and in addition it plays an important role in proper membrane function [53, 80, 133]. Moreover, sphingomyelin is the predominant source for bioactive sphingomyelin metabolites, such as ceramide and sphingosine 1-phosphate (S1P). Evidence is now emerging that alterations in sphingolipid metabolism, leading to enhanced proinflammatory ceramide production, occur in several neurological disorders [31, 42, 55, 61, 81, 115, 116]. Importantly, inflammatory mediators, including TNF-α, ROS, and IL-1β, induce the production of ceramide through activation of acid sphingomyelinase (ASM), which in turn amplifies the inflammatory cascade either by direct activation of downstream targets or by affecting membrane organization [70, 122, 126].

Recently, we demonstrated an increase in the production of ceramide in reactive astrocytes in active MS lesions. Interestingly, astrocytes isolated from active MS lesions maintain increased ASM mRNA expression in culture which may be the result of continuous ceramide-induced autocrine activation through proinflammatory cytokines. During MS pathogenesis, stress signals such as ROS, TNF-α, and IFN-γ are present in the inflamed brain parenchyma and may be responsible for the observed increase in astrocytic ceramide. In addition, ceramide induces IL-6 mRNA and protein levels in a human astrocytoma cell line and ASM is able to induce release of microparticles containing IL-1β in astrocytes most likely mediated through ceramide formation [15, 46]. In turn, ceramide was found to

impair the function of the BBB in vitro [144], illustrating the impact of the reactive astrocyte phenotype on the barrier properties in MS.

Strikingly, reactive astrocytes were found to have an induced expression of the S1P receptors which after triggering with the S1P analogue fingolimod (FTY-720P) resulted in a diminished production of pro-inflammatory mediators [145, 144]. Together, these data indicate that the dampening of the pro-inflammatory response in the reactive astrocyte phenotype is an attractive new therapeutic strategy [83].

Altogether, astrocytes show a high degree of control of BBB function, both under healthy and disease conditions. Despite the fact that altered astrocyte–endothelial interaction might contribute significantly to MS pathogenesis, this role is far from understood. A better understanding of the changes that are related to astrocyte–endothelial crosstalk will enhance our ability to intervene in their communication in future therapeutic approaches.

3 Future Perspectives

The BBB is specialized to function as a barrier to protect the CNS by restricting entry of unwanted molecules and immune cells into the brain. An important hallmark of MS pathology is a dysfunctional BBB and consequent loss of the imperative CNS homeostasis. The unrestrained access of immune cells and harmful compounds into the CNS play a central role in demyelination and axonal damage, two hallmarks of MS pathology strongly contributing to the clinical symptoms of MS.

Strategies aimed at restoring the impaired function of the BBB in MS are therefore a promising new tool to combat disease progression, together with the dampening of the inflammatory phenotype and enhancing the protective response of reactive astrocytes

3.1 Developmental Pathways in BBB Protection

As discussed in this chapter, the astrocytic response to neuroinflammation is not restricted to detrimental effects on the surrounding cells, but also reflects protective aspects. Therefore, dampening the reactive state of astrocytes to reduce detrimental effects might also result in the reduction of protective and anti-inflammatory effects, necessary for regeneration and repair. A better understanding of the inflammatory pathways resulting in the various astrocytic responses is therefore warranted to separate the detrimental and beneficial effects of the reactive phenotype on the BBB, as well as on other neuronal cell types. Interestingly, developmental pathways involved in BBB development are now emerging as possible protective mechanisms to reduce BBB damage in neuroinflammation, as illustrated by the increased expression of sHh. Recently, retinoic acid (RA), an important astrocyte-derived morphogen in CNS development, has been shown to play a role in the

induction of the BBB [100]. Unpublished data from our group indicates that, similar to the expression of sHh, RA production reemerges during neuroinflammation in MS pathology. Although the effect of RA at the disrupted BBB remains to be investigated, recent reports show anti-inflammatory [161] and neuroprotective effects [73] of RA in the CNS. The association of other pathways that have been associated with BBB development, the Wnt/β-catenin pathway [28, 91] and the early association of CNS pericytes with the developing BBB [29], with MS or EAE pathology remains to be investigated. Restarting developmental programs at the disrupted BBB might be an intrinsic mechanism to reinstate the barrier during or after neuroinflammation. Interestingly PLVAP expression in the CNS microvasculature has been described as a marker for BBB disruption in acute brain ischemia, Alzheimer's disease, and malignant brain tumors in both human and mice studies [23, 131, 163]. Since normal PLVAP function involves promoting transendothelial transport, it is surprising that BECs respond to neuroinflammation by re-expressing this marker for the immature brain microvasculature and non-CNS endothelium. This putative immature state of the CNS endothelium might reflect the need for developmental programs, but warrants further research. Unraveling ways of boosting the self-regenerative capacity of the CNS to repair BBB disruption shows significant promise as a possible therapeutical avenue in MS, working side by side with the current immune-dampening therapeutic strategies.

