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REVIEW

The blood–brain barrier in health and disease: Important unanswered questions

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The blood vessels vascularizing the central nervous system exhibit a series of distinct properties that tightly control the movement of ions, molecules, and cells between the blood and the parenchyma. This "blood–brain barrier" is initiated during angiogenesis via signals from the surrounding neural environment, and its integrity remains vital for homeostasis and neural protection throughout life. Blood–brain barrier dysfunction contributes to pathology in a range of neurological conditions including multiple sclerosis, stroke, and epilepsy, and has also been implicated in neurodegenerative diseases such as Alzheimer's disease. This review will discuss current knowledge and key unanswered questions regarding the blood–brain barrier in health and disease.

Blood vessels provide the vital infrastructure for delivery of oxygen and essential nutrients throughout the body, and the term "blood–brain barrier" (BBB) is used to describe the unique characteristics of the blood vessels that vascularize the central nervous system (CNS; Saunders et al., 2008; Zlokovic, 2008; Obermeier et al., 2013). The BBB is not a single physical entity but rather the combined function of a series of physiological properties possessed by endothelial cells (ECs) that limit vessel permeability. The BBB tightly regulates the movement of ions, molecules, and cells between the blood and the parenchyma and is thus critical for neuronal function and protection. The interaction of ECs with different neural and immune cells is commonly referred to as the neurovascular unit (NVU; Fig. 1 A). The complex properties that define the BBB are often altered in disease states, and BBB dysfunction has been identified as a critical component in several neurological conditions. This review will discuss BBB development, regulation, and dysfunction, emphasizing important unanswered questions.

The NVU

ECs

A cross-section of an artery or vein might contain dozens of ECs, while in the smallest capillaries, a single EC forms the vessel circumference (Aird, 2007). In all tissues, adherens junctions, composed of vascular endothelial cadherin and catenins, comprise the basic cellular adhesions between ECs, supporting the integrity of the vascular tube and regulating tensile forces. PECAM1 is a critical regulator of EC adhesion, promoting adherens junction formation (Biswas et al., 2006; Privratsky and Newman, 2014). CNS ECs are further specialized to restrict paracellular and transcellular movement of solutes.

Tight junctions (TJs). TJs are cell adhesions consisting of multiple transmembrane proteins that directly interact via their extracellular components, linking two cells' membranes together (Furuse, 2010; Fig. 1 B). CNS TJs are specialized in their molecular and structural P-face composition to form a high-resistance electrical barrier, and the specific combination of TJ proteins at the BBB determines its paracellular permeability.

The composition of claudins, a family of 27 four-pass transmembrane proteins, within a TJ is thought to determine the size and charge selectivity of paracellular permeability (Amasheh et al., 2005; Hou et al., 2006; Furuse et al., 1999). Claudin 5 (CLDN5) is the most abundant claudin at the BBB, and *Cldn5* knockout mice exhibit size-selective leakage of the BBB and die at birth (Morita et al., 1999; Nitta et al., 2003). ECs in peripheral vascular beds also express CLDN5, and thus its expression alone is not sufficient for barrier formation. Other key components of TJs include claudin 12, occludin, and junctional adhesion molecules. Cytoplasmic proteins including ZO-1, ZO-2, ZO-3, cingulin, JACOP, MAG1, and MUPP1 aid TJ formation, binding TJs to the cytoskeleton, adherens junctions, and polarity complexes (Umeda et al., 2004; Tietz and Engelhardt, 2015; Sawada, 2013). It is still unknown why CLDN5 and ZO-1 expression does not confer the same low paracellular permeability in peripheral vessels as in the CNS. Expression data suggest that the answer might lie in the CNS-specific enrichment of certain cytoplasmic adaptors (e.g., JACOP, MPP7) and tricellular TJ molecules such as LSR and MARVELD2 (Daneman et al., 2010a; Sohet et al., 2015).

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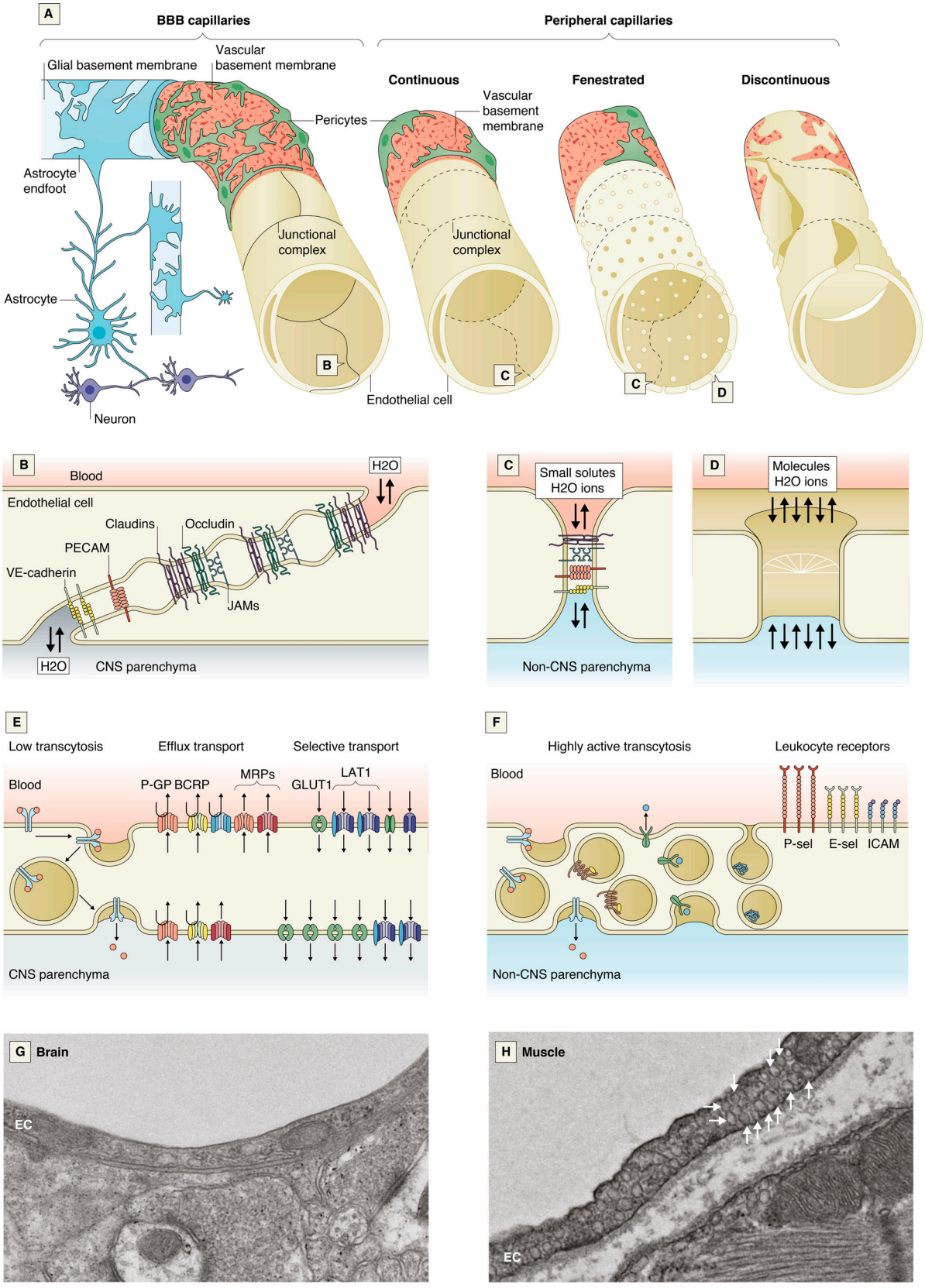


