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Development and Cell Biology of the Blood-Brain Barrier

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Abstract

The vertebrate vasculature displays high organotypic specialization, with the structure and function of blood vessels catering to the specific needs of each tissue. A unique feature of the central nervous system (CNS) vasculature is the blood-brain barrier (BBB). The BBB regulates substance influx and efflux to maintain a homeostatic environment for proper brain function. Here, we review the development and cell biology of the BBB, focusing on the cellular and molecular regulation of barrier formation and the maintenance of the BBB through adulthood. We summarize unique features of CNS endothelial cells and highlight recent progress in and general principles of barrier regulation. Finally, we illustrate why a mechanistic understanding of the development and maintenance of the BBB could provide novel therapeutic opportunities for CNS drug delivery.

Contents

1. INTRODUCTION

The blood-brain barrier (BBB) is the interface that separates neural tissue from circulating blood. The BBB comprises a single layer of endothelial cells that forms the blood vessel wall and maintains a safe and homeostatic milieu for proper neuronal function and synaptic transmission. Impaired barrier function is associated with neurodegenerative diseases, including multiple sclerosis, Alzheimer's disease, and Parkinson's disease (Sweeney et al. 2018, Zhao et al. 2015). Conversely, the BBB is a major obstacle for drug delivery into the CNS, impeding the treatment of neurological diseases, including neurodegenerative diseases, psychiatric disorders, brain infections, and brain tumors (Banks 2016, Pardridge 2012). Despite the importance of the BBB, our understanding of the fundamental mechanisms underlying the formation and maintenance of the BBB remains limited.

Our limited understanding of the BBB can be attributed to its complexity. The BBB is localized to CNS endothelial cells that build the vessel wall and display unique cell biological features. These features distinguish these cells from peripheral endothelial cells. First, CNS endothelial cells have specialized tight junctions (TJs) to prevent free paracellular passage through the vessel wall. Second, they express designated transporters to regulate the dynamic influx and efflux of specific substrates. Third, they display extremely low rates of transcellular vesicle trafficking, termed transcytosis, to limit transcellular transport through the vessel wall. Lastly, CNS endothelial cells have low expression levels of leukocyte adhesion molecules (LAMs) to limit the entry of immune cells into the brain. Recent studies have also implicated the glycocalyx, a negatively charged dense layer of carbohydrates on the luminal side, in preventing immune cell entry, acting as the first line of defense in protecting the brain (Kolarova et al. 2014, Kutuzov et al. 2018) (**Figure 1**).

However, barrier properties are not intrinsic to endothelial cells. BBB formation and maintenance depend on local perivascular cells and the neuronal environment of the CNS (Armulik et al. 2010, Daneman et al. 2010, Janzer & Raff 1987, Stewart & Wiley 1981). Blood vessels in the brain are surrounded by pericytes and astrocytes, which serve as the interface between endothelial cells and neurons. Together, these cells form the neurovascular unit (NVU) (Iadecola 2004, 2017; Zhao et al. 2015) (**Figure 1**).

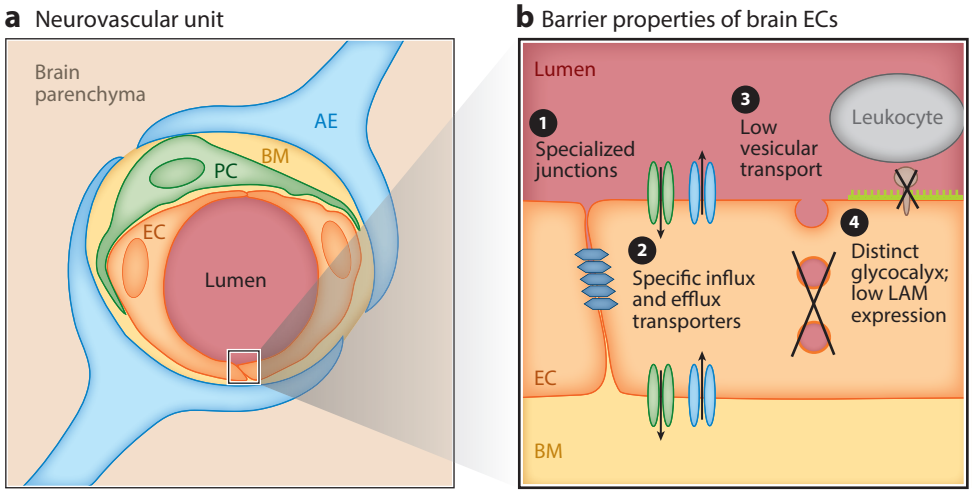

Figure 1

The neurovascular unit. (*a*) Schematic illustration of a cross section through the neurovascular unit. Capillary endothelial cells (EC) forming the vessel walls are tightly wrapped by pericytes (PC). These cells are embedded in the basement membrane (BM) and are ensheathed by astrocytic endfeet (AE). (*b*) Schematic of the CNS endothelium highlighting the four unique properties of the blood-brain barrier. LAM denotes leukocyte adhesion molecule.

In this review, we provide an overview of the cellular and molecular features of the BBB. We first elucidate the specialization of CNS endothelial cells and discuss the cell biology underlying their unique characteristics. We then illustrate the role of other cell types in the NVU that contribute to barrier function. Next, we lay out the development of the BBB, focusing on the signaling pathways and mechanisms that establish the barrier. Finally, we discuss critical questions in the field that require further investigation.

2. UNIQUE BARRIER PROPERTIES OF CNS ENDOTHELIAL CELLS

2.1. Specialized Tight Junctions

Initial tracer injection experiments revealed that brain endothelial cells have specialized TJs that prevent the intercellular passage of tracers from the circulation into the brain parenchyma (Reese & Karnovsky 1967). Using ultrastructural electron microscopy analysis, this study revealed that the entry of the injected tracer is sharply halted at TJs between two neighboring endothelial cells, highlighting two aspects: (*a*) CNS endothelial cells are the site of the BBB, and (*b*) specialized TJs are important for BBB integrity. Subsequent studies revealed that CNS TJs are specialized to seal contact sites between endothelial cells to prevent paracellular transport of molecules into the CNS and that adherens junctions promote cell-cell adhesion (Brightman & Reese 1969, Lampugnani et al. 1992). Earlier studies focusing on junctions in epithelial cells informed much of our understanding about TJs in general and formed the foundation for our understanding of TJs in CNS endothelial cells. Epithelial cells lining peripheral tissues have three kinds of junctions: TJs generally found on the apical side of epithelial cells, desmosomes on the basolateral side, and adherens junctions found in between desmosomes and adherens junctions (Farquhar & Palade 1963). In contrast, CNS endothelium lacks desmosomes and has a different spatial distribution of junctions

than do epithelial cells. Specifically, TJs are embedded within adherens junctions and are found throughout the cleft between endothelial cells (Schulze & Firth 1993).

