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Review

The Molecular Constituents of the Blood–Brain Barrier

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The blood–brain barrier (BBB) maintains the optimal microenvironment in the central nervous system (CNS) for proper brain function. The BBB comprises specialized CNS endothelial cells with fundamental molecular properties essential for the function and integrity of the BBB. The restrictive nature of the BBB hinders the delivery of therapeutics for many neurological disorders. In addition, recent evidence shows that BBB dysfunction can precede or hasten the progression of several neurological diseases. Despite the physiological significance of the BBB in health and disease, major discoveries of the molecular regulators of BBB formation and function have occurred only recently. This review highlights recent findings describing the molecular determinants and core cellular pathways that confer BBB properties on CNS endothelial cells.

History of the BBB

The **BBB** (see [Glossary](#)) partitions the brain from circulating blood and functions to: (i) shield the brain from potential blood-borne toxins; (ii) meet the metabolic demands of the brain; and (iii) regulate the homeostatic environment in the CNS for proper neuronal function [1]. The functional BBB comprises CNS **endothelial cells**, pericytes, astrocytes, and neurons that collectively form a functional ‘**neurovascular unit**’ (NVU) ([Figure 1](#)) [2].

The BBB was first observed over a century ago. Pioneering physiologists studying the cerebrospinal fluid (CSF) noticed that water-soluble dyes injected in the peripheral circulation stained several tissues except the brain [3]. Ehrlich argued that this phenomenon occurred because the CNS had low affinity for the dye [4]. However, Goldmann questioned this argument, as injection of the same dyes in the subarachnoid space colored the brain but not peripheral tissues [5]. Continuing from these studies, Lina Stern and colleagues performed experiments in which they injected several vehicles into the brain parenchyma and blood. The results from these dye studies prompted Stern to introduce the term ‘blood–brain barrier’ and suggest its physiological function in maintaining brain homeostasis [6]. Over the years, the concept of the BBB fascinated physiologists but the anatomical site of the BBB was highly disputed; specific possibilities included the endothelium, astrocytic end-feet, and the basement membrane. A seminal study by Reese and Karnovsky using electron microscopy (EM) and injection of electron-dense horseradish peroxidase (HRP) resolved this dispute [7]. In this work, ultrastructural analysis by EM was used to delineate astrocytic end-feet and the luminal, abluminal, and basement membrane. Results revealed that the HRP was confined to the lumen of the CNS endothelium. Furthermore, EM revealed that the CNS endothelial cells are joined continuously by **tight junction** complexes and have limited intracellular vesicles [7]. Similar to Goldman's experiments, HRP injected into the brain parenchyma diffused past astrocytic end-feet and halted at the abluminal membrane of the endothelium, demonstrating that astrocytic end-feet do not significantly contribute to the physical barrier [8]. Thus, the site

Trends

The BBB comprises CNS endothelial cells that display specialized molecular properties essential for BBB function and integrity.

These molecular BBB properties are not intrinsic to CNS endothelial cells but have to be induced by the environment.

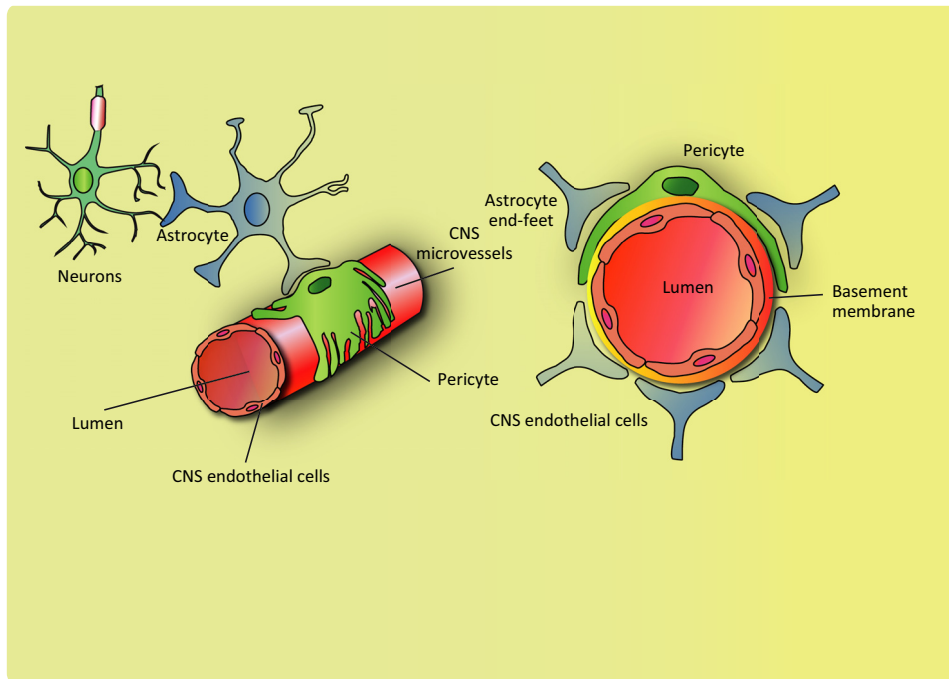
The formation, function, and maintenance of the BBB require functional interaction between CNS endothelial cells and NVUs.