Figure 1. **Cellular and molecular properties of the BBB. (A)** A schematic comparison of the BBB capillaries with the continuous nonfenestrated, continuous fenestrated, and discontinuous capillaries found in peripheral organs. **(B–F)** Schematics of the molecular composition of junctional complexes of BBB ECs (B) and of ECs in peripheral organs (C), peripheral endothelial fenestra (D), and transport mechanisms in CNS ECs (E) and peripheral ECs (F). **(G and H)** Electron micrographs of a mouse brain EC (G) and a mouse muscle EC, which is densely packed with vesicles (arrows; H). BCRP, breast cancer resistance protein; GLUT1,

glucose transporter 1; ICAM, intercellular adhesion molecule; JAM, junctional adhesion molecule; LAT1, L-type amino acid transporter 1; MRP, multidrug resistance-associated protein; PECAM-1, platelet and EC adhesion molecule 1; P-GP, p-glycoprotein; VE-cadherin, vascular endothelial cadherin; P-sel, P-selectin; E-sel, E-selectin.

Transcellular permeability. Peripheral ECs possess properties that confer transcellular permeability, including high rates of caveolin-mediated transcytosis, diaphragm-containing pores termed fenestrae, or large discontinuities or gaps in the endothelial layer (Aird, 2007; Fig. 1 A). In contrast, CNS ECs form a continuous lining that lacks fenestrations and has low levels of transcytosis, properties that greatly limit transcellular permeability (Fig. 1, A–H). MFSD2A, enriched in CNS ECs, limits caveolin-dependent transcytosis by regulating EC lipid composition (Ben-Zvi et al., 2014; Nguyen et al., 2014; Andreone et al., 2017). Plasmalemma vesicle-associated protein (PLVAP) is important both for vesicle formation and fenestrations. Its down-regulation in CNS ECs, along with up-regulation of MFSD2A, coincides with BBB formation during embryogenesis (Hallmann et al., 1995; Hnasko et al., 2002; Chow and Gu, 2017).

Transporters. Numerous transporters are enriched in brain ECs, which generally fall into two categories: efflux and solute transporters (Miller, 2015; Nałęcz, 2017; Strazielle and Ghersi-Egea, 2015; Fig. 1 E).

Efflux transporters, concentrated on the luminal side of the membrane, use ATP hydrolysis to transport a wide range of small molecules up their concentration gradients back into the blood (Shen and Zhang, 2010). MDR1/P-glycoprotein (PGP) and breast cancer resistance protein are the most abundant BBB efflux proteins and limit entry of many xenobiotics and endogenous molecules, including steroids such as aldosterone (Hindle et al., 2017).

Solute transporters carry specific substrates down their concentration gradients, ensuring barrier passage to specific nutrients, such as glucose, that are vital for energy and homeostasis (Simpson et al., 2007). Transport of glucose, lactate, amino acids, and fatty acids occurs via GLUT1 (*Slc2a1*), MCT1 (*Slc16a1*), LAT1 (*Slc7a5*), and MFSD2A, respectively (Boado et al., 1999; Cornford et al., 1994; Kido et al., 2000; Nguyen et al., 2014). Other transporters provide receptor-mediated vesicular transport, including the transferrin receptor (TFR1) and low-density lipoprotein receptors (Jefferies et al., 1984; Méresse et al., 1989). Substrate-specific solute transporters can also be important for removing molecules from the CNS; lipoprotein receptor-related protein-1 (LRP1) is a critical transporter for eliminating β-amyloid (Shibata et al., 2000; Storck et al., 2016).

Leukocyte adhesion molecules. Leukocyte adhesion molecules on EC surfaces initiate binding of leukocytes, the first step of their entrance into tissues (Bevilacqua, 1993). Healthy CNS ECs exhibit lower leukocyte adhesion molecule expression compared with peripheral ECs (Daneman et al., 2010a), and thus there is minimal leukocyte crossing of the BBB in health (Fig. 1, E and F; and Fig. 2 A). Instead, CNS immune surveillance by lymphocytes in health occurs primarily at the blood–CSF interfaces of the meninges and choroid plexus (Ransohoff and Engelhardt, 2012; Kipnis et al., 2012; Shechter et al., 2013; Box 1).

The NVU

The luminal surface of the capillary endothelium is covered by the EC glycocalyx (Ausprunk et al., 1981a, b; Pillinger and Kam, 2017). Brain ECs have a denser glycocalyx than peripheral vasculature; average glycocalyx coverage is 40.1% in brain vessels compared with 15.1% and 3.2% in cardiac and pulmonary vessels, respectively (Ando et al., 2018). This dense network of luminal glycoproteins prevents larger molecules from interacting with the EC. While small dyes such as fluorescein (376 daltons) and Alexa Fluor (643 daltons) permeate the glycocalyx, dextrans (40–150 kD) penetrate <60% of its volume (Kutuzov et al., 2018). In disease, glycocalyx degradation is associated with more severe BBB leakage in models of multiple sclerosis (MS) and cardiac arrest (DellaValle et al., 2018; Zhu et al., 2018).

The abluminal surface of the EC is covered by the basal lamina (Fig. 1 A), a structural matrix of laminins, fibronectin, collagens, tenascin, and proteoglycans. This basement membrane (BM) surrounds ECs and pericytes, acting as an interface for the binding of molecules and migration of cells, while also limiting passage of macromolecules (Del Zoppo et al., 2006). The BM consists of two layers: the inner vascular BM secreted by ECs and pericytes, and the outer glial BM secreted by astrocytes (Sorokin, 2010). These BMs are merged surrounding capillaries but separate at post-capillary venules, creating a CSF-drained perivascular space for immune surveillance (Engelhardt and Ransohoff, 2012).