The four classes of TJ proteins in the CNS are occludin, tricellulins, claudins, and junctional adhesion molecules (JAMs). Occludin (encoded by *Ocln*) is enriched in CNS endothelium and was the first transmembrane protein shown to localize exclusively to TJs (Furuse et al. 1993). However, $Ocln^{-/-}$ mice develop normal TJs, despite having other abnormalities like calcification of the brain (Saitou et al. 1998, 2000). A closer examination of $Ocln^{-/-}$ mice revealed mislocalization of tricellulin proteins from tricellular TJs (where three cells meet) to bicellular TJs (between two cells) (Ikenouchi et al. 2008). These findings suggest that the role of Occludin in TJ formation is complex, and given the structural similarities between tricellulins and Occludin (Ikenouchi et al. 2005), tricellulins may compensate for Occludin deficiency, providing an explanation for the apparently normal TJs in $Ocln^{-/-}$ mice. Consistent with this idea, recent work has demonstrated a role for Lsr, a tricellulin protein enriched at the BBB, in the formation of a functional barrier (Sohet et al. 2015).

The surprising finding of functional barriers in $Ocln^{-/-}$ mice fueled the continued search for other TJ proteins, leading to the discovery of claudins (Furuse et al. 1998). Of the 27 mammalian claudin genes, the gene encoding Claudin-5 (*Cldn5*) is highly enriched in CNS endothelial cells (Morita et al. 1999). $Cldn5^{-/-}$ mice exhibit a size-selective increase in barrier permeability (Nitta et al. 2003), implicating Claudin-5 as a key component of BBB regulation. These mice display normal vascular development and show no gross abnormalities in peripheral tissues but die shortly after birth, likely due to an impaired BBB (Nitta et al. 2003). Finally, JAMs are transmembrane TJ proteins belonging to the immunoglobulin superfamily that regulate leukocyte transmigration across cellular barriers (Martin-Padura et al. 1998).

While several TJ proteins are decreased in pathological conditions, our understanding of the basic biology of these proteins in the context of the BBB is still rudimentary. Intracellular trafficking patterns of these proteins and their turnover rates in the CNS endothelium are unknown. Generating CNS endothelium–specific conditional knockout mouse models for these proteins to determine their contribution to BBB formation and maintenance will provide insight into the specific roles of such proteins in barrier function.

2.2. Transporters at the Blood-Brain Barrier

The highly selective nature of the BBB and the high metabolic demand of the brain necessitate other routes of entry for various nutrients to feed and nurture the brain. Metabolic supply is achieved via several influx and efflux transporters that are expressed on the surface of CNS endothelial cells and that drive the active transport of specific solutes and metabolites into the brain. Transport across the BBB can generally be classified into four categories: (*a*) passive diffusion of lipid-soluble molecules such as oxygen, (*b*) carrier-mediated transport via solute carrier transporters (SLCs), (*c*) selective transport via ATP-binding cassette transporters (ABCs), and (*d*) vesicular trafficking by transcytosis (Section 2.3). Here we briefly discuss transporters expressed at the BBB. Many of these transporters are ATPases and ATP-binding pumps, indicating a high energy demand to maintain the barrier. Consistent with this, CNS endothelial cells have four- to fivefold more mitochondria relative to peripheral endothelial cells (Oldendorf et al. 1977).

SLCs are a large class of transporters that facilitate the uptake of nutrients, including glucose, amino acids, ions, and fatty acids (Campos-Bedolla et al. 2014). Owing to the structural complexity and the genetic redundancy of the more than 400 genes in the SLC family, SLCs have been relatively difficult to study. One of the best-studied SLCs is SLC2A1, or Glut1 (glucose transporter 1), which transports glucose. The BBB is highly enriched in Glut1, and patients with Glut1

deficiency present with seizures, microcephaly, developmental delays, and ataxia (De Vivo et al. 1991). While *Slc2a1*$^{-/-}$ mice die at embryonic stages, mice haploinsufficient for *Slc2a1* recapitulate the features seen in human patients (Wang et al. 2006). Recent work has also shown that lack of Glut1 causes barrier breakdown and prevents clearance of amyloid plaques, thus contributing to the progression of Alzheimer's disease (Winkler et al. 2015).

SLC7A5, or LAT1 (large neutral amino acid transporter 1), is another well-studied transporter in this family. LAT1 transports branched amino acids into the brain and, like Glut1, is highly enriched in CNS endothelial cells. Recent identification of mutations in *LAT1* associated with autism spectrum disorder gave further insight into the role of LAT1. Mice lacking endothelial LAT1 have developmental and neurological abnormalities (Tarlungeanu et al. 2016). These mice also display altered amino acid levels in the brain, resulting in reduced mRNA translation with slower protein synthesis. Besides amino acid uptake transporters, the BBB also expresses SLCs that drive the net efflux of amino acids like glutamate and aspartate, which are excitotoxic at high levels. These SLCs are excitatory acidic amino acid transporters (EAATs) that are expressed on the abluminal side of CNS endothelial cells (O'Kane et al. 1999) and that export glutamate from the brain parenchyma to maintain low levels of glutamate in the brain extracellular fluid. Thus, the roles of these transporters highlight the importance of regulating amino acid concentrations within the brain for neuronal homeostasis.

In contrast to most nutrient transporters, ABC efflux pumps actively clear out metabolic waste from the CNS and hence are usually considered molecular defense systems that protect the brain from exogenous and toxic substances. Among these transporters, P-glycoprotein (P-gp) has been investigated extensively owing to its role in amyloid β (Aβ) efflux from the brain parenchyma. Aβ are small peptides that can form plaques in the brain if they are not cleared, a phenomenon seen widely in Alzheimer's patients. Initial studies observed that P-gp is on the luminal side of BBB capillaries (Beaulieu et al. 1997, Cordon-Cardo et al. 1989) and promotes the secretion of Aβ (Lam et al. 2001). Mice lacking P-gp not only have increased deposition of Aβ (Cirrito et al. 2005) but also display increased sensitivity to several drugs (Schinkel et al. 1996), highlighting the role of P-gp in preventing drugs from entering the brain. Consistently, multiple studies have reported decreased levels of P-gp and other ABC efflux pumps in postmortem brain tissue samples of Alzheimer's disease patients (Jeynes & Provias 2011, Wijesuriya et al. 2010), implicating the decreased levels of those pumps in promoting disease progression.

Overall, our understanding of the transporters at the BBB is still in the early stages. SLCs are thought to make up approximately 10% of the human genome (Hediger et al. 2013), yet the precise roles of most of these proteins are not known. In the case of efflux pumps, it is unclear what triggers their initial loss contributing to disease progression. General questions that remain in this field include: What portion of the nutrients provided by the transporters is consumed by endothelial cells themselves versus other cell types in the NVU? Furthermore, what is the fate of these nutrients once taken up by endothelial cells, and how are such nutrients transported to other cell types in the brain? These aspects are completely unknown for most transporters at the BBB.