3.2 Involvement of Neurons and Pericytes in BBB Disruption

Due to the high metabolic need of neurons and the dynamic pattern of neural activity, the CNS requires a tight regulation of the microcirculation which provides the necessary nutrients and means of waste transport. The coupling of brain activity and CNS blood flow is therefore crucial for normal neuronal functioning. Although the cellular aspect of this coupling is not fully understood, the involvement of all components of the neurovascular unit seems to be necessary for the regulation of CNS blood flow by neurons [54]. Besides the indirect regulation of blood flow, neurons are also found to directly innervate BEC or BEC-associated astrocytes functioning as a liason for neuronal–endothelial coupling. Because disruption of BBB integrity is often found to accompany pathological changes in CNS blood flow, it was suggested that the observed BBB permeability changes were due to active involvement of neurons in BBB integrity [86]. Indeed, noradrenergic [26], serotonergic [25], cholinergic [138], and GABA-ergic [148] neurons have been found to directly contact the microvascular endothelium. Although the mechanism of action is unknown, neurons innervating the neurovascular unit are thought to regulate BBB permeability [63, 113, 114]. An example of this regulation is shown by the loss of cholinergic innervation of the CNS microvasculature, resulting in impaired cerebrovascular functioning in AD [138]. In short, neurons in the NVU do not only play an active part in the regulation of CNS blood flow, but also seem able to directly influence BBB permeability, through direct innervations of BEC. The

extent of BBB disruption caused by decreased neuronal input or neurodegeneration in MS has not been investigated thus far. Furthermore, the loss of BBB integrity in grey matter has only recently been reported in EAE, where subtle TJ complex changes and leakage of FITC-dextran into the parenchyma was associated with areas of cortical demyelination, as was reactive astrogliosis and microgliosis [41]. To date, similar findings have not been reported in MS grey matter pathology.

Pericytes are perivascular, contractile cells that closely associate with capillary walls and directly contact the BEC membrane [79]. Pericytes are thought to exert influences on the BEC, through their specialized junctions, involving gap junctions, TJs, and AJs [130, 151]. Although the molecular mechanism by which pericytes mediate vascular integrity is not yet understood, perivascular pericytes are known to release growth factors and angiogenic molecules which are able to regulate microvascular permeability and angiogenesis [35]. Besides influencing BEC function, pericytes also contribute to the stability of microvessels and cover a large part of the abluminal BEC surface, further influencing BBB permeability [106, 151].

Reductions in the number of CNS pericytes have been linked to neurovascular disruption in both AD [128] and ALS [158–160] but the mechanism of pericyte detachment or disappearance from the BBB remains unknown. Considering the embedded location of pericytes within the endothelial basement membrane, and their extensive coverage of the CNS microvasculature, pericytes seem to be ideal candidates to monitor endothelial cell function and to communicate with perivascular astrocytes. This "tripartate" regulation of the BBB warrants further investigation in both animal models of neuroinflammation and in in vitro models of the BBB.

The BBB endothelium, together with all cell types involved in the NVU, ensures a tightly regulated CNS homeostasis that is crucial for normal brain function. A dysfunctional BBB is an early hallmark of MS lesion formation and therefore represents an important target structure for the discovery of new disease modifying drugs for MS. A better understanding of the process leading to BBB dysfunction and the resulting alterations, as well insights in the mechanisms underlying BBB development and maintenance in the adult brain are therefore crucial to discover pharmaceutical targets to improve BBB function in MS.

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Drug Delivery Across the Blood–Brain Barrier with Focused Ultrasound and Microbubbles

Stephen Meairs

Abstract Medical treatment options for central nervous system (CNS) diseases are limited due to the inability of most therapeutic agents to penetrate the blood–brain barrier (BBB). Neuropeptides, proteins, and chemotherapeutic agents are notable examples of potential therapeutics where the intact BBB is the major obstacle to their use. Indeed, all large-molecule products of biotechnology such as monoclonal antibodies, recombinant proteins, antisense, or gene therapeutics do not cross the BBB.

Although a variety of approaches have been investigated to open the BBB for facilitation of drug delivery, none has achieved clinical applicability. Recent studies suggest that ultrasound in combination with microbubbles might be useful for delivery of drugs to the brain region through transient opening of the BBB. This technique offers a unique noninvasive avenue to deliver a wide range of drugs to the brain and promises to provide treatments for CNS disorders with the advantage of being able to target specific brain regions without unnecessary drug exposure. Clearly, if this method could be applied for different drugs, new CNS therapeutic strategies could emerge at an accelerated pace that is not currently possible in the field of drug discovery and development. This chapter will review both the merits and possible harmful bioeffects of this new approach. It will assess methods used to verify disruption of the BBB with MRI and examine the results of studies aimed at elucidating the mechanisms of opening the BBB with ultrasound and microbubbles. Moreover, possible interactions of this novel delivery method with brain disease as well as safety aspects of BBB disruption with ultrasound and microbubbles will be addressed.