Advances in gene profiling and cell-type purification methods have progressed the identification of the molecular mediators and core cellular pathways involved in BBB function and integrity.

A comprehensive understanding of the key molecules and cellular pathways involved in BBB function would offer novel strategies for CNS therapeutics.

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Trends in Neurosciences

Figure 1. The Functional Blood–Brain Barrier (BBB) is Dependent on the Neurovascular Unit (NVU). The BBB is localized at central nervous system (CNS) microvessels, comprising a single layer of continuous, non-fenestrated endothelial cells. Surrounding the abluminal surface of the CNS endothelial cells are the basement membrane, pericytes, and astrocyte end-feet, collectively known as the NVU. BBB properties are not intrinsic to CNS endothelial cells but require continuous functional interactions with the NVU.

of the BBB is CNS capillaries comprising a single, non-fenestrated, continuous endothelial cell layer.

Molecular Properties of the BBB

CNS endothelial cells are highly polarized with distinct luminal (apical) and abluminal (basolateral) compartments [9]. The polarized nature of CNS endothelial cells is reflected in their four fundamental barrier properties that contribute to BBB function and integrity (Figure 2) [10]. First, circumferential tight junction complexes at the lateral, apical membrane between CNS endothelial cells establish a high-resistance paracellular barrier to small hydrophilic molecules and ions [8,11]. Tight junction complexes comprise: (i) tight junction proteins such as claudins and occludin; (ii) adhesion molecules such as VE-cadherin and E-cadherin; and (iii) junctional adhesion molecules [12,13]. These transmembrane proteins are further linked and stabilized to the cytoskeleton via multiple cytoplasmic adaptor proteins such as zonula occludens proteins [14]. Emerging studies have demonstrated that there is significant crosstalk among these tight junction complex proteins to regulate the restrictive barrier junction [15]. Second, in contrast to the peripheral endothelium, CNS endothelial cells display minimal vesicular trafficking, limiting the vesicle-mediated transcellular movement of cargo known as **transcytosis** [16]. Although CNS endothelial cells display limited transcytosis, it remains the preferred pathway for the selective transport of plasma macromolecules such as albumin and low-density lipoprotein [17]. Third, the establishment of the restrictive paracellular and transcellular barriers allows CNS endothelial cells to use highly polarized cellular transporters to dynamically regulate the influx of nutrients and efflux of metabolic waste and toxins between the blood and the brain parenchyma. The major class of known efflux transporters is the ATP-binding cassette (ABC) transporters – including Pgp, BCRP, and MRP – mostly localized at the luminal membrane [18–20]. These efflux

Glossary

Angiogenesis: the development of new vessels from proliferation of pre-existing endothelial cells.

Blood–brain barrier (BBB): a physiological barrier comprising a thin layer of continuous, non-fenestrated CNS endothelial cells that regulates the brain microenvironment for proper neuronal function.