Mural cells—vascular smooth muscle cells (VSMCs) and pericytes—are found on the abluminal side of blood vessels in all tissues. VSMCs line all larger vessels but are more abundant on arteries and arterioles, forming a complete layer around them (Smyth et al., 2018; Vanlandewijck et al., 2018; Armulik et al., 2011). VSMC myosin fibers regulate blood flow via vasoconstriction and vasodilation (Aird, 2007). Pericytes are embedded in the BM and form an incomplete layer on the surface of CNS micro-vessels (Fig. 1 A). Pericytes play a key role in the regulation of angiogenesis, vascular remodeling, vascular tone, and BBB formation (Daneman et al., 2010a; Armulik et al., 2005, 2010; Winkler et al., 2011). Perivascular fibroblasts are found in the walls of large vessels (Vanlandewijck et al., 2018); however, their role in cerebrovascular function remains unexplored.

Astrocytes extend cellular processes terminating in endfeet that ensheath synapses, nodes of Ranvier, and ECs, contacting the BM around parenchymal vessels (Fig. 1 A). This astrocyte–endothelial interaction is critical in regulating blood flow (Mishra et al., 2016). Several groups have shown that CSF flows between the BM and astrocyte endfeet of arteries and capillaries, with arteriole pulsations driving bulk fluid flow through the parenchyma, although others have argued about the extent of bulk flow (Abbott et al., 2018; Hladky and Barrand, 2019). This "glymphatic" system helps to clear interstitial solutes such as amyloid via paravenous drainage pathways (Iliff et al., 2012; Xie et al., 2013; Mestre et al., 2018) and has been visualized in human

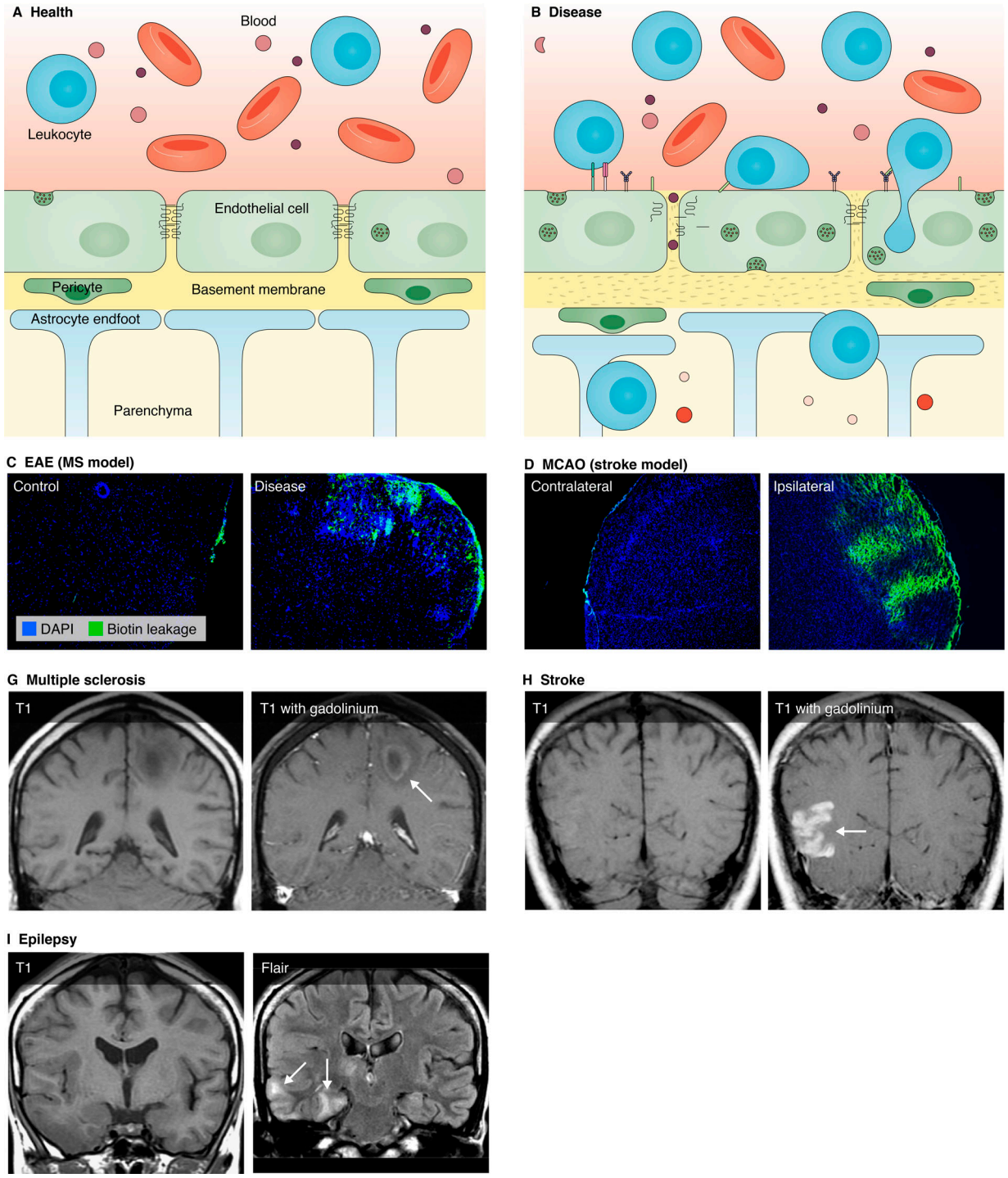
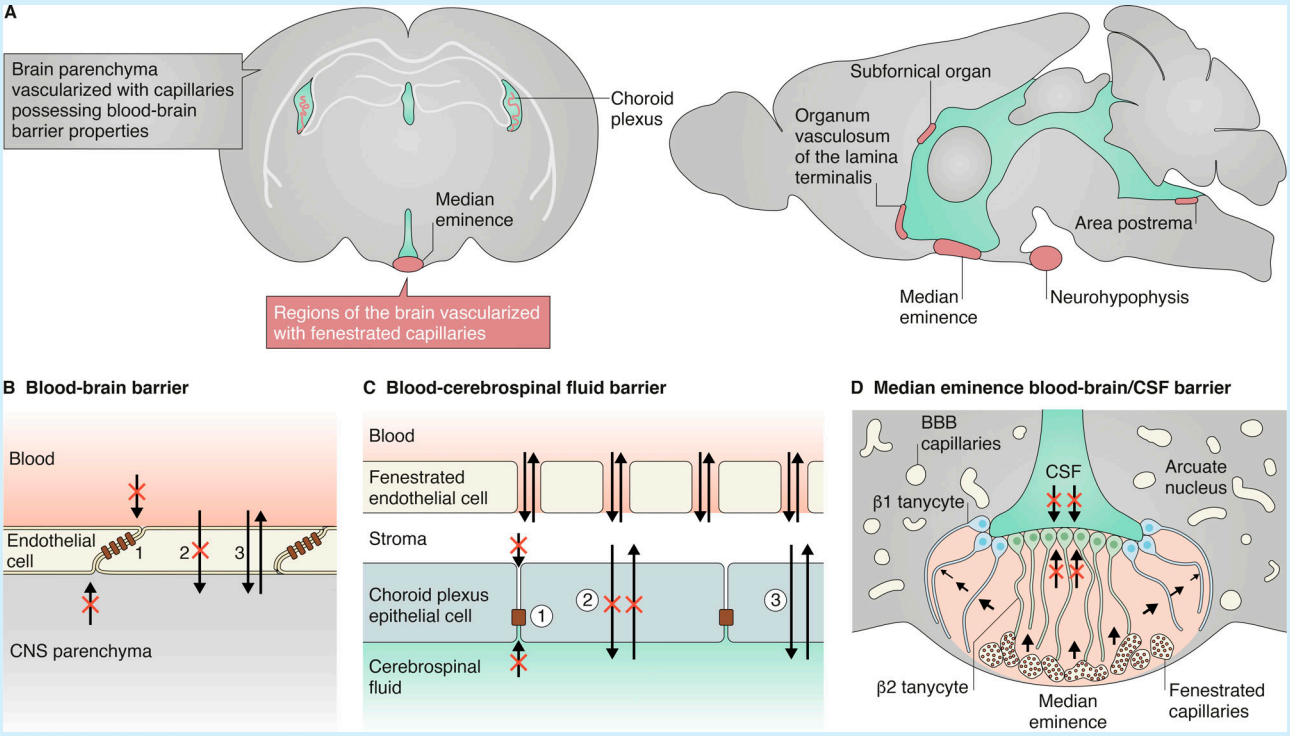