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Contents

1 Factors Influencing BBB Transport

Methods aimed at facilitating drug delivery across the BBB must address highly complex issues regarding BBB transport mechanisms. Indeed, the ability of a particular substance to cross the BBB and enter the brain depends on a multitude of factors. These include the concentration between compartments, the size, flexibility, and conformation of the molecule, amino acid composition, lipophilicity, cellular enzymatic stability, and cellular sequestration. Moreover, the affinity for efflux mechanisms, hydrogen bonding potential, and affinity for carrier mechanisms are further factors regulating the permeability of the BBB. Other factors that affect transport across the BBB include systemic enzymatic stability, plasma protein binding affinity, cerebral blood flow, uptake into other tissues, clearance rate, and effects of existing pathological conditions [1]. A number of different mechanisms are available for transport of a substance across the BBB: simple diffusion, facilitated diffusion, carrier-mediated transport, receptor-mediated endocytosis, absorptive-mediated transport, and carrier-mediated efflux.

2 Methods for Overcoming the BBB

2.1 Chemical Opening of the BBB

Intra-arterial injection of hyperosmotic solutions such as mannitol has been used to facilitate drug delivery to the brain. This causes the endothelial cells to shrink, which results in an opening of the tight junctions that lasts for a few hours. Both osmotic and chemical methods require invasive intra-arterial catheterization and produce diffuse, transient blood–brain barrier opening within the entire tissue volume supplied by the arterial branch that is injected. This method can enhance delivery of therapeutic agents to brain tumors, which has been demonstrated in several promising clinical trials [2–4]. Likewise, solvents such as high-dose ethanol or DMSO, alkylating agents like etoposide and melphalan, immune adjuvants, and cytokines have all been used to disrupt the BBB [1]. While such approaches can be effective for delivering drugs to large brain regions, they are invasive procedures that can require general anesthesia and lead to serious side effects such as seizures, bradycardia, and hypotension.

2.2 Modifying Drugs to Cross the BBB

There are a number of ways to modify drugs so that they may cross the BBB. While these methods are very promising, they require expensive development of new agents. Delivery is consequent to the entire brain, which may not always be desirable.

One method is to convert water-soluble molecules that would not ordinarily cross the BBB into lipid-soluble molecules through addition of lipid groups or functional groups such as acetate to block hydrogen bonding. The molecule then undergoes passive diffusion across the BBB. Another approach utilizes the solute carrier proteins (SLC) on the endothelial surface that transport many essential polar and charged nutrients such as glucose, amino acids, vitamins, small peptides, and hormones transcellularly across the BBB. An example of using SLC to deliver drugs to the brain is the amino acid transporter type 1 (LAT1), which transports L-dopa across the BBB for therapy of Parkinson's disease.

Endothelial-surface receptors can be targeted using the "Trojan horse" approach to transport drugs across the BBB. A targeting ligand, e.g., a serum protein or monoclonal antibody, binds to its receptor to activate endocytosis. A drug is then linked to this ligand, thus allowing it to be transported across the BBB. This technique has been used to transport antineoplastic drugs, fusion proteins, growth factors, plasmid vectors, RNAi, liposomes, and nanoparticles into the brain [5–8].

2.3 Bypassing the BBB for Drug Delivery

Localized drug delivery can be accomplished by injecting a drug through a needle or catheter directly into the targeted brain area. Such direct injections are invasive and require opening the skull. They also cause penetration of nontargeted brain tissue and carry the risk of brain damage, bleeding, and infection. Control of the drug distribution can be difficult with this method, since drug concentrations decrease exponentially from the injection or implantation site [9].

Drugs can be introduced into the cerebrospinal fluid (CSF) via intrathecal or intraventricular routes to enter the brain parenchyma via diffusion. This approach can be useful when the target is in the subarachnoid space [10], but penetration into the brain parenchyma can be limited because drug diffusion drops off exponentially from the brain surface [11]. An alternative approach is to deliver drugs transnasally from the submucous space into the olfactory CSF [12, 13]. This application of drug delivery is noninvasive and relatively easy to administer. Only small amounts of drug can be delivered and there is a significant interindividual variability when using this procedure [14].

3 Hazards of Opening the BBB?

An essential question that arises when discussing methods to open the BBB is whether such a procedure is not fundamentally dangerous. Certainly the fact that the blood–brain barrier excludes many different kinds of molecules and drugs from entering the brain from the vasculature suggests that increased BBB permeability would be harmful. From a clinical perspective, increased BBB permeability is usually a consequence of brain pathology. This is true, for example, in ischemic stroke. Cerebral ischemia is a complex pathophysiologic event that involves a loss of blood flow as well as depletion of oxygen and essential nutrients to the brain. Cerebral ischemia and hypoxia lead to increased permeability and disruption of BBB tight junctions. Recent animal experiments have demonstrated that serum proteins leaking into the brain may serve as a direct signaling mechanism resulting in the activation of astrocytes and the brain immune system, with consequent neuronal hyperexcitability and delayed neurodegeneration [74]. In this context one could argue that even transient opening of the BBB allowing leakage of proteins into the brain could result in brain disease.