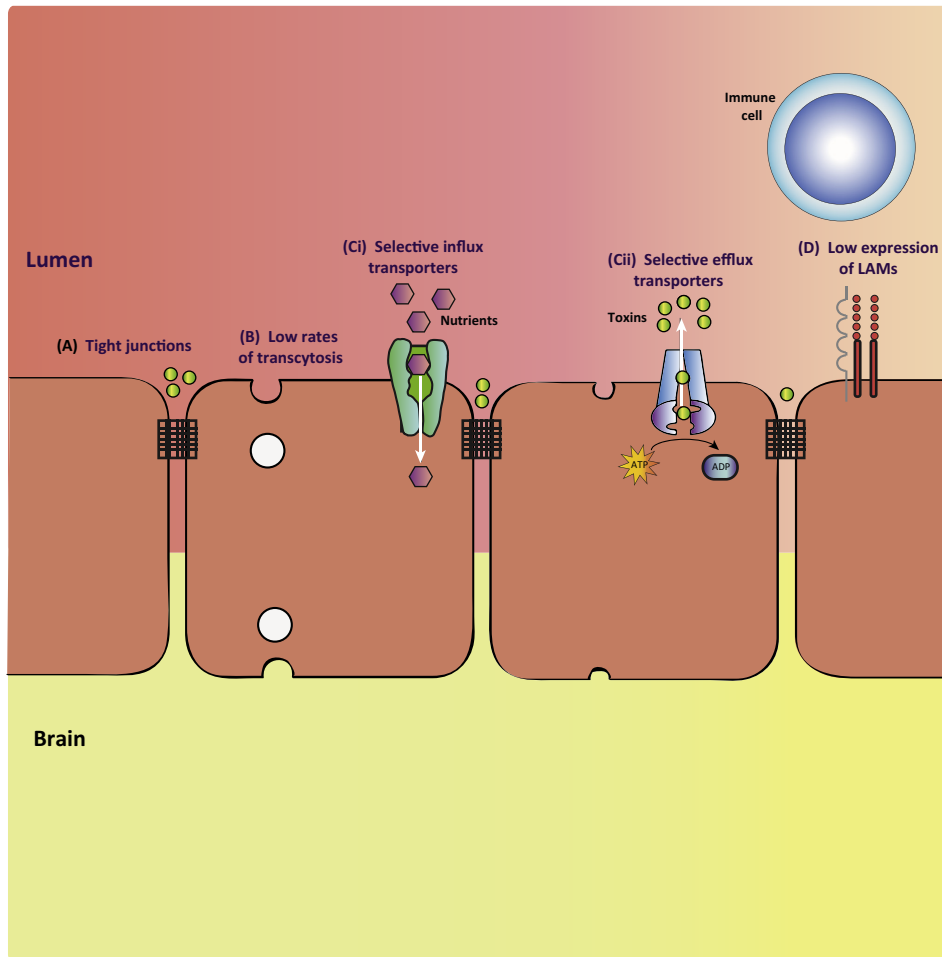
Endothelial cells: mesoderm-derived cells that line the vasculature of the circulatory system.

Immune privilege: the introduction of antigens without eliciting an inflammatory adaptive immune response.

Neurovascular unit (NVU): the functional interactions among neurons, glia, pericytes, and endothelial cells.

Tight junction: a junctional complex between two cells that is essential for cell polarity, barrier functions, and cell adhesion.

Transcytosis: vesicular trafficking from the luminal to the abluminal plasma membrane and vice versa.



Trends in Neurosciences

Figure 2. The Four Fundamental Molecular Properties of Central Nervous System (CNS) Endothelial Cells that Contribute to Blood–Brain Barrier (BBB) Integrity and Function. (A) Specialized tight junction complexes between endothelial cells prevent paracellular flux. (B) CNS endothelial cells have low rates of transcytosis, limiting transcellular flux. CNS endothelial cells mediate (Ci) the selective uptake of nutrients and molecules from the blood using selective influx transporters and (Cii) efflux of toxins against their concentration gradient with ATP-dependent selective efflux transporters. (D) The low expression of leukocyte adhesion molecules (LAMs) contributes to the low level of immune surveillance in the CNS.