Figure 2. **Dysfunction of the BBB in disease. (A and B)** Schematic representation of the NVU in health and disease. **(A)** In health, CNS ECs exhibit TJs, low rates of transcytosis, and low expression of leukocyte adhesion molecules. Pericytes embedded in the BM help to maintain the barrier, and astrocyte endfeet contact the BM. **(B)** In disease, TJs are internalized or down-regulated, rates of transcytosis increase, increased leukocyte adhesion molecule expression leads to increased leukocyte extravasation, the BM degrades, and pericytes and astrocytes less tightly cover the ECs. Made with BioRender. **(C–F)** BBB disruption in models of MS, traumatic brain injury, and stroke. Sections showing BBB leakage to a sulfo-*N*-hydroxysulfosuccinimide-biotin tracer (green) in three disease models. **(C and D)** A section of spinal cord from a healthy mouse (C) and from the EAE model of MS (D). **(E and F)** The contralateral (E) and ipsilateral (F) hemispheres in a coronal section of the middle-cerebral artery occlusion model of ischemic stroke. **(G–I)** BBB leakage and edema in human cases of MS, stroke, and epilepsy. T1 weighted MRI images with gadolinium enhancement to show BBB leakage in (G) MS lesions and (H) stroke infarct. **(I)** T1 weighted and fluid attenuation inversion recovery (FLAIR) MRI images showing edema in epilepsy. Images courtesy of Dr. John Hesselink, University of California, San Diego, San Diego, CA.

Box 1. Barriers of the CNS



There are small regions of the brain that lack an endothelial BBB and are instead vascularized by permeable fenestrated capillaries. In these regions, a specialized glial barrier takes on the role of the endothelial BBB. **(A)** Among these regions is the choroid plexus, the structure that generates most of the cerebrospinal fluid. The specialized ependymal epithelial cells of the choroid plexus surround its fenestrated capillaries and filter the fluid that enters through fenestrae to generate the cerebrospinal fluid. The choroid plexus epithelial cells possess similar properties as the ECs of the BBB. **(B and C)** These properties include (compare panel B to C): (1) a dense formation of junctional complexes that restrict paracellular diffusion of hydrophilic solutes; (2) expression of efflux transporters and low rates of transcytosis that limit transcellular movement of molecules; and (3) expression of selective transporters that import necessary nutrients or export wastes (Marques et al., 2017). CVOs are vascularized by fenestrated capillaries and allow a small subset of neurons and glia to sense blood-derived signals or secrete hormones into the blood to regulate peripheral processes such as fluid homeostasis, osmoregulation, body temperature, energy balance, and inflammation. The subfornical organ, area postrema, and organum vasculosum of the lamina terminalis are the sensory CVOs, while the median eminence and neurohypophysis are the secretory CVOs. Each of these CVOs possess a glia-derived, cellular barrier generated by tanycytes or tanycyte-like cells that limit further diffusion of blood-derived solutes into neighboring regions or the cerebrospinal fluid (Ganong, 2000; Miyata, 2015). **(D)** In the median eminence, β1 tanycytes limit diffusion of solutes originating from the ventrally localized fenestrated capillaries into the arcuate nucleus while β2 tanycytes restrict chemical exchange between the median eminence and CSF (Miyata, 2015; Langlet et al., 2013).

patients via magnetic resonance imaging (MRI; Meng et al., 2019; Fultz et al., 2019). Expression of water channel aquaporin-4 in astrocyte endfeet has been reported to play a critical role in the movement of CSF into the parenchyma (Haj-Yasein et al., 2011; Iliff et al., 2012; Mestre et al., 2018).

CNS-associated macrophages, which express a gene signature of *Mrc1* (CD206), *Pf4*, *Cbr2*, *Ms4a7*, and *Stab1*, include choroid plexus, dural, leptomeningeal, and perivascular macrophages (Kierdorf et al., 2019; Jordão et al., 2019). Perivascular macrophages are elongated cells residing between the astrocytic endfeet and parenchymal vessels (primarily arteries and veins). While nonmotile, they extend processes along the perivascular space, providing the first line of defense by collecting debris (Hickey and Kimura, 1988; Prinz et al., 2017). Microglia, derived from yolk-sac progenitor cells (Takahashi et al., 1989; Alliot et al., 1999), reside within the CNS parenchyma. They possess a highly ramified morphology and perform immune surveillance, phagocytosing infectious agents that evade the barrier (Streit et al., 2005; Prinz et al., 2011). Microglia have also been shown to regulate BBB resealing following vascular injury and disease (Fernández-López et al., 2016; Lou et al., 2016). In disease states, leukocytes such as neutrophils and T cells can interact with the BBB, increasing permeability via release of cytokines, reactive oxygen species, and other mediators of barrier dysfunction (Hudson et al., 2005; Persidsky et al., 1999).