Inflammatory mediators are known modulators of BBB permeability. Indeed, compromised BBB tight junctions are a hallmark of neuroinflammatory disease states [15]. BBB disruption is well established as an early event in the progression of MS. In experimental models of MS, BBB disruption is induced by T-cells and monocytes. MS lesions are associated with loss of occludin and ZO-1 in the microvasculature [16] that is likely mediated by cytokines. Similar observations have been made in postmortem examinations of brains from HIV encephalitis [17].

Several authors have suggested a role of the BBB in disease initiation or progression. BBB disruption may be a precipitating event in multiple sclerosis [18] and encephalitis. Another hypothesis suggests that blood–brain barrier dysfunction, with leakage of plasma components into the vessel wall and surrounding brain tissue leading to neuronal damage, may contribute to the development of several overlapping and disabling cerebrovascular conditions: lacunar stroke, leukoaraiosis, and dementia [19]. This hypothesis might explain the link between ischemic cerebral small-vessel disease and several apparently clinically distinct dementia syndromes.

Because the BBB plays critical roles in maintaining CNS homeostasis, its dysfunction can contribute to multiple diseases. Types of BBB dysfunction include (1) BBB disruption, which results in leakage of circulating substances into the CNS that can be neurotoxic; (2) transporter dysfunction, which has consequences such as inadequate nutrient supply, buildup of toxic substances in the CNS, and increased entry of compounds that are normally extruded; and (3) altered protein expression and secretions by endothelial cells and other cell types of the NVU that can result in inflammatory activation, oxidative stress, and neuronal damage. All three effects have been reported in Alzheimer's disease (AD) [20].

The possibility that the BBB is leaky in AD, that is, it does not prevent the uncontrolled entry into the brain of blood proteins and other molecules, has been investigated for many years. This is clearly an important question as disruption of even a transient or localized nature could have devastating consequences for brain function, inducing a cascade of events involving neurotoxicity, neuroinflammation, and oxidative stress that eventually could produce the AD phenotype. Indeed, some, but not all, animal models of AD exhibit BBB disruption. However, there is conflicting evidence on whether BBB disruption is actually a feature of AD. At any rate, any method utilizing BBB opening to foster drug delivery must take every effort to rule out a possible impact of this procedure on initiation or worsening of brain disease.

4 Imaging BBB Disruption

In most studies, the confirmation of BBB disruption has been obtained with MR contrast imaging at targeted locations [21–23] or with postmortem histology [24, 25]. Standard imaging of BBB integrity is performed with small, water-soluble, contrast agents with short plasma half-lives. Iodinated contrast agents produce enhancement in the brain on computed tomographic (CT) scans, which indicates where there is a loss of BBB integrity. Such enhancement is commonly found for malignant tumors, abscesses, or other lesions that cause vasogenic edema. The degree of enhancement on CT scans increases linearly with the amount of contrast agent entering the brain. For magnetic resonance imaging, chelated gadolinium is used as a water-soluble, paramagnetic, contrast agent. As with enhanced CT scanning, BBB breaches can be observed as enhancement on T1-weighted MRI scans, but with greater sensitivity than on CT scans. Signal

intensity changes attributable to gadolinium enhancement on MRI scans are not linear, unlike CT scanning results. Superparamagnetic iron oxide compounds (ultra-small-particle iron oxide) are now being used to assess BBB integrity. One such agent, ferumoxtran-10, has a long plasma half-life of 1–2 days and is taken up by phagocytic cells, but generally not by tumor cells. Therefore, despite their large size, relative to standard gadolinium contrast agents, these compounds facilitate imaging of brain tumors with slow leakage into the tumor and brain tissue around the tumor and uptake (trapping) by reactive cells in and around the tumor. These agents may also facilitate imaging of inflammatory brain lesions, including multiple sclerosis and stroke.

Small molecules with similar molecular weights have been used to obtain complimentary data on pharmacodynamics behavior of BBB opening. Gd-DTPA provides both contrast in MRI and semiquantitative verification of biodistribution in vivo, while Evans blue (EB) dye can be used as a measure of drug accumulation after animal sacrifice. These two molecules, which normally do not enter the brain parenchyma from the bloodstream, can potentially be used as surrogate markers for drug delivery. Although the dynamic distribution of Gd-DTPA may differ from that of Evans blue, AUC accumulation of Gd-DTPA analyzed by MRI was highly correlated with EB accumulation in the brain [26], implying that MRI AUC analysis of Gd-DTPA could predict the concentration of EB accumulating in the brain. Gd-DTPA may thus have the potential to predict the pharmacodynamics behavior and biodistribution of therapeutic agents delivered through the BBB.