2.3. Low Rates of Transcytosis

Besides having designated transporters to deliver specific nutrients to the brain, CNS endothelial cells also have very low rates of vesicle-mediated transcellular trafficking, a process termed transcytosis (Reese & Karnovsky 1967). Recent work demonstrated that these low rates result from the active inhibition of transcytosis in CNS endothelial cells. The mechanisms regulating this inhibition at the BBB were largely unknown until recent studies established Mfsd2a as an inhibitor of transcytosis in regulating permeability of the BBB (Andreone et al. 2017, Ben-Zvi et al. 2014,

Chow & Gu 2017). Mfsd2a is a lipid transporter (Nguyen et al. 2014) enriched in CNS endothelial cells, and *Mfsd2a*$^{-/-}$ mice have increased caveolae-mediated vesicular trafficking, causing leaky barriers (Andreone et al. 2017, Ben-Zvi et al. 2014, Chow & Gu 2017). Andreone et al. (2017) investigated the specific mechanism by which Mfsd2a inhibits transcytosis: Intriguingly, Mfsd2a, by its lipid transport function, establishes a unique lipid composition in the CNS endothelial cell plasma membrane that inhibits caveolae vesicle formation, thereby suppressing transcytosis.

Although CNS endothelial cells have limited transcytosis, some macromolecules, such as insulin, albumin, and iron-bound transferrin, lack specific transporters at the BBB and thus enter the brain by transcytosis. The passage of these macromolecules demonstrates how the BBB can precisely regulate the transcytotic pathway to allow for the transendothelial passage of specific molecules via either receptor-mediated transcytosis or adsorptive transcytosis. In the former, receptors on the luminal cell surface bind to cargo or their ligand. This complex is then endocytosed and trafficked within the endothelial cell. Adsorptive transcytosis, in contrast, is receptor-independent vesicular trafficking and depends on charged interactions between the ligand and the glycocalyx of endothelial cells. In both cases, after the first step of vesicle formation, the vesicles are trafficked through endosomes and exocytosed into the brain parenchyma. While clathrin-mediated transcytosis and caveolae-mediated transcytosis are the two major endocytic pathways, other kinds of vesicular structures have been reported at the ultrastructural level by using electron microscopy analyses (Hansen & Nichols 2009). Furthermore, various adaptor proteins play essential roles in each step of these processes. However, it is unclear what determines whether a molecule will be transcytosed and delivered into the brain or sorted into lysosomes for degradation.

On the therapeutic front, researchers have sought to harness receptor-mediated transcytotic pathways, particularly the transferrin receptor (TfR)-mediated transcytosis pathway, to facilitate drug delivery into the brain (Yu & Watts 2013). Tfr-mediated transcytosis has been an attractive system to explore the possibilities of facilitating CNS drug delivery. Antibodies targeting TfR preferentially bind to brain capillaries (Jefferies et al. 1984). After binding, the antibody-TfR complex is shuttled into the brain parenchyma via transcytosis (Friden et al. 1991). Bispecific antibodies targeting TfR and Aβ have been employed to promote the delivery of these antibodies into the CNS and to promote the clearance of Aβ plaques (Bien-Ly et al. 2014, Niewoehner et al. 2014). These studies revealed that both the affinity and the valency of antibody binding to the receptor are important in determining the efficient delivery of the antibody into the CNS.

Recent studies have also demonstrated that upregulation of transcytosis is an initial step in certain models of brain disorders. Studies investigating the BBB in stroke and brain injury models have revealed that a few hours post–brain insult, transcytotic vesicles in endothelial cells are upregulated, causing barrier breakdown (Haley & Lawrence 2017, Knowland et al. 2014, Sadeghian et al. 2018). Consistent among all these studies are also the observations that leakage via transcellular pathways precedes breakdown of TJs. While these results may apply only to specific models such as stroke, it is worth examining what factors cause the early increase in transcytosis at the BBB. Identification of these factors will allow for the development of strategies to prevent barrier breakdown.

On the CNS drug delivery front, the discovery of BBB regulators like Mfsd2a provides an entry point for the development of compounds that specifically target proteins to open the barrier for CNS drug delivery. For example, inhibiting Mfsd2a to upregulate transcytosis at the BBB could provide a platform for drug delivery into the brain. Thus, identification of molecules unique to CNS barriers will provide a parts list to effectively harness transcytosis pathways for drug delivery.

2.4. Limited Immune Cell Trafficking at the Blood-Brain Barrier

The CNS has low levels of immune cell surveillance relative to peripheral tissues. Initial studies revealed that extremely low numbers of leukocytes, particularly T cells, enter the brain (Hauser et al. 1983, Hickey et al. 1991). Further experiments demonstrated that CNS endothelial cells normally express low levels of LAMs (Rossler et al. 1992) and that LAMs are significantly upregulated in autoimmune diseases such as multiple sclerosis (Cannella et al. 1990, Raine et al. 1990).

Leukocyte entry into tissues is a highly orchestrated process involving multiple steps and is an early event of pathogenesis in autoimmune diseases. Briefly, leukocytes first survey the vascular wall, and signaling molecules such as selectins mediate their initial capture onto the vessel wall. This capture triggers the firm adhesion of the leukocyte to the endothelium via interaction of leukocyte integrins with endothelial membrane proteins such as ICAM-1, ICAM-2, and VCAM-1. Following leukocyte adhesion, diapedesis through the vessel wall occurs through either the paracellular or the transcellular route, leading to their entry into the CNS parenchyma. The entry of leukocytes into the CNS and their subsequent binding to antigen-presenting cells within the brain cause the release of cytokines and chemokines. These molecules in turn recruit more leukocytes, thus acting in a positive feedback manner for further infiltration of these cells into the CNS. For detailed reviews on immune cell entry into and trafficking at the BBB, please see Engelhardt & Wolburg (2004) and Ransohoff & Engelhardt (2012).

Recent studies have begun to address which cell types in the brain first sense inflammation signals and how these processes impact the BBB. With the initiation of infection, mural cells of blood vessels (pericytes and smooth muscle cells) release the CCL2 chemokine, triggering neuroinflammatory responses acting as sensors of the initial infiltration of immune cells (Duan et al. 2018). Another recent study exploring the effects of stress on neuroinflammation and the BBB revealed that, in mouse models of social defeat, there is a decrease in Claudin-5 expression in some parts of the brain; this decrease in turn promotes the infiltration of cytokines such as interleukin-6 into the CNS parenchyma (Menard et al. 2017).

Collectively, these studies highlight the complexity of the neuro-immune axis. We currently know just a fragment of the multistep, highly coordinated process of how initial leukocyte entry into the CNS causes neuroinflammation and pathology. Further studies are required to delineate the manifold cell types and multiple signaling components that regulate the neuro-immune axis.