Thus, the BBB is a series of structural, transport, and metabolic barriers that together limit CNS entry of nonspecific molecules while ensuring the delivery of specific nutrients, thereby controlling the extracellular environment. Several important questions remain. What exactly gets through the barrier, how much, and by which route(s)? The barrier is not absolute. Small, nonpolar molecules enter unrestricted through passive diffusion unless they are substrates of efflux transporters. In contrast, large or polar molecules are greatly restricted in access unless they are substrates of specific nutrient transporters. However, even large molecules enter the CNS parenchyma at 0.1% of their blood concentration through an unsaturable mechanism (Yu and Watts, 2013; Poduslo et al., 1994), likely via nonspecific transcytosis, which occurs at low rates. Future work fully characterizing the substrate specificity

of BBB transporters and their dynamic response to various stimuli may enable manipulation of these transporters for CNS drug delivery.

There is heterogeneity of gene expression among different branches of the vascular tree (Macdonald et al., 2010; Vanlandewijck et al., 2018; Murugesan et al., 2011). It is thought that this heterogeneity enables capillaries, arterioles, and venules to be specialized for regulation of solute transport, blood flow, and inflammation, respectively. But what is the relevance of this arteriovenous zonation in terms of barrier function? How is this phenotypic continuum programmed during development?

It is also currently unknown whether there is regional heterogeneity of the BBB. Several regions of the CNS termed circumventricular organs (CVOs)—the area postrema, subfornical organ, pineal gland, and median eminence of the hypothalamus—have fenestrated capillaries that lack BBB properties (Box 1; Gross, 1992). This vascular permeability allows for the exchange of sensory or secretory signaling molecules between the brain and blood, enabling CVO-mediated regulation of body homeostasis. Much less is known about whether there are region-specific differences among areas with a functional BBB, including the cortex, hippocampus, cerebellum, and white matter tracks, and whether BBB heterogeneity might contribute to the specialized function of a particular brain region or render that region more vulnerable to disease.

BBB formation and regulation

How BBB properties are regulated in development and maintained in adulthood remains a fundamental field of study (Blanchette and Daneman, 2015). Transplanted CNS tissue is sufficient to induce BBB-like properties in the gut endothelium in vivo (Stewart and Wiley, 1981), suggesting a role for the neural microenvironment in BBB formation. Transplantation of astrocytes into nonneural tissues of adult rats induces barrier properties in local ECs (Janzer and Raff, 1987), and several astrocyte-secreted proteins are sufficient to induce EC barrier properties in vitro and in vivo, including Sonic hedgehog, angiotensin, and basic fibroblast growth factor (Alvarez et al., 2011; Sobue et al., 1999; Wosik et al., 2007). However, barrier properties arise during development before astrogliogenesis takes place (Ben-Zvi et al., 2014; Daneman et al., 2010a; Sohet et al., 2015; Sauvageot and Stiles, 2002), delaying astrocytic contact with ECs does not affect barrier formation (Saunders et al., 2016), and laser ablation of astrocyte endfeet in adult mice does not induce BBB leakage (Kubotera et al., 2019). These data suggest that astrocytes are not necessary for BBB formation, but perhaps provide dynamic BBB regulation in response to specific stimuli. For instance, reactive astrocytes have been shown to be critical for BBB repair following neurological disease (Bush et al., 1999).

Neural progenitor–derived Wnt signaling induces BBB properties during the angiogenic program (Daneman et al., 2009; Liebner et al., 2008; Stenman et al., 2008; Ye et al., 2009; Wang et al., 2012; Zhou and Nathans, 2014; Cho et al., 2017). Loss of Wnt signaling disrupts angiogenesis specifically in the CNS, reducing the expression of TJ proteins and solute transporters while increasing PLVAP (Daneman et al., 2009; Liebner et al., 2008; Stenman et al., 2008). Interestingly, β-catenin activation in the more permeable CVO vessels is sufficient to induce BBB properties (Benz et al., 2019; Wang et al., 2019). These data suggest that the same signal that drives angiogenic invasion of the CNS also induces initial BBB properties within the endothelium.

Pericytes are also essential in BBB development, and EC recruitment of pericytes is concomitant with development of barrier properties. The BBB fails to completely seal in mice lacking CNS pericytes, as they inhibit nonspecific transcytosis and leukocyte adhesion molecule expression (Daneman et al., 2010b; Armulik et al., 2010).

Thus, the BBB is regulated by a series of different cellular interactions: BBB "tight" properties are induced during the angiogenic program by Wnt signaling, "leaky" properties are inhibited by pericytes, and the overall phenotype of the BBB can be influenced by astrocytes, pericytes, and other cell types throughout life.

Important questions still remain. How is the induction of different BBB properties coordinated? Interestingly, Wnt signaling induces endothelial secretion of platelet-derived growth factor B, the key ligand for pericyte recruitment (Reis et al., 2012), suggesting that induction of different BBB properties is tightly coordinated via Wnt-mediated pericyte recruitment. Are the same signals required for induction also responsible for regulating BBB maintenance in adulthood? Although Wnt signaling decreases in ECs after angiogenesis, this pathway is critical for BBB maintenance; disruption of Wnt signaling in adulthood leads to cell-autonomous loss of TJ integrity and an increase in PLVAP in the retina and cerebellum (Wang et al., 2012). Additionally, pericytes are important for BBB function throughout life (Armulik et al., 2010), suggesting that similar signals are required for BBB formation and maintenance. Do region-specific differences in signaling influence BBB heterogeneity? Different Wnt ligands and receptor complexes have been shown to promote BBB formation in different regions of the CNS (Daneman et al., 2009; Wang et al., 2012, 2018; Zhou et al., 2014); however, it is not clear whether this induces regional heterogeneity or is merely a remnant of dorsal–ventral and rostral–caudal axis specification.

How dynamic is each BBB property in a healthy CNS? Are properties modulated by neural activity or environmental stimuli such as exercise and diet? Single-cell sequencing has revealed vascular changes in response to neural activity (Hrvatin et al., 2018), and neuronal activity has been shown to modulate BBB insulin-like growth factor 1 (Nishijima et al., 2010). However, whether neural activity dynamically regulates specific properties of the BBB to modulate circuit function remains unknown. While exercise might help to protect against BBB dysfunction in aging or disease, solid evidence is still forthcoming (Małkiewicz et al., 2019). A high-fat diet can increase BBB permeability (de Aquino et al., 2019; Salameh et al., 2019; Yamamoto et al., 2019), but the specific BBB properties affected have not been thoroughly characterized. Not only can diet affect the BBB, but the BBB can in turn dynamically regulate nutrient availability; animals entering hibernation up-regulate ketone transporters at the BBB to modulate energy utilization

during inactivity (Andrews et al., 2009). How dynamic are BBB properties over the course of 24 h, and how might these fluctuations influence brain microenvironment and waste clearance? PGP expression levels follow a diurnal pattern (Savolainen et al., 2016; Kervezee et al., 2014), and a circadian clock in glial cells of the *Drosophila melanogaster* BBB regulates xenobiotic efflux (Cuddapah et al., 2019; Zhang et al., 2018), but the extent and functional implications of circadian oscillations at the BBB remain unclear.