5 Focused Ultrasound Therapy

Ultrasound can be used to induce a broad range of bioeffects through thermal or mechanical mechanisms. Focused ultrasound (FUS) is a special ultrasound technology that can be focused deep into the body. FUS has been investigated since the 1940s for noninvasive ablation in the brain as a potential alternative to surgical resection and radiosurgery [27]. Until recently the technique required removal of the skull bone for its application, since bone absorption of ultrasound led to severe heating of the skull and unacceptable beam aberration occurred due to the irregular shape of the skull and high acoustic impedance of bone. In the past decade great technical progress has been made to allow FUS to overcome these obstacles for completely noninvasive application to the brain [28–30]. These methods use acoustic simulation based on CT scans of the skull bone to determine the phase and amplitude corrections for the phased array [31–33] and MR temperature imaging (MRTI) to monitor the heating [34]. These systems for thermal ablation are currently being tested in clinical trials [35, 36].

6 Using Focused Ultrasound with Microbubbles to Transiently Open the BBB

There is a good deal of evidence showing that ultrasound can be used to permeate blood–tissue barriers. Large molecules and genes can cross the plasma membrane of cultured cells after application of acoustic energy [37]. Indeed, electron microscopy has revealed ultrasound-induced membrane porosity in both in vitro and in vivo experiments [38]. High-intensity focused ultrasound has been shown to allow selective and nondestructive disruption of the BBB in rats [25]. If microbubbles are introduced to the blood stream prior to focused US exposure, the BBB can be transiently opened at the ultrasound focus without acute neuronal damage [21]. Thus, the introduction of cavitation nuclei into the blood stream can confine the ultrasound effects to the vasculature and reduce the intensity needed to produce BBB opening (Fig. 1). This can diminish the risk of tissue damage and make the technique more easily applied through the intact skull.

6.1 Mechanisms of Ultrasound/Microbubble BBB Disruption

Several hypotheses on the mechanism of BBB disruption with microbubbles and ultrasound have been proposed [39]. Since an ultrasound wave causes bubbles to expand and contract in the capillaries, the expansion of larger bubbles could fill the entire capillary lumen, resulting in a mechanical stretching of the vessel wall. This in turn could result in the opening of the tight junctions. This interaction could create a change in the pressure in the capillary to evoke biochemical reactions that trigger the opening of the BBB. Moreover, bubble oscillation may also reduce the local blood flow and induce transient ischemia, which could trigger BBB opening. Finally, the bubbles could collapse during sonication, causing localized shock waves and fluid jets. Such mechanical effects may be responsible for the opening of the BBB and could play an important role in tissue damage induced at high-pressure amplitudes. In a recent study, focused ultrasound pulses in the presence of Optison® resulted in disruption of the BBB without indicators for inertial cavitation in vivo [23]. These results suggest other mechanisms of ultrasound and microbubble interactions in opening the BBB.

6.2 Morphology of BBB Opening

At the morphological level several avenues of transcapillary passage after ultrasound sonication have been identified. These included transcytosis, passage through endothelial cell cytoplasmic openings, opening of tight junctions, and free passage

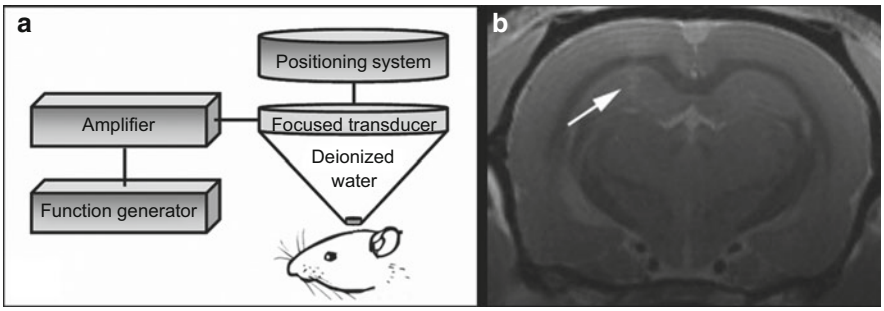

Fig. 1 Example of blood–brain disruption by ultrasound and microbubbles in a rat brain. (**a**) The right hemisphere of a male Wistar rat was insonated with a 500 kHz transducer adapted to a stereotactic positioning system. The transducer was driven by a function/arbitrary waveform generator and amplifier. (**b**) To demonstrate successful opening of the BBB, rats underwent magnetic resonance imaging 30 min after insonation. Gadolinium-enhanced T1-weighted images showed a slight contrast enhancement in the focus of the insonation site (see *arrow*)