3. BARRIER REGULATION BY THE NEUROVASCULAR UNIT

The characteristics described above reflect unique properties of the BBB in endothelial cells. However, early transplantation experiments have shown that these properties are not intrinsic to the CNS endothelium (Stewart & Wiley 1981). Rather, such properties are induced by the CNS environment. Indeed, while the BBB is localized to CNS endothelial cells, it requires multiple cell types, such as pericytes and astrocytes, working in concert to form a functional barrier. Together, these cells form the NVU, which induces and regulates barrier formation (**Figure 1**) and which we discuss below.

3.1. Pericytes

Pericytes are mural cells enwrapping capillary blood vessels on their abluminal side. Structurally, pericytes extend processes from their cell body, covering several endothelial cells. In contrast to peripheral tissues, the brain and the retina have the highest pericyte–to–endothelial cell ratio (Frank et al. 1987, 1990). Pericytes are embedded within the basement membrane (BM) of capillary endothelial cells and are thus centrally positioned between endothelial cells, astrocytes,

and neurons. Although pericytes were first described in the 1870s (Eberth 1871, Rouget 1873), studying them has been difficult due to the lack of specific markers. It is now generally accepted that pericytes are NG2 positive, platelet-derived growth factor receptor β (PDGFRβ) positive, and alpha smooth muscle actin negative (Armulik et al. 2011). Recently, the fluorescent Nissl dye NeuroTrace 500/525 was shown to specifically label pericytes in live mice, allowing for in vivo imaging (Damisah et al. 2017).

The physical apposition of pericytes and endothelial cells facilitates signaling between them. This signaling plays a role in vessel formation, vessel maturation, pericyte recruitment, and pericyte coverage (Gaengel et al. 2009). PDGF-B signaling is one such pathway. PDGF-B secreted by endothelial cells recruits pericytes to blood vessels by binding to PDGFRβ, a cell surface protein on pericytes (Hellstrom et al. 1999, Lindahl et al. 1997). While deletion of either gene (*Pdgfb* and *Pdgfrb*) is perinatally lethal (Leveen et al. 1994, Soriano 1994), studies using viable mouse models defective in PDGF-B signaling demonstrated a critical role for pericytes in the formation of the BBB (Armulik et al. 2010, Daneman et al. 2010). These mice have decreased pericyte coverage and continue to have a dysfunctional barrier through adulthood (Bell et al. 2010). These studies also revealed that an intact barrier is highly reliant on the appropriate pericyte coverage of endothelial cells. Consistent with this notion, overexpression of PDGF-B causes an increase in pericyte numbers and results in impaired development of the retinal vasculature, resembling proliferative retinopathy (Edqvist et al. 2012, Seo et al. 2000).

While the role of pericytes in barrier formation is now well acknowledged, their role in the maintenance of the barrier through adulthood remained unknown until a recent study examined the role of PDGF-B signaling in adult mice (Park et al. 2017). In contrast to the role of this pathway in recruiting pericytes during development, endothelium-specific deletion of PDGF-B during adulthood did not cause either (*a*) loss of pericytes from the endothelium or (*b*) the breakdown of the blood-retinal barrier (BRB) or the BBB. Park et al. (2017) also induced diphtheria toxin expression in adult pericytes to specifically cause their ablation and found that pericyte depletion had no effect on the adult BRB. However, the authors did note that pericyte-ablated adult retinas were more susceptible to leakage under stress or injury conditions. These findings suggest that, although pericytes are required for barrier formation, they may be dispensable for barrier maintenance through adulthood.

All mouse models used in these studies are mutants causing reduction or ablation of an entire cell type (pericytes) in the NVU. Identifying pericyte genes that do not affect pericyte recruitment to capillaries but play a role in barrier formation will be crucial in dissecting the specific mechanisms of how pericytes confer barrier properties to CNS endothelium. One known regulator is the transcription factor Foxf2, which is specifically expressed in CNS pericytes (Reyahi et al. 2015). *Foxf2*$^{-/-}$ mice have decreased PDGFRβ signaling, yet they have significantly increased pericyte density and fail to form a functional BBB. In stark contrast to the study by Park et al. (2017), Reyahi et al. (2015) found that Foxf2 deletion in adult mice resulted in severe BBB leakage, revealing the role of a pericyte-specific gene in BBB maintenance. Additionally, Reyahi et al. (2015) made the intriguing observation that BBB integrity is not affected during the first 3 weeks after *Foxf2* deletion but is severely compromised after 6 weeks. These findings implicate a role for pericytes in barrier maintenance as well.

Collectively, these studies reveal the following compelling ideas. First, pathways in addition to PDGFRβ signaling promote pericyte migration and recruitment. Second, BBB breakdown weeks after Foxf2 deletion suggests a gradual deterioration of the components regulating barrier integrity, likely due to slow turnover rates of these molecules. In light of these findings, the timing of analysis for a functional barrier after temporal gene/pericyte ablation could impact the conclusion. For example, although Park et al. (2017) did not observe a barrier defect at 3 weeks after

gene/pericyte ablation, the barrier may break down several weeks after pericyte ablation. Third, pericyte loss could make CNS barriers susceptible to breakdown under stress, as shown by Park et al (2017). Together, all these ideas call for a careful investigation into the role of pericytes in barrier function.

Pericyte loss is also correlated with BBB breakdown in Alzheimer's disease (Sagare et al. 2013). Pericytes play an active role in the clearance of amyloid aggregates via expression of LRP1 (Kanekiyo et al. 2012), an Aβ clearance receptor (Deane et al. 2004, Shibata et al. 2000). CNS pericytes also have a remarkable concentration of acid phosphatase–positive lysosomes (Broadwell & Salcman 1981), which likely indicates robust phagocytic and degradative capabilities. Consistent with this, studies have demonstrated clearance of cellular debris by pericytes (Castejon et al. 2005, Mazlo et al. 2004). Lastly, several studies have indicated the multipotent differentiation capacity of pericytes (Birbrair et al. 2015, Dore-Duffy 2008). However, we know very little about pericyte differentiation and turnover rates. The last decade in barrier research has uncovered a significant role for pericytes in barrier function. It is becoming increasingly clear that we are only beginning to appreciate the role of pericytes in the CNS, and future studies will further evolve our understanding of pericytes in terms of their physiological functions and their role in pathophysiology.

3.2. Astrocytes

Astrocytes ensheathing capillaries constitute the most abluminal layer of the NVU. These cells contact the outer BM of the brain vasculature via polarized endfeet that express the water channel aquaporin 4 (Aqp4). Astrocyte endfeet produce extracellular matrix (ECM) proteins that contribute to the unique BM of brain capillaries (see Section 3.3). Given the close contact of astrocyte endfeet with brain capillaries, it is intuitive to assume that they play a role in BBB formation or maintenance. However, it is unlikely that astrocytes contribute to BBB formation in development, because they appear postnatally in the brain, well after the barrier has sealed (Yang et al. 2013). Rather, they are likely important for barrier maintenance.