Are there differences in the BBB across individuals? Are there sex differences in BBB properties? There is evidence for variation in male and female patient CSF/serum albumin ratio (Parrado-Fernández et al., 2018), and BBB sexual dimorphism has been proposed to underlie differences in response to traumatic brain injury and infection and in proclivity to autoimmune disease (Cruz-Orengo et al., 2014; Jullienne et al., 2018; Maggioli et al., 2016).

How do BBB properties change in age? Several studies have reported age-related decline in BBB function (Mooradian, 1988; Montagne et al., 2015; Erdő et al., 2017), and age-related pericyte dysfunction contributes to BBB permeability (Bell et al., 2010). VCAM1 up-regulation at the BBB is a crucial step in age-related cognitive deficits and increased inflammatory tone (Yousef et al., 2019), highlighting VCAM1 as a potential therapeutic target for age-related neurodegeneration.

BBB dysfunction

BBB dysfunction occurs in a number of diseases, including MS, epilepsy, and stroke. In these conditions, BBB dysfunction is a central element of the pathology, whereas in others, such as Alzheimer's disease (AD), the incidence and extent of breakdown are more controversial and an area of burgeoning research. BBB disruption causes ion dysregulation, edema, and neuroinflammation, which can lead to neuronal dysfunction, increased intracranial pressure, and neuronal degeneration. However, the mechanisms underlying BBB dysfunction and its role in the onset and progression of disease or recovery are not fully understood.

The phrase "BBB breakdown" conjures images of the destruction of a physical wall, allowing an unabated flow of molecules from the blood into the brain. However, the BBB is not a wall but a series of physiological properties, and a change in just one property (transcytosis, transport) can significantly alter the neural environment (Fig. 2). For instance, dysfunction of GLUT1 glucose transport, LAT1 amino acid transport, and MCT8 thyroid hormone transport across the BBB leads to seizure, autism spectrum, and psychomotor retardation syndromes, respectively (Seidner et al., 1998; Tărlungeanu et al., 2016; Vatine et al., 2017).

Importantly, leakage of nonspecific molecules is distinct from leukocyte extravasation, which occurs via an active trafficking process. Single-cell sequencing has identified many subsets of immune cells with distinct roles in neuroinflammation that likely interact with the BBB in disease (Mrdjen et al., 2018; Jordão et al., 2019; Kierdorf et al., 2019; Masuda et al., 2019; Mundt et al., 2019). Parenchymal ECs up-regulate leukocyte adhesion molecules, thus increasing leukocyte trafficking. P-selectin and E-selectin mediate the rolling of leukocytes along the endothelium, ICAM1 and VCAM1 mediate firm adhesion, and proteins like PLVAP—also up-regulated in disease—aid in transmigration across ECs (Engelhardt and Ransohoff, 2012; Ioannidou et al., 2006). Leukocyte extravasation across the BBB can be either transcellular or paracellular (Carman et al., 2007; Winger et al., 2014). Levels of ICAM1 and PECAM1 can influence T cell diapedesis route (Abadier et al., 2015; Wimmer et al., 2019), and specific subsets of T cells prefer different routes (Lutz et al., 2017).

Thus, the BBB is not an on–off switch, and it is critical to understand the specificities and consequences underlying each instance of dysfunction.

BBB dysfunction in CNS disorders

MS. BBB dysfunction is a central feature of MS, and the time course of leakage has been studied with dynamic contrast-enhanced MRI (Bastianello et al., 1990; Harris et al., 1991; Guttmann et al., 2016; Gaitán et al., 2011; Fig. 2 G). While barrier leakage is almost always present in new lesions, it is rarely observed in older lesions (Bastianello et al., 1990, Harris et al., 1991). Interestingly, MRI evidence suggests that BBB permeability is the initial event in the formation of a subset of lesions, but in others, lesion formation occurs before barrier dysfunction (Guttmann et al., 2016).

CNS immune infiltration is a critical step in MS pathophysiology, and the dynamics of this process have been primarily studied in experimental autoimmune encephalomyelitis (EAE), a rodent model of MS. The primary sites of CNS immune surveillance in health are the blood–CSF barriers of the choroid plexus and meninges, and both are important sites of initial lymphocyte activation in EAE (Bartholomäus et al., 2009; Schläger et al., 2016; Mundt et al., 2019; Engelhardt et al., 2001, 2017; Carrithers et al., 2000; Reboldi et al., 2009). These immune cells first enter the perivascular space surrounding postcapillary venules (Greter et al., 2005) and gain parenchymal access after breaking down the BM (Song et al., 2017; Wu et al., 2009). Leukocyte-derived cytokines activate CNS ECs, inducing expression of leukocyte adhesion molecules (Carrithers et al., 2000; Barkalow et al., 1996; Lou et al., 1996), which leads to massive parenchymal infiltration of immune cells. Limiting immune cell trafficking across the BBB has proven effective in treating MS. Natalizumab, which targets the α4 integrin on immune cells, preventing their interaction with endothelial VCAM1, greatly reduces new lesion formation (Miller et al., 2003).

It is critical to note that while leukocyte invasion is often assumed to be detrimental, leukocyte trafficking is required at low levels in order to limit infections. Of great interest is the identification of leukocyte adhesion molecules that facilitate the extravasation of only certain subsets of immune cells (Steinman, 2015). This could enable targeting pathological inflammation without rendering patients more vulnerable to infection. Indeed, ninjurin1 (NINJ1; monocytes), activated leukocyte cell adhesion molecule (ALCAM; $CD4^+$ T cells, monocytes), junction adhesion molecule–like (JAML; monocytes, $CD8^+$ T cells), and melanoma cell adhesion molecule (MCAM; CD8, T helper cell 17)

regulate the entry of specific immune cell populations into the CNS (Alvarez et al., 2015; Cayrol et al., 2008; Flanagan et al., 2012; Ifergan et al., 2011; Larochelle et al., 2015). It will be necessary to ensure that targeting these molecules does not produce secondary effects; *Alcam* knockout mice develop more severe EAE as ALCAM also enforces TJ integrity (Lécuyer et al., 2017).