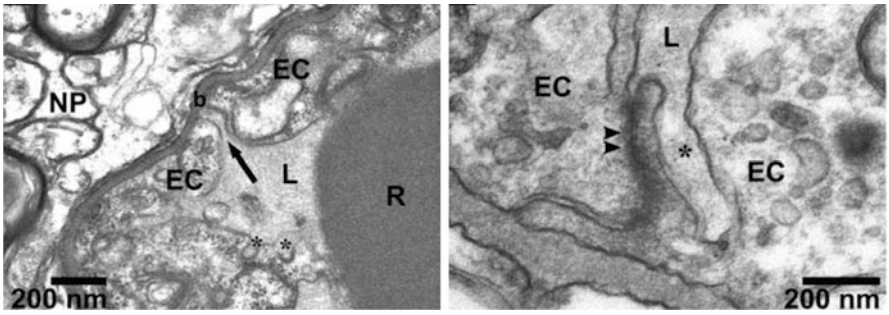

Fig. 2 Electron micrographs of BBB disruption with ultrasound (0.55 W) in the presence of microbubbles. *Left*: A transendothelial channel (*arrow*) exposes the basement membrane (b) to the lumen (L). (*) 2 plasmalemmal pits at the luminal surface of the endothelial cell are shown. *Right*: Deep channel-like invagination (*) in an edematous-looking endothelial cell (EC, *right*). The interendothelial cleft (*arrowheads*) near the invagination does not appear to be widened. *EC* endothelial cell, *NP* neuropil, *R* red blood cell. Adapted from Sheikov et al. [39]

through injured endothelium [39] (Fig. 2). One study investigated the integrity of the tight junctions (TJs) in rat brain microvessels after BBB disruption by ultrasound bursts (1.5-MHz) in combination with Optison [40]. BBB disruption, as evidenced by leakage of i.v. administered horseradish peroxidase (HRP) and lanthanum chloride, was paralleled by the apparent disintegration of the TJ complexes, the redistribution and loss of the immunosignals for occludin, claudin-5, and ZO-1. At 6 and 24 h after sonication, no HRP or lanthanum leakage was observed and the barrier function of the TJs, as indicated by the localization and density of immunosignals, appeared to be completely restored. The results of these studies demonstrate that the effect of ultrasound upon TJs is very transient, lasting less than 4 h.

6.3 Kinetics of BBB Opening

Information on how long the BBB remains open after sonication with ultrasound and microbubbles has been variable. This may be due to the different methods used to demonstrate BBB opening. In one study, BBB opening with HIFU was reported to occur at up to 72 h after sonication. Light microscopy was used to demonstrate either entirely preserved brain or tissue damage in a small volume within the region of BBB opening. Electron microscopic examinations in this study showed opening of capillary endothelial cell tight junctions [25]. Using acoustic power levels ranging from 0.2 to 11.5 W with a burst length of 10 or 100 ms and repetition frequency of 1 Hz another group reported that BBB opening as documented with MRI contrast imaging declined after 6 h and was not demonstrable after 24 h [21].

Recently BBB opening and closure was studied under magnetic resonance imaging (MRI) guidance in a rat model [41]. MRI contrast agents (CA) of different hydrodynamic diameters (1–65 nm) were employed to estimate the largest molecular size permissible across the cerebral tissues. To estimate the duration of the BBB opening, CA was injected at various times post-BBB disruption (12 min to 24 h). A T(1) mapping strategy was developed to assess CA concentration at the ultrasound (US) focal point. Based on the experimental data and BBB closure modeling, a calibration curve was obtained to compute the half closure time as a function of CA hydrodynamic diameter. These findings provide an important basis for optimal design and delivery of nanoparticles to the brain.

6.4 Safety of Opening the BBB

The effect of peak rarefactional pressure amplitudes up to 3.1 MPa have been evaluated in rabbit brains [42]; 10-ms exposures with a frequency of 690 kHz and a repetition frequency of 1 Hz over a duration of 20 seconds were used. Using contrast-enhanced MR images to detect localized BBB disruption after sonication, BBB disruption was demonstrated at pressure amplitudes starting at 0.4 MPa. At 0.8 MPa 90 % and at 1.4 MPa, 100% of the sonicated locations showed enhancement. The histological findings following 4 h survival indicated that brain tissue necrosis was induced in approximately 70–80% of the sonicated locations at a pressure amplitude level of 2.3 MPa or higher. At lower pressure amplitudes, small areas of erythrocyte extravasation were seen. In another recent study, pulsed ultrasound exposures using a frequency of 1.63 MHz, a burst length of 100 ms, pulse repetition frequency of 1 Hz, and duration of 20 s with pressure amplitudes ranging from 0.7 to 1.0 MPa were performed in the brains of 24 rabbits [24]. MRI was used to document BBB disruption through documentation of contrast enhancement with gadolinium. Whole brain histological examination was performed using hematoxylin and eosin staining for general histology, vanadium acid fuchsin-toluidine blue staining for ischemic neurons, and TUNEL staining for apoptosis. The study was able to show that only a few cells in

some of the sonicated areas showed evidence for apoptosis or ischemia. No ischemic or apoptotic regions were detected that would indicate a compromised blood supply. Importantly, no delayed effects were observed either by MRI or histology up to 4 weeks after sonication. These results demonstrate that ultrasound-induced BBB disruption is possible without inducing substantial vascular damage that would result in ischemic or apoptotic death to neurons. However, the fact that red blood cell extravasation into tissue follows ultrasound exposure indicates that BBB injury has occurred and that the method cannot be considered as totally harmless. This must be taken carefully into account when considering this technique for therapeutic applications of brain disease.