transporters hydrolyze ATP to transport a wide array of substrates into the blood against their concentration gradients [19,20]. CNS endothelial cells also express specialized nutrient transporters that facilitate the transport of ions, macromolecules, and proteins from the blood to the brain. Many of these transporters belong to the superfamily solute carrier proteins (SLCs) of facilitative transporters that includes sugar transporters such as SLC2A1 (GLUT1) and cationic amino acid transporters such as SLC7A1 [21,22]. It is surprising to note that although SLCs play a vital role in metabolism and nutrition, they are particularly understudied [23]. Fourth, CNS endothelial cells lack expression of leukocyte adhesion molecules (LAMs) such as E-selectin and Icam1 [24]. The lack of these luminal surface molecules prevents the entry of immune cells from the blood into the parenchyma, resulting in a paucity of immune cells in the brain microenvironment [25]. As a result, the healthy brain is **immune privileged**, whereby introduced antigens do not elicit the development of adaptive immune responses [26]. These fundamental molecular characteristics confer BBB properties on CNS endothelial cells to regulate brain homeostasis.

Identifying Molecular Regulators of BBB Function and Integrity in CNS Endothelial Cells

The four fundamental BBB properties listed above are not intrinsic to CNS endothelial cells but are induced and regulated by the neural environment [27]. Transplantation studies using chick/quail chimeras have demonstrated that nonvascularized brain fragments transplanted into the coelomic cavity were soon vascularized by abdominal vessels that developed BBB characteristics such as exclusion of circulating dye and low number of vesicles [28]. By contrast, nonvascularized embryonic mesoderm tissues grafted in the CNS were soon vascularized by neural vessels that failed to display BBB properties [28]. This seminal experiment demonstrated that: (i) BBB properties are not inherent to CNS endothelial cells and (ii) the neural environment provides inductive cues to CNS endothelial cells to activate genetic programs and thus acquire BBB properties. Although the identities of these signals and genetic programs have been elusive, recent advances in the purification and gene expression profiling of CNS endothelial cells have elucidated novel molecular mediators that confer barrier properties on CNS endothelial cells.

The most well-characterized genetic program inducing BBB properties in CNS endothelial cells (Table 1) is β -catenin signaling [29–31]. Daneman *et al.* purified CNS and peripheral endothelial cells using fluorescence-activated cell sorting (FACS) from Tie2GFP mice, a transgenic GFP reporter for endothelial cells [31]. Microarray analysis indicated that many downstream effectors of Wnt/ β -catenin signaling are enriched in CNS endothelial cells, suggesting that β -catenin signaling may mediate CNS vasculature functions. Various studies have demonstrated that canonical Wnt signaling is essential for both CNS-specific **angiogenesis** and **barriergeneration**. For example, endothelial cell-specific deletion of β -catenin disrupts CNS angiogenesis, resulting in gross vascular malformations and hemorrhages, whereas peripheral angiogenesis remains largely undisrupted [31]. In addition, endothelial cell-specific deletion of β -catenin disrupts barriergeneration, downregulating expression of Glut1, a marker commonly used for BBB formation, in the CNS vasculature [29–31]. Zhou *et al.* [28] demonstrated that postnatal endothelial cell-specific deletion of β -catenin results in BBB breakdown, exemplified by extravasation of dyes and downregulation of tight junction protein expression, showing that barrier dysfunction is not a consequence of disrupted angiogenesis. Recently, many of the receptors upstream of β -catenin – including Frizzled receptors, coreceptors LRP5/LRP6, and auxiliary receptor GPR124 – as well as the Wnt ligands (see next section) necessary for β -catenin activation have been

Table 1. CNS Endothelial Cell Genes that Mediate Angiogenesis and Barriergeneration^a

CNS Endothelial Cell Genes	Vascular formation	Barrier integrity		Refs
		Tight Junctions	Transcytosis	
Wnt Signaling Components		Tight Junctions	Transcytosis	[29–40]
β -catenin	Yes	Yes	Yes ^c	[29–31]
Frizzled4	Yes	Yes	Yes ^c	[32–34,80]
GPR124	Yes	Yes	Yes ^c	[35–39]
LRP5/LRP6	Yes	Yes	Yes ^c	[32–34,40]
TROY/DR6	Yes	Yes	ND	[41]
Mfsd2a	No	No	Yes ^c	[42–45,47–48]
LSR	No ^b	Most likely ^d	ND	[51–53]

^aAbbreviation: ND, not determined.