Many questions remain unanswered. How much of MS pathophysiology directly results from BBB dysfunction? Is there a subset of lesions caused by leakage while others have a different etiology? If these lesion subsets exist, do they vary with respect to severity and repair processes? Does the BBB interact with the lymphatic system to regulate leukocyte efflux during remission?

Ischemia/stroke. BBB dysfunction during stroke follows a biphasic time course. Leakage is evident within hours of the primary insult, is subsequently reduced, and then reappears the day after (Huang et al., 1999; Kuroiwa et al., 1985; Fig. 2, E, F, and H). An increase in transcytosis of nonspecific molecules is the first stage of dysfunction, followed by structural alteration of TJs (Knowland et al., 2014). Questions still remain regarding the importance of leukocyte infiltration in pathogenesis. Several reports have shown that leukocyte adhesion molecule knockouts or antibodies directed against leukocyte adhesion molecules minimize infarct volume (Bowes et al., 1993; Connolly et al., 1996; Mayadas et al., 1993), whereas others have not been able to replicate this effect (Enzmann et al., 2018).

Much of the cell death that leads to neurological deficits occurs in the days following a stroke; thus, the second phase of BBB leakage may be an important therapeutic target. Major outstanding questions in stroke research surround the relevance of this biphasic BBB dysfunction. It is unknown whether the first and second openings are mechanistically different; perhaps the first opening is due to dynamic signaling while the second results from changes in BBB gene expression.

Epilepsy. There is a clear association between epilepsy and BBB dysfunction. BBB leakage in epilepsy patients is visible with contrast-enhanced MRI (Horowitz et al., 1992; Alvarez et al., 2010; Rüber et al., 2018; Fig. 2 I), and analysis of brain tissue from epileptic patients shows increased parenchymal albumin (Cornford et al., 1998a; Mihály and Bozóky, 1984), implicating blood-to-brain extravasation of large molecules. Furthermore, patient samples exhibit regional reduction in GLUT1 (Cornford et al., 1998b), and positron emission tomography scans demonstrate decreased uptake and metabolism in seizure foci (Cornford et al., 1998a; Janigro, 1999).

BBB dysfunction itself may be epileptogenic or may help propagate seizures. Experimental disruption of the BBB with osmotic shock leads to seizures in patients (Marchi et al., 2007), and diseases in which the BBB is compromised such as infection, inflammation, stroke, and traumatic brain injury can lead to seizures and epilepsy (Oby and Janigro, 2006; van Vliet et al., 2007). Furthermore, neuroinflammation has been hypothesized to be involved in seizure etiology; blockage of leukocyte–vascular interactions either pharmacologically or by genetic knockout inhibits both induction and recurrence of seizures (Fabene et al., 2008). Interestingly, patients with a BBB-GLUT1 deficiency develop epilepsy (De Vivo et al., 1991; De Vivo et al., 2002), demonstrating a critical role for BBB transport in normal brain function.

AD. The extent of BBB dysfunction in AD and its role in etiology are an important ongoing focus of research. Several techniques have been used to examine BBB function in AD patients, including staining postmortem brain tissue for serum components, measurement of blood/CSF albumin concentrations, and various imaging modalities. Histological analyses have shown increased albumin and immunoglobulins in areas of heavy plaque burden (Wisniewski et al., 1997) as well as increased levels of fibrinogen (Ryu and McLarnon, 2009). A three-dimensional in vitro AD model has shown evidence of BBB dysfunction, phenocopying vascular changes reported in patients (Shin et al., 2019). Additionally, several imaging studies have found evidence of a leakier BBB in AD patients and propose BBB dysfunction as an early biomarker of AD (Starr et al., 2009; Montagne et al., 2015; van de Haar et al., 2016; Nation et al., 2019). While many older reports found no change in CSF albumin levels or contrast-enhanced imaging (Alafuzoff et al., 1987; Frölich et al., 1991; Kay et al., 1987; Mecocci et al., 1991; Bronge and Wahlund, 2000; Dysken et al., 1990; Schlageter et al., 1987), several of these studies did find evidence of BBB leakage in patients with vascular disease, suggesting that even in the absence of widespread leakage, there is a crucial vascular component to pathology (Erickson and Banks, 2013; Farrall and Wardlaw, 2009; Mecocci et al., 1991; Alafuzoff et al., 1983). As new imaging technology with greater resolution has gained wider use, BBB dysfunction has been further implicated in the pathogenesis of AD (Montagne et al., 2015; van de Haar et al., 2016; Nation et al., 2019). With these new tools, it will be vital to perform a more detailed analysis to determine at what stage and in which brain regions BBB dysfunction occurs, whether leakage is transient or chronic, and which cellular BBB properties are affected.

Regardless of the extent of widespread BBB leakage, there are several links between BBB dysfunction and AD pathology (Petersen et al., 2018). Fibrin accumulates in amyloid-positive vessels in AD patients and mouse models, and fibrin depletion protects against cognitive deficits in mice (Paul et al., 2007; Cortes-Canteli et al., 2010). Perhaps small amounts of BBB leakage related to injury, infection, or aging increase fibrin deposition, setting in motion an inflammatory cascade that plays an important role in AD pathology (Petersen et al., 2018; Kumar et al., 2016; Kinney et al., 2018).

In addition to nonspecific leakage, dysfunction of BBB Aβ transport may drive AD pathology (Bell and Zlokovic, 2009; Erickson and Banks, 2013). LRP1, a cell-surface receptor expressed on ECs, regulates Aβ clearance from the parenchyma (Shibata et al., 2000). EC-specific *Lrp1* knockout increases levels of soluble brain Aβ and the severity of learning and memory deficits in an AD mouse model (Storck et al., 2016). A phosphatidylinositol binding clathrin assembly protein (PICALM)/PGP-dependent mechanism also aids in the clearance of Aβ across the BBB. PICALM regulates clathrin-dependent internalization of Aβ, guiding receptor-mediated transcytosis and clearance of Aβ, potentially presenting Aβ to efflux transporters (Zhao et al., 2015). PGP deficiency in an AD mouse model cuts Aβ

clearance rate in half and increases CNS Aβ deposition (Cirrito et al., 2005), and Aβ40 triggers ubiquitination and internalization of PGP (Hartz et al., 2016), suggesting a dangerous feedback cycle. Conversely, receptor for advanced glycation endproducts (RAGE) imports Aβ into the CNS (Deane et al., 2003), and alterations in LRP:RAGE activity are hypothesized to drive CNS amyloid deposition in AD patients (Jeynes and Provias, 2008).