An early transplantation study suggests that an astrocytic environment is sufficient to induce barrier properties in newly forming vessels (Janzer & Raff 1987). In this study, astrocytes were transplanted into the eyes of rats. Two weeks after transplantation, newly formed vessels in astrocyte aggregates on the surface of the iris contained a functional barrier. In contrast, transplantation of meningeal cells also led to vascularization of cell aggregates, but these vessels displayed a leaky phenotype (Janzer & Raff 1987). Yet it is unclear whether the astrocytes themselves induced barrier properties in endothelial cells or whether they recruited additional cells that induced the functional barrier. Along the same line, blood vessels in the retina develop a functional barrier postnatally (Chow & Gu 2017), when astrocytes are already present there. The growing vessels in the retina display a leaky barrier phenotype in the presence of astrocytes (Chow & Gu 2017), indicating that astrocytic signals alone are not sufficient to induce barrier properties.

To date, most evidence for astrocyte contribution to the BBB has been generated in vitro. For example, an unspecified astrocyte-derived factor induces endothelial polarization and production of the distinct glycocalyx of CNS endothelium (Yamagata et al. 1997). Furthermore, astrocyte-derived Sonic hedgehog (Shh) signaling (*a*) stimulates the expression of TJ proteins such as Claudin-5 and Occludin in endothelial cells (Alvarez et al. 2011) and (*b*) reduces the expression of LAMs and chemokines, which results in the immune quiescent phenotype of brain endothelial cells (Alvarez et al. 2011).

In vivo evidence for the contribution of astrocytes to BBB formation and maintenance is sparse. During embryonic development, before astrocytes are present in the brain, Shh is highly expressed by neuroprogenitors, and Shh depletion leads to reduced expression of Claudin-5 in endothelial

cells at embryonic day (E)13.5 (Alvarez et al. 2011). A recent in vivo study investigated another pathway of brain endothelial cell communication with glial cells during BBB development (Segarra et al. 2018). This study showed that neuronal Reelin activates Dab1 in endothelial cells, which causes endothelial cells to deposit laminin α4 to the ECM. *Dab1* conditional knockout mice show barrier leakage and reduced astrocyte endfeet coverage on brain capillaries. Segarra et al. (2018) concluded that Dab1-activated endothelial cells communicate to glial cells via laminin α4 to seal the barrier. Overall, this model sheds light on the interaction between endothelial and glial cells in BBB development. However, further investigation is needed to rule out that endothelial Dab1 deletion causes cell-autonomous impairment of the BBB.

Regarding postnatal barrier maintenance by astrocytes, current literature also implies a role for Shh signaling. Endothelium-specific deletion of the Shh receptor Smoothened reduces barrier integrity but does not affect vessel patterning in the brain (Alvarez et al. 2011). However, although Alvarez et al. (2011) suggested that Shh in the postnatal brain is derived from astrocytes, recent transcriptional profiling studies of different cell types in the CNS showed very low mRNA expression of Shh in astrocytes (Zeisel et al. 2018, Zhang et al. 2014), challenging the proposed mechanism.

Another contribution of astrocytes to barrier maintenance was demonstrated by Yao et al. (2014), who showed that laminins specifically secreted by astrocytes maintain BBB integrity. Deletion of astrocytic laminins leads to decreased Aqp4 expression in astrocytes and to reduced TJ protein levels in endothelial cells. Mechanistically, Yao et al. (2014) suggest that astrocytic laminins inhibit pericyte differentiation via integrin α2 activation, which results in a leaky barrier.

In summary, there are many indications for a contribution of astrocytes to BBB formation and maintenance, in addition to the intriguing close proximity of astrocytic endfeet to the vasculature. However, the exact signaling pathways and mechanisms require a more detailed examination.

3.3. Basement Membrane of the Neurovascular Unit

The BM is the ECM that provides structural support for the cells of the NVU and is a hub for intercellular communication and signaling pathways between these cells. Structural proteins that make up the ECM of the BM are type IV collagens, fibronectin, laminins, and other glycoproteins. Collagen IV and fibronectin are secreted by all three cell types of the NVU, and deletion of either of these genes is embryonic lethal (George et al. 1993, Poschl et al. 2004). Laminins, in contrast, have several isoforms, and the appropriate balance of these isoforms is important in vessel formation (Thyboll et al. 2002), barrier formation (Menezes et al. 2014), and the regulation of leukocyte infiltration into the brain (Wu et al. 2009).

The BM has two main families of ECM receptors: dystroglycans and integrins. Together, these receptors enable cell-ECM interactions and link the ECM to the cytoskeleton. The ligands within the ECM bind to these matrix receptors to trigger signaling cascades regulating cell migration, proliferation, and cell survival. These signaling cascades in turn regulate barrier function. Integrins are ligand-binding membrane proteins that are heterodimers comprising α and β subunits. The αv subunit is one of the best-studied integrin subunits in the field owing to its role in angiogenesis. *Itgav*$^{-/-}$ mice die of cerebral hemorrhages (Bader et al. 1998), and interestingly, conditional knockouts revealed that deletion of *Itgav* from neurons and glia, but not from endothelial cells or pericytes, recapitulated the phenotype (McCarty et al. 2002, 2005). Furthermore, studies have also shown that αv integrins regulate the brain vasculature through activation of TGF-β signaling (reviewed in Munger & Sheppard 2011, Sheppard 2004). There is also growing evidence for integrin-mediated and ECM-driven regulation of Wnt signaling (Astudillo & Larrain 2014), which in turn modulates barrier properties. For example, deletion of integrin β1 in endothelial

cells impairs VE-cadherin signaling, and such impairment causes disorganization of TJs, resulting in a leaky barrier (Yamamoto et al. 2015).

Thus, the signaling pathways and cellular interactions occurring within the BM of the NVU are coordinated to regulate barrier function. Not surprisingly, BM breakdown is seen in several CNS disorders and diseases (Thomsen et al. 2017). This degradation is thought to be due to matrix metalloproteinases (MMPs), which are upregulated in disease conditions. Specifically, increased MMP-2 and MMP-9 have consistently been associated with stroke, ischemia, and Alzheimer's and Parkinson's diseases (Rempe et al. 2016). Although targeting MMPs in disease to impede protein degradation is an attractive idea from a therapeutic standpoint, studying MMPs has been difficult owing to the lack of suitable reagents to examine their localization or measure their proteolytic activities in vivo. New tools and further research are needed to understand the functional roles of these molecules in BBB regulation.

4. DEVELOPMENT OF THE BLOOD-BRAIN BARRIER

4.1. Barriergenesis

When brain endothelial cells first enter the CNS, they do not inherently display barrier properties. Instead, BBB formation is a gradual process that occurs during embryonic development after exposure to the neural environment. The process begins with vascularization of the brain at approximately E11.5. Blood vessels from the perineural vascular plexus (PNVP) invade the brain in a stereotypical manner by sprouting angiogenesis (Daneman et al. 2010, Risau 1997). Vascularization is initiated by the release of growth factors from the neural tube. These factors attract vessels to invade via binding to specific receptors on the endothelial cell surface. Shortly after vascular sprouts invade the neural tube, they begin to connect and form a vessel plexus in the brain (Engelhardt 2003).