^bNo vascular defects were observed during embryonic analysis of *lsl* knockout mice.

^cLoss-of-function of Wnt signaling results in elevated expression of PLVAP, a marker for fenestrated endothelial cells and transcytosis.

^dAlthough ultrastructural analysis by EM revealed no defects in tight junction complexes, loss of LSR affects tight junctions.

identified [32–40]. Loss of function of these genes results in CNS vasculature dysfunction and largely resembles β -catenin mutants [33,34].

A recent study identified novel downstream targets of β -catenin signaling that mediate both CNS angiogenesis and barrierogenesis. Tam *et al.* used antibody-based FACS to isolate endothelial cells from CNS and non-CNS tissues at three developmental ages [41]. Microarray analysis indicated that *dr6*, *troy*, and *spock2* are highly enriched in CNS endothelial cells. These genes are essential for CNS vasculature function, as *dr6*, *troy*, and *spock2* knockdown in zebrafish resulted in vascular malformation and barrier dysfunction. By contrast, loss of function of two other genes enriched in CNS endothelial cells, *abcyap1r1* and *tspn5*, resulted in vascular morphogenesis defects but exhibited no barrier dysfunction. The authors demonstrated that *in vitro* activation of β -catenin with recombinant Wnt ligands upregulates the expression of *dr6* and *troy*, suggesting that these genes are downstream effectors of β -catenin. However, it is difficult to determine whether these genes specifically regulate barrierogenesis or whether barrier dysfunction is a consequence of vascular malformations. Temporal deletion of these genes after CNS angiogenesis can clarify this point.

Genes that specifically regulate BBB function and integrity independent of CNS angiogenesis have been identified via gene profiling of purified CNS endothelial cells. Ben-Zvi *et al.* mapped the development of mouse cortical barrierogenesis at E15.5 and performed microarray analysis from FACS-purified Tie2GFP⁺ CNS and lung endothelial cells at E13.5, a time when barrier properties are actively forming [42]. The microarray analysis indicated that major facilitator domain containing protein 2A (*Mfsd2a*) is enriched in CNS endothelial cells. *Mfsd2a* is expressed specifically in the CNS vasculature and not in the choroid plexus, a structure that lacks a BBB. *Mfsd2a* knockout mice display BBB dysfunction due to unregulated bulk flow of transcytosis. However, vascular development and patterning remain unaffected, suggesting that *Mfsd2a* specifically regulates BBB integrity independent of angiogenesis. Surprisingly, *Mfsd2a* has putative dual physiological functions [43,44]. Not only is *Mfsd2a* essential for the CNS endothelium to maintain low rates of transcytosis, but also Nguyen *et al.* reported that *Mfsd2a* is a transporter for omega-3 fatty acids across the CNS endothelium [45]. Lipidomics revealed that the brains of *Mfsd2a* knockout mice exhibit decreased levels of docosahexaenoic acid (DHA), an omega-3 fatty acid essential for neuronal function, and elevated levels of arachidonic acid, an omega-6 fatty acid. Furthermore, *Mfsd2a* knockout mice display fewer neurons in the hippocampus and cerebellum, microcephaly, and other neurological deficits. These altered brain fatty acids and behavioral abnormalities are reminiscent of omega-3 fatty acid deficiency [46]. Recent human genetics studies identified loss-of-function missense mutations in *MFSD2A* as a recessive cause of microcephaly [47,48]. One study identified two *MFSD2A* missense mutations that result in severe intellectual disability, seizures, and early lethality, whereas a second study identified a milder *MFSD2A* missense mutation that results in intellectual disability alone. Similar to GLUT1, *Mfsd2a* has dual physiological functions at the BBB: maintaining barrier integrity and transporting nutrients across the barrier [49,50]. It will be essential for future studies to determine whether *Mfsd2a*'s dual physiological functions of (i) maintaining BBB integrity and (ii) transporting essential nutrients act simultaneously or whether one function is required for the other.