Other studies have addressed the question of whether burst ultrasound in the presence of a US contrast agent using parameters similar to those used in diagnostic transcranial Doppler examinations in humans can cause tissue damage. In one experiment, rabbit brains were sonicated with 1.5-MHz, 10-μs bursts repeated at a frequency of 1 kHz at temporal peak acoustic pressure amplitudes ranging from 2 to 12.7 MPa for 20-s duration [43]. Results of MRI contrast enhancement and histological findings showed that brain tissue damage was induced at a pressure amplitude level of 6.3 MPa. This consisted of vascular wall damage, hemorrhage, and, sometimes, necrosis. The authors observed occasional mild vascular damage in about 50% of the sonicated locations at all pressure values tested. However, signs of ischemia or apoptosis were not found. These results provide good evidence that US exposure levels currently used for blood flow measurements in the brain are below the threshold of blood–brain barrier opening or brain tissue damage.

Further work investigated the integrity of the BBB in humans after bubble destruction of two ultrasound contrast agents (Levovist™ and Optison™) with transcranial color-coded sonography [44]. MRI examinations with gadolinium (Gd-MRI) were performed during both early and late phases after insonation. Ultrasound transmission power levels were kept within diagnostic limits and resembled standard settings in brain perfusion studies. Using a triple dose of gadolinium to increase sensitivity and considering the potential time dependence of BBB changes, the authors showed that insonation of Levovist and Optison did not lead to any detectable difference in T1 signal intensities in 2 defined brain regions in Gd-MRI. Moreover, they found no signs of focal signal enhancement or focal brain damage. This study provides further evidence for the safety of these contrast agents and of the exposure levels of current ultrasonic equipment used for transcranial investigations. The results are reassuring but not totally conclusive in terms of ultrasound safety, since hypothetically more subtle effects of ultrasound and microbubbles on the BBB might be missed by Gd-MRI. MRI performed with an ultrasmall particle of iron oxide may be an alternative to triple-dose Gd-MRI in detecting such an effect.

Although much effort has been undertaken to demonstrate the safety of BBB opening with ultrasound and microbubbles, further work is needed to elucidate the molecular effects of this application. Recent data demonstrate that at the upper thresholds of acoustic pressure for safe BBB opening, a reorganization of gap-junctional plaques in both neurons and astrocytes may occur [45]. This is important because gap junctions allow transfer of information between adjacent

cells and are responsible for tissue homeostasis. Likewise, there is evidence that focused ultrasound-induced opening of the BBB in the presence of ultrasound contrast agents can lead to increased ubiquitinylation of proteins in neuronal cells [46], indicating that brain molecular stress pathways are affected by this treatment. Further studies have concentrated on whether leakage of albumin during transient BBB opening with ultrasound could be potentially dangerous. This is because albumin uptake into neurons has been shown to be neurotoxic. Fortunately, ultrasound-induced BBB opening leads to albumin extravasation which is phagocytized predominantly by activated microglia, astrocytes, and endothelial cells [47]. This rapid albumin clearance by microglia likely prevents neuronal cell injury after BBB opening.

6.5 MRI-Guided Focused Ultrasound BBB Opening in Nonhuman Primates

The BBB in monkeys has been opened transcranically using focused ultrasound in conjunction with microbubbles [48]. A passive cavitation detector was used to identify and monitor the bubble behavior. During sonication, the cavitation spectrum was found to be region-, pressure-, and bubble-dependent, providing real-time feedback regarding the opening occurrence and its properties. These findings demonstrate feasibility of transcranial, cavitation-guided BBB opening using FUS and microbubbles in noninvasive human applications [48]. Similar experiments in nonhuman primates indicate that harmonic emissions can be used to control focused ultrasound-induced BBB disruption [49].

A recent study determined whether targeted drug delivery can be applied safely and reliably and in a controlled manner on rhesus macaques using a focused ultrasound system [50]. The results identified a clear safety window during which BBB disruption could be produced without evident tissue damage. The acoustic pressure amplitude where the probability for BBB disruption was 50% was half of the value that would produce tissue damage. Acoustic emission measurements were used for predicting BBB disruption and damage. In addition, repeated BBB disruption to central visual field targets was performed over several weeks in animals trained to conduct complex visual acuity tasks [50]. All animals recovered from each session without behavioral deficits, visual deficits, or loss in visual acuity. Together, the findings show that BBB disruption can be reliably and repeatedly produced without evident histological or functional damage in a clinically relevant nonhuman primate animal model.

7 Drugs Delivered to the Brain with Focused Ultrasound

A large number of therapeutic agents have been delivered to the brain using focused ultrasound and microbubbles. Dopamine D(4) receptor-targeting antibody has been injected intravenously and shown to recognize antigen in the murine brain following

disruption of the BBB with ultrasound [22]. Likewise, doxorubicin, a chemotherapeutic drug that does not cross the BBB, has been administered to the brain using ultrasound and microbubbles [51, 52]. Different levels of doxorubicin in the brain were accomplished through alteration of the microbubble concentration [51]. Other chemotherapeutic agents such as BCNU [53], methotrexate [54], cytarabine [55], and temozolomide [56] have been administered to the brain with focused ultrasound and microbubbles. Ultrasound-enhanced chemotherapy has also been packaged in liposomes [51, 57], targeted liposomes [58], and magnetic particles [59], which allow for MRI-based tracking and enhanced delivery via magnetic targeting.