Numerous signaling pathways such as the VEGF, Notch, and ephrin signaling pathways play crucial roles in CNS angiogenesis (Engelhardt & Liebner 2014). However, these pathways are not specific to CNS vascularization. Perturbations in these signaling pathways lead to severe vascular defects both in the CNS and in peripheral organs, including the most severe form of a barrier breakdown: hemorrhage. Consequently, these phenotypes are likely due to angiogenic defects throughout all tissues as opposed to CNS-specific angiogenic mechanisms.

An elegant model to study barriergenesis specifically in the CNS is the mouse retina. The retina is vascularized radially from the central nerve from postnatal day (P)0 to P10 (Chow & Gu 2017, Pitulescu et al. 2010, Stahl et al. 2010). Chow & Gu (2017) recently showed by ultrastructural and molecular investigation that invading vessel sprouts already have functional TJs yet have active transcytosis and therefore do not have a functional barrier. During vascular outgrowth, the immature distal vessels in the retina are leaky, while the mature proximal vessels have a sealed barrier. Interestingly, expression of Mfsd2a, a molecule that suppresses transcytosis, correlates with functional barrier formation (Chow & Gu 2017). This model proposes a gradual sealing of the barrier, with manifestation of TJs first and inhibition of vesicular transport later (**Figure 2**).

While the brain is much more complex than the retina and the chronology of events that seal the barrier has been debated, barriergenesis appears to follow a similar gradual process. Initial sprouts invading into the neural tube from the PNVP display TJs (Risau 1991, Risau et al. 1986) and already show expression of genes associated with barrier function such as *Slc2a1*, *Cldn5*, and *Abcb1a* (Dermietzel et al. 1992, Morita et al. 1999, Qin & Sato 1995). At the same time, murine brain vessels contain transcytotic vesicles before E15.5 (Ben-Zvi et al. 2014). Moreover, up to E15.5 capillaries express Plvap, a marker associated with transcytosis and endothelial fenestrae

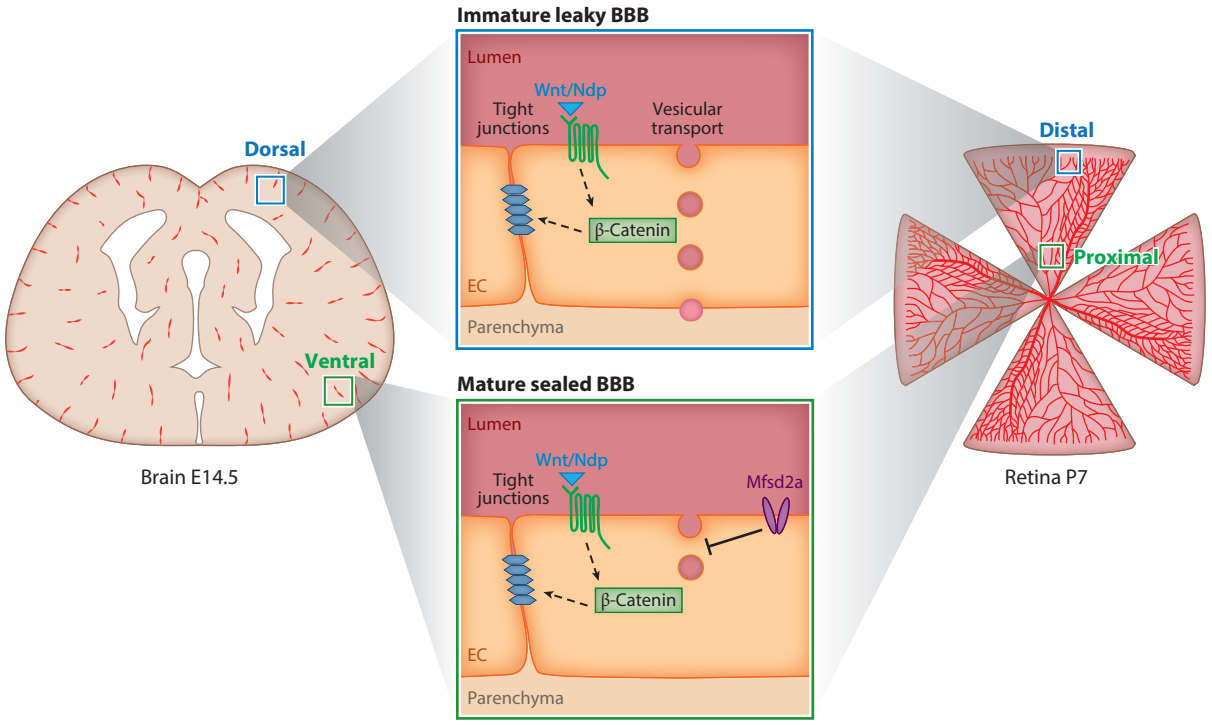

Figure 2

Gradual formation of the blood-brain barrier (BBB) and blood-retina barrier. Depiction of a coronal brain section at E14.5 (*left*) and a flat-mounted retina at P7 (*right*) highlighting the spatiotemporal gradient in functional barrier formation in the CNS endothelial cells (ECs). The top inset (outlined in *blue*) illustrates features of the immature, leaky CNS barriers found in dorsal brain regions and the distal retina. Canonical Wnt signaling induces tight junction formation and prevents paracellular flow. However, transcellular flow via transcytosis remains active. The bottom inset (outlined in *green*) shows features of the mature, sealed blood-CNS barriers of ventral embryonic brain and the proximal vessels of the developing retina. Here, in addition to Wnt signaling, Mfsd2a expression inhibits transcellular flow through transcytosis.

(Daneman et al. 2010, Hallmann et al. 1995, Stan et al. 2012). Tracer injections have shown that brain vessels show leakage of tracers only until E15.5 (Ben-Zvi et al. 2014, Risau et al. 1986). Interestingly, the functional BBB forms in a ventral-to-dorsal fashion (Ben-Zvi et al. 2014, Daneman et al. 2010), indicating spatiotemporal maturation of the barrier (**Figure 2**).

To summarize, when blood vessels first enter the CNS, they immediately acquire functional TJs. This process is mainly controlled by the canonical Wnt signaling pathway, discussed below. In contrast, transcellular transport through endothelial cells via transcytosis is active during earlier stages of development and is then gradually suppressed. Thus, the formation of a functional barrier coincides with the suppression of transcytosis (**Figure 2**).

Interestingly, while the brain is growing and angiogenesis is still occurring, newly forming vessels immediately display the barrier phenotype. This phenomenon is best demonstrated in the retina. When the intermediate plexus is vascularized between P12 and P17, vessel sprouts invading from the deeper plexus into the intermediate plexus are not leaky, although they are still undergoing angiogenesis (Chow & Gu 2017). Thus, newly formed endothelial cells after initial barrier formation inherit barrier properties from their mother cell, and they depend on the local microenvironment to maintain these barrier properties (Janzer & Raff 1987, Stewart & Wiley 1981).