Another factor that might contribute to BBB dysfunction in AD is apolipoprotein E (*APOE*) genotype. Transgenic mice expressing human APOE4, the AD risk allele, exhibit cerebral vasculature with a thinner BM and BBB dysfunction due to cyclophillin/MMP9 signaling in pericytes (Bell et al., 2012; Alata et al., 2015). Further, postmortem AD tissue has revealed decreased TJ proteins and MMP9 elevation along with pericyte degeneration in *APOE4* carriers (Bell et al., 2012; Halliday et al., 2016; Nishitsuji et al., 2011). However, there are conflicting reports; others show no changes in BBB function in *Apoe4* knockout or *APOE4* transgenic mice (Bien-Ly et al., 2015). One possible explanation is that APOE4 might cause minor, highly localized BBB leakage while not disrupting global BBB integrity (Ulrich et al., 2015).

To address the outstanding questions in the field, a deeper understanding of the association between vascular damage and AD pathology is necessary. This will require a focus on finding causal rather than correlational information linking BBB leakage, inflammation, and AD pathology. For instance, a recent study found that BBB dysfunction is an early marker of cognitive decline independent of Aβ or tau accumulation (Nation et al., 2019), but more details are needed regarding the extent of BBB dysfunction at various points during the AD time course. Furthermore, it is critical to understand how the BBB, glymphatics, and lymphatics cooperate to remove Aβ and other waste products from the CNS parenchyma, and what role this plays in AD pathophysiology (Stower, 2018; Rasmussen et al., 2018; Sweeney and Zlokovic, 2018; Da Mesquita et al., 2018).

Looking forward. Several important questions remain regarding the BBB in the context of disease. How is each BBB property altered in neurological diseases, and how do these changes affect the extracellular environment of the CNS? One problem is that different studies in humans or mouse models often use a single modality to detect BBB breakdown, whether sampling postmortem tissue, measuring markers in the CSF or blood, quantifying leakage of an exogenous tracer, or performing live imaging with a contrast agent. The BBB is not a single entity that is "open" or "shut," and moving forward, it is imperative to understand exactly how the complex physiology of the BBB changes in each disease. It is especially important to consider whether alterations are induced by the same or different signals across neurological conditions. If mechanistic similarities exist, it might be possible to design a therapeutic strategy applicable to a wide range of disorders (Munji et al., 2019). Indeed, several molecular factors regulate BBB dysfunction in multiple diseases, including vascular endothelial growth factor (Argaw et al., 2009, 2012), inflammatory cytokines (tumor necrosis factor α [Nishioku et al., 2010], interleukins 1 and 6 [Chiaretti et al., 2005; Paré et al., 2018; Wang et al., 2014]), reactive oxygen species (Maier et al., 2006; Pun et al., 2009; Relton et al., 1997), and matrix metalloproteinases (Gidday et al., 2005; Ugarte-Berzal et al., 2018). However, there is also evidence that barrier dysfunction is due not only to "breakdown signals" but also to disrupted maintenance signals. Disruption of Wnt signaling can lead to vascular permeability and worse disease outcomes (Wang et al., 2012; Chang et al., 2017); thus, increasing CNS EC Wnt signaling might have therapeutic potential.

Can subtle changes in different BBB properties cause specific neurological symptoms? Dysfunction in several BBB transporters causes specific developmental disorders (Seidner et al., 1998; Tărlungeanu et al., 2016; Vatine et al., 2017), and there may be more undiscovered instances of this pattern. It is possible that regional heterogeneity at the BBB renders particular brain regions vulnerable to certain disease pathologies. For instance, if the BBB is indeed specialized to cater to the distinct nutrient and signaling needs of individual brain regions, loss of one of those BBB specializations might lead to deficits in local circuit function.

It is also important to also think beyond ECs. Disruption of pericyte coverage leads to an increase in EC nonspecific transcytosis and leukocyte adhesion molecules expression, and it is unclear to what extent this drives neurological disease. Furthermore, disruption of astrocyte endfeet at the NVU would decrease glymphatic clearance, potentially contributing to pathological accumulation of proteins including Aβ. Future work analyzing how each cell type of the NVU, and the glycocalyx and BMs, is altered will be critical to understand the pathophysiology of different neurological diseases.

Another fundamental question is how the BBB is repaired. While the BBB becomes less permeable to molecular tracers at chronic phases of disease models, it is unclear whether there are functional or structural compromises made in the process of reversing leakiness. More work is needed to fully characterize the repaired BBB at the levels of physical integrity and transcriptomics. It is also unknown what endogenous signals induce BBB repair, and whether repair occurs cell-autonomously within ECs or with mediation from other cell types. Interestingly, both microglia and reactive astrocytes regulate repair of the BBB in response to injury, highlighting the importance of the interactions of cells within the NVU (Lou et al., 2016, Bush et al., 1999, Fernández-López et al., 2016).

Concluding remarks

The BBB is not a single entity, but rather a complex series of physiological properties allowing CNS ECs to tightly regulate the extracellular environment of the parenchyma. These properties are vital for proper neural function, and dysfunction of the BBB can lead to critical pathology in many neurological diseases. However, more work is needed in order to understand exactly what crosses the healthy BBB, the degree to which the BBB dynamically responds to environmental stimuli, the extent of its regional heterogeneity, and the signaling mechanisms underlying its maintenance, disruption, and repair (Box 2). As future research answers these questions and further reveals the cellular and molecular intricacies underlying the BBB, the clinical advantages will be twofold: a deeper knowledge of the BBB will provide therapeutic targets for BBB repair in a range of

Box 2. Important unanswered questions

BBB function
- What endogenous and exogenous molecules permeate the barrier, how much, and by which route(s)?
- Is there regional heterogeneity of the BBB? Do regional specifications of the BBB regulate circuit function?
- What is the relevance of arteriovenous zonation in terms of barrier function?
- Are there sex differences in BBB function?
- How dynamic is each BBB property in a healthy CNS?
- Is the BBB modulated by neural activity, diet, or environmental stimuli?

BBB function
- How is the induction of different BBB properties coordinated?
- How is the seamless phenotypic continuum of arteriovenous zonation programmed?
- Do differences in developmental signaling influence BBB heterogeneity?
- Are the same signals required for induction also responsible for regulating BBB maintenance in adulthood?
- To what extent are BBB properties regulated by a circadian clock?
- How do BBB properties change in age?

BBB dysfunction
- How is each BBB property altered in neurological diseases?
- How do these changes affect the CNS extracellular environment?
- Are these alterations induced by the same or different signals across neurological conditions?
- Can subtle changes in different BBB properties cause specific neurologic symptoms?
- How does the BBB interact with the lymphatic and glymphatic clearance pathways?
- What endogenous signals induce repair?
- Is BBB repair occurring cell-autonomously within ECs or with mediation from other cell types?

neurological conditions and will also enable more effective strategies for delivering drugs to the CNS.

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