Others have delivered trastuzumab, an antibody-based agent used for HER2-positive breast cancer [60, 61], and boronophenylalanine, which is used for boron neutron capture therapy, to the brain and to brain tumor models [62, 63]. FUS-induced BBB disruption has also been shown to improve the delivery of natural killer cells in a brain tumor model [64].

7.1 BBB Opening and Sonoporation for Gene Therapy to the Brain

Ultrasound may be a valuable tool in gene therapy by virtue of its ability to enhance transgene expression through a process termed sonoporation. Simple exposure to ultrasound has been shown to enhance transgene expression in vascular cells by up to tenfold after naked DNA transfection. Likewise, transfection studies performed using marker genes that do not exert a fluorescent protein demonstrated that ultrasound consistently increased gene expression in cell lines such as HeLa, NIH t-3, and COS-1 cells [65]. The enhancement of transfection occurred at levels of ultrasound of about 0.5 W/cm^2 and duration of exposure of only about 15 s and did not appreciably heat the cells or adversely affect their survival. Depending on the type of cell and conditions of sonoporation the transfection efficacy has been as high as 20% [66]. Recently, chimeric adeno-associated virus 2/1 (AAV2/1) particles containing the coding region for the LacZ gene were efficiently delivered into the rat brain upon intravenous (IV) administration after BBB opening by focused ultrasound and microbubbles [67]. Histochemical LacZ staining combining double immunofluorescence with antibodies against tubulin III allowed identification of large amounts of neurons expressing the enzymatically active protein. It is likely that BBB opening with ultrasound is synergistic with sonoporation in achieving effective gene transduction.

7.2 Targeted Drug Delivery

Not only can microbubbles be used to enhance the effects of ultrasound, they may also be employed as carriers of therapeutic agents [65, 68]. Several recent studies have loaded chemotherapy and other agents into the microbubbles used for the BBB

disruption [59, 69, 70], which offers the possibility of achieving even higher local payload at the targeted region.

There are a number of ways to entrap different drugs with microbubbles. One technique is to incorporate them into the membrane- or wall-forming materials that stabilize microbubbles. Charged drugs can be stabilized in or onto the surfaces of microbubbles by virtue of electrostatic interactions. In this way, cationic lipid-coated microbubbles can bind DNA, which is a polyanion and binds avidly to cationic (positively charged) microbubbles. Drugs can also be incorporated into the interior of microbubbles (gas-filled microspheres). Another way to entrap drugs in microbubbles is to create a layer of oil (e.g., triacetin) to stabilize the outer surface of the bubble. Hydrophobic drugs can then be incorporated into the oil layer. Regardless of the technique used to incorporate the drugs, they are released when ultrasound energy cavitates the microbubble. These methods for making drug-carrying microbubbles are most applicable to drugs that are highly active. This is the case for gene-based drugs, in which the amount of gene injected is usually on the order of micrograms or milligrams. Therefore, large volumes of bubbles are not required to deliver highly active drugs such as genes.

Ultrasound may also be used to target liposomal drug delivery. Mechanisms of enhancement include acoustic cavitational effects and acoustic radiation force [71]. Novel developments include the combination of nanotechnology with microbubbles for drug delivery [72, 73].

8 Conclusion

There is significant evidence that ultrasound and microbubbles can be used to open the BBB for targeted delivery of macromolecular agents to the brain. Possible ways in which substances cross the BBB after application of this novel approach include transcytosis, passage through endothelial cell cytoplasmic openings, opening of tight junctions, and free passage through injured endothelium. The exact mechanism by which ultrasound and microbubbles exert this effect remains unclear. Although cavitation was previously thought to be primarily responsible for opening the BBB, recent work has demonstrated disruption in the absence of indicators for inertial cavitation. Several studies have addressed the safety of this method for opening the BBB. Although relatively little tissue damage occurs at low acoustic intensities capable of opening the BBB, no investigation has demonstrated a total lack of BBB injury when using ultrasound and microbubbles. Further experiments that address the effect of ultrasound and microbubbles upon the various routes of transport across the BBB are necessary. In particular, an understanding of how they may influence transport mechanisms such as receptor-mediated endocytosis, absorptive-mediated transport, and carrier-mediated efflux would be helpful. Moreover, investigations aimed at elucidating how ultrasound and microbubbles interact at the molecular level of the BBB could provide information for design of new drugs that could be targeted with ultrasound to treat a variety of brain diseases. Such

studies could also provide valuable information on possible molecular bioeffects of ultrasound on the BBB, thus contributing to our understanding of whether ultrasound and microbubbles may influence CNS disease processes, both in states with and without previous BBB disruption.

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