4.2. Wnt Signaling

Canonical Wnt signaling is, as currently known, the major pathway specifically regulating brain angiogenesis and barriergenesis, but not peripheral angiogenesis (Daneman et al. 2009, Liebner et al. 2008, Moro et al. 2012, Stenman et al. 2008). During development, neural progenitor cells secrete Wnt ligands, which act on Frizzled receptors on endothelial cells, activating canonical Wnt signaling (Daneman et al. 2009, Stenman et al. 2008). In this pathway, Wnt ligands binding to their receptors induce β-catenin stabilization and downstream gene induction via TCF/LEF transcription factors.

Endothelium-specific knockout mice for the canonical Wnt signaling mediator β-catenin (*Ctnnb1*) show embryonic lethality associated with severe hemorrhaging specifically in the CNS (Daneman et al. 2009). Early postnatal endothelial cell–specific deletion of *Ctnnb1* disrupts barrier integrity, which correlates with reduced Claudin-5 expression (Liebner et al. 2008, Zhou et al. 2014). Multiple studies have illustrated that the expression of marker genes for a functioning barrier such as *Slc2a1* and *Cldn5* and the absence of *Plvap* are directly regulated by Wnt signaling (Daneman et al. 2009, Liebner et al. 2008, Zhou et al. 2014).

To date, at least two ligand-receptor signaling complexes have been identified that mediate β-catenin stabilization and thereby CNS vascularization and barriergenesis. These pathways show partially redundant functions in activating canonical Wnt signaling.

In the first pathway, Wnt7a/b ligands signal through Frizzled receptors, which are in complex with their coreceptors Lrp5/6, GPR124, and Reck to induce canonical Wnt signaling (**Table 1**). Wnt7a and Wnt7b are expressed by astroglia, oligodendrocytes, and neurons in the forebrain and ventral neural tube (Daneman et al. 2009, Wang et al. 2018, Zhang et al. 2014). They act on Frizzled receptors, which are located in complex with their coreceptors on endothelial cells (C. Cho et al. 2017, Eubelen et al. 2018, Zhou et al. 2014). Knockout mice for components of this pathway show CNS angiogenic defects and leaky CNS barriers. However, these effects occur in distinct regions of the CNS, namely the cerebral cortex and dorsal spinal cord (C. Cho et al. 2017, Daneman et al. 2009, Stenman et al. 2008, Wang et al. 2012, Zhou et al. 2014).

The role of GPR124, the coactivator in this pathway, has been well studied (Anderson et al. 2011, Chang et al. 2017, Cullen et al. 2011, Kuhnert et al. 2010, Vanhollebeke et al. 2015, Zhou et al. 2014). Interestingly, while GPR124 plays a major role in BBB development, with knockout mice displaying severe angiogenic defects and tracer leakage in the forebrain, endothelium-specific deletion in adult mice does not result in barrier breakdown (Chang et al. 2017). In contrast, postnatal deletion of either *Ctnnb1* or *Fzd4* (which encodes Frizzled4) results in a compromised BBB (Tran et al. 2016, Wang et al. 2012). However, under challenge, mouse models of ischemic stroke or glioblastoma GPR124 deficiency show rapid barrier breakdown via loss of canonical Wnt signaling (Chang et al. 2017).

The second described Wnt pathway in barriergenesis is activated by Norrin (encoded by *Ndp*), a ligand that belongs to the TGFβ family and that binds to Frizzled4 (Smallwood et al. 2007). Norrin is expressed by astroglia and, to some extent, by oligodendrocytes in the CNS (Zhang et al. 2014). Binding of Norrin to Frizzled4, which acts with its coreceptors Lrp5 and Tspan12, leads to downstream β-catenin stabilization (Junge et al. 2009, Wang et al. 2012, Ye et al. 2010) (**Table 1**). Knockout mice for *Ndp*, *Fzd4*, or *Tspan12* show severe angiogenic phenotypes and have a dysfunctional barrier in the retina, cerebellum, and ventral spinal cord (Junge et al. 2009, Wang et al. 2012). In the retina, the Wnt inhibitor Apcdd1 modulates the Norrin pathway and barrier maturation (Mazzoni et al. 2017). Depleting components of both the Norrin and Wnt7a/b pathways during development results in severe leakage throughout the brain, including regions

Table 1 Determinants of blood-brain barrier physiology

Cell type	Protein (gene)	Function	Reference(s)
Endothelial cells	Lsr (*Lsr*)	Formation of tricellular junctions	Ikenouchi et al. 2005, Sohet et al. 2015
	Claudin-5 (*Cldn5*)	Formation of tight junctions	Morita et al. 1999, Nitta et al. 2003
	Glut1 (*Slc2a1*)	Transport of glucose across the barrier	De Vivo et al. 1991, Wang et al. 2006, Winkler et al. 2015
	LAT1 (*Slc7a5*)	Transport of branched amino acids across the barrier	Tarlungeanu et al. 2016
	P-glycoprotein (*Abcb1*)	Export of amyloid β and efflux of drugs from the brain	Cirrito et al. 2005, Cordon-Cardo et al. 1989, Schinkel et al. 1996
	Mfsd2a (*Mfsd2a*)	Suppression of transcytosis	Andreone et al. 2017, Ben-Zvi et al. 2014
	Transferrin receptor (*Tfrc*)	Receptor for transferrin; undergoes transcytosis upon binding of transferrin	Friden et al. 1991, Jefferies et al. 1984, Yu & Watts 2013
	PDGF-B (*Pdgfb*)	Recruitment of pericytes	Armulik et al. 2010, Hellstrom et al. 1999, Lindahl et al. 1997
	Smoothened (*Smo*)	Activation by Sonic hedgehog induces *Cldn5* and *Ocln* expression and induces immune quiescence	Alvarez et al. 2011
	Dab1 (*Dab1*)	Activation by Reelin induces laminin α4 localization to ECM and astrocyte endfeet attachment	Segarra et al. 2018
	Integrin β1 (*Itgb1*)	Required for VE-cadherin localization to adherens junctions	Yamamoto et al. 2015
	Lrp5/6 (*Lrp5*, *Lrp6*)	Coreceptors of Frizzled receptors; required to induce tight junction formation via β-catenin stabilization	Chen et al. 2012, Junge et al. 2009, Zhou et al. 2014
	GPR124 (*Adgra2*)	Coreceptor of Frizzled receptors; required to induce tight junction formation via β-catenin stabilization	Anderson et al. 2011, Chang et al. 2017, C. Cho et al. 2017
	Reck (*Reck*)	Coreceptor of Frizzled receptors; required to induce tight junction formation via β-catenin stabilization	C. Cho et al. 2017, Vanhollebeke et al. 2015
	Frizzled4 (*Fzd4*)	Frizzled receptor mediating Norrin signal to induce tight junction formation via β-catenin stabilization	Smallwood et al. 2007, Wang et al. 2018
	Tspan12 (*Tspan12*)	Coreceptor of Frizzled4; required to induce tight junction formation via β-catenin stabilization	Junge et al. 2009, Wang et al. 2018
Pericytes	PDGFRβ (*Pdgfrb*)	Localization of pericytes to endothelial cells	Daneman et al. 2010, Hellstrom et al. 1999
	Foxf2 (*Foxf2*)	Stimulates *Pdgfrb* expression and TGFβ signaling	Reyahi et al. 2015
Astrocytes	Sonic hedgehog (*Shh*)	Activates Smoothened on endothelial cells and induces *Cldn5* and *Ocln* expression and immune quiescence	Alvarez et al. 2011
	Laminin γ1 (*Lamc1*)	Induces tight junction protein expression in endothelial cells and Aqp4 expression in astrocytes	Yao et al. 2014

(*Continued*)

Table 1 (*Continued*)

Cell type	Protein (gene)	Function	Reference(s)
Other cell types	Reelin (*Reln*)	Secreted by neuronal cells; activates Dab1 in endothelial cells	Segarra et al. 2018
	Wnt7a and -b (*Wnt7a*, *Wnt7b*)	Secreted by Bergmann glia and neuronal cells; activate Frizzled receptors, inducing canonical β-catenin signaling	Daneman et al. 2009, Wang et al. 2018
	Norrin (*Ndp*)	TGFβ family ligand; secreted by neuronal cells and astroglia; activates Frizzled4	Smallwood et al. 2007; Wang et al. 2012, 2018
	Sonic hedgehog (*Shh*)	Secreted by neural progenitors and astrocytes; activates Smoothened on endothelial cells	Alvarez et al. 2011

such as the brain stem that are not affected by perturbation of either pathway alone (C. Cho et al. 2017, Wang et al. 2018, Zhou et al. 2014).

The two different pathways of canonical Wnt activation are regionally active in both mutually exclusive and redundant patterns. The Wnt7a/b pathway is exclusively active in the forebrain and ventral spinal cord, while the norrin pathway is exclusive to the cerebellum, retina, and dorsal spinal cord. In the brain stem, both pathways are active (C. Cho et al. 2017, Wang et al. 2018, Zhou et al. 2014). This local regulation of Wnt signaling may provide avenues to manipulate CNS barriers in a spatially selective manner. Future studies are required to explore this idea further.

Canonical Wnt signaling is the best-characterized pathway in BBB formation. The expression of certain transporters and TJ proteins such as Glut1 and Claudin-5 also correlates with canonical Wnt activation (**Figure 2**). However, the mechanisms of how canonical Wnt activation leads to formation of a functional barrier are not well understood. For example, it is not known whether Wnt signaling also plays a role in the inhibition of transcytosis, although mutants for Wnt components show increased Plvap expression. Plvap expression commonly correlates with permeable vasculature and has been associated with transcytosis; however, overexpression of Plvap does not increase brain vessel permeability (Stan et al. 2012).

While we are beginning to understand the contribution of canonical Wnt signaling to BBB development, there are likely other involved pathways that are locally integrated into the formation of a functional barrier. One task for future research will be to understand how these pathways collaborate throughout the CNS and locally in defined brain regions.

5. OUTLOOK

Other mammalian tissues, such as the gut, skin, placenta, testis, and kidney, also contain barriers. These barriers regulate the molecular exchange between neighboring compartments, although with various levels of permeability, depending on the function of the tissue. Because each barrier operates in a distinct tissue environment, the site of the barrier is formed by different cell types in each tissue. For example, while endothelial cells form the BBB, epithelial cells form the gut barrier, and Sertoli cells form the testis barrier. However, these barriers and the BBB also exhibit many similarities. For example, these barriers utilize common mechanisms to regulate paracellular and transcellular passage, such as by the formation of TJs and control of the transcytotic pathway. Moreover, the cells that compose each of these barriers are polarized and rely on mechanisms of apical-basolateral sorting. Furthermore, the interaction of each barrier with the ECM and local cellular microenvironment dynamically regulates the function of each of these organ barriers.

Within the field of BBB research, we envision several new avenues that will unravel previously unknown modes of BBB regulation. The advent of new single-cell transcriptomic analyses has allowed researchers to begin to identify the various cell types in the brain and their transcriptional signatures (Saunders et al. 2018, Vanlandewijck et al. 2018, Zeisel et al. 2018). Our next challenges will be to utilize these data sets to identify the specific molecules and signaling pathways that mediate BBB formation and function across cell types. Additionally, identifying endothelial subtype–specific markers might help in understanding the spatial distribution of cell subtypes and in dissecting regional differences in barrier induction. The development of genetic tools to target pericytes, astrocytes, or subtypes of endothelial cells will be critical to advancing our understanding of barrier function.

As discussed in this review, the BBB is a highly dynamic structure regulated by multiple cell types. The latest research has found that BBB properties in fruit flies underlie the circadian rhythm (Zhang et al. 2018). While the structure of the barrier is very different in flies than in mammals (O'Brown et al. 2018), it will be interesting to see whether the capacity of barrier modulation by circadian signals is conserved across species. Although we are beginning to dissect specific roles of different cell types during barrier development, their differential contributions to barrier maintenance through adulthood are unclear. Furthermore, the physiology of the barrier and the roles of pericytes and astrocytes in disease progression are unknown. Although barrier breakdown has been observed in several pathologies, it is unclear whether breakdown is the initial step of pathogenesis or a consequence of disease progression.

The discovery of novel BBB regulators and a deeper understanding of the mechanisms mediating barrier function will likely provide new targets to pharmacologically modulate the barrier in disease conditions. After this initial identification, reliable and robust in vitro systems are needed to develop high-throughput screening of molecules that can change barrier properties. In recent years, a few models have been successful in capturing most, if not all, properties of the NVU. These models include inducible pluripotent stem cells (Lippmann et al. 2012), self-assembling multicellular BBB spheroids (C.-F. Cho et al. 2017), and microfluidic organ chips (Maoz et al. 2018). It remains to be seen whether we can use these model systems to screen molecules for drug delivery into the brain and to study the basic cell biology of the NVU, which can then be investigated in vivo.

Finally, the heterogeneity of the BBB across various brain regions is an unexplored area of research that needs investigation. The phenotypes of mouse mutants for the Wnt signaling components described above demonstrate that there is molecular heterogeneity in BBB formation across different brain regions. It is presently unknown whether differential development translates into distinct barrier properties during adulthood. Moreover, there are regions in the brain with permeable vessels serving nearby neurons to sense components in the bloodstream and to secrete compounds into circulation. Understanding the molecular and cellular basis of a functional barrier in different parts of the brain not only will identify new players in BBB function but also will uncover unique themes of barrier regulation. Together, achieving such advances would have the potential to provide novel ways to manipulate the barrier globally as well as in a region-specific manner. Over the next few decades, we anticipate all these research areas to reveal new aspects of barrier regulation that will provide a detailed understanding of the BBB in both health and disease.

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