Another recently discovered gene that mediates barrierogenesis independent of angiogenesis is lipolysis-stimulated lipoprotein receptor (LSR). Sohet and Daneman *et al.* purified TIE2GFP⁺ CNS and peripheral endothelial cells and performed microarray analysis to identify *lsr*, another gene enriched in CNS endothelial cells [51,52]. Although *lsr* is expressed in many cell types in peripheral tissues, it is expressed specifically in endothelial cells in the brain. LSR was initially reported to mediate the clearance of triglyceride-rich lipoproteins and low-density lipoproteins but the physiological function of LSR in the BBB was only recently explored [53]. *lsr* knockout mice were reported not to display any vascular malformations or hemorrhage, but still display

BBB dysfunction. LSR is essential for BBB integrity, as *lssr* knockout embryos exhibit extravasation of low molecular weight tracers but not higher molecular weight tracers, a phenotype that is reminiscent of the *claudin5* knockout mouse [54]. This size-selective permeability dysfunction may be a common phenotype observed on the disruption of tight junction molecules. The extravasation of small tracers is most likely mediated via paracellular entry. By contrast, larger molecules most likely leak out through transcytosis, as observed in *Mfsd2a* knockout mice where HRP (44 kDa) and 70-kDa dextran tracers leak out of the CNS vasculature. However, the molecular mechanism underlying LSR regulation of BBB integrity remains unknown. Although LSR is localized at tricellular tight junctions (where two bicellular tight junctions meet) in the functional BBB, *lssr* knockout mice display no obvious disruption in tight junctions complexes on EM. Because *lssr* has known physiological functions in peripheral tissues, conditional deletion of LSR will determine whether a cell-autonomous function of LSR regulates BBB integrity.

Identifying Inductive Signals that Confer BBB Properties

Recent studies have identified key inductive signals in the CNS microenvironment that confer BBB properties on CNS endothelial cells (Table 2). It is evident that these inductive signals originate from the NVU. As mentioned above, the most well-characterized signal that mediates BBB function is canonical Wnt signaling [29–31,33]. Neural progenitors in the neuroepithelium secrete Wnt7a/Wnt7b, whereas in the cerebellum Bergmann glia secrete Norrin. These secreted ligands bind to classical components of canonical Wnt signaling such as the Frizzled receptors and co-receptors LRP5/LRP6, which are expressed on CNS endothelial cells to drive β -catenin signaling. Disruption of these Wnt ligands phenocopies β -catenin mutants, impairing CNS angiogenesis and displaying reduced vessel numbers, vascular malformations, hemorrhages, and BBB dysfunction [31–33].

Another inductive signal essential for barrierogenesis is Sonic hedgehog (Shh) signaling. Although Hedgehog signaling has been well-characterized for neuronal development and angiogenesis, Alvarez *et al.* demonstrated that astrocyte-secreted Shh is essential for BBB integrity and CNS immune quiescence [55,56]. Astrocytes express *shh* whereas CNS endothelial cells robustly express the Hedgehog signaling components Patched-1, Smoothed, and Gil. Astrocyte-conditioned medium or recombinant Shh were sufficient to: (i) elevate tight junction protein expression and transendothelial electrical resistance (TEER), a technique used to measure the integrity of tight junctions dynamics in cell culture and (ii) suppress permeability to various tracers *in vitro*. Furthermore, endothelial cell-specific disruption of Hedgehog signaling *in vivo* results in normal vascular formation but BBB dysfunction through suppressed expression of tight junction proteins and extravasation of plasma proteins. Hedgehog signaling is also essential to establish immune quiescence in the CNS. For example, Hedgehog signaling is sufficient to suppress chemokines and LAM expression in ECs *in vitro*. Furthermore, Hedgehog signaling in leukocytes suppresses the expression of proinflammatory cytokines such as tumor necrosis factor (TNF),

Table 2. Inductive Signals that Mediate CNS Angiogenesis and Barrierogenesis^a

Inductive Signals	Secreted by	Vascular Formation	Barrier Integrity		Refs
			Tight Junctions	Transcytosis	
Wnt7a/7b	Neural progenitors; Bergmann glia	Yes	Yes	Yes ^b	[29–40]
Shh	Astrocytes	ND	Yes	ND	[55]
Short chain fatty acids	Gut microbiota	No	Yes	ND	[57]

^aAbbreviation: ND, not determined.

^bLoss-of-function of Wnt signaling results in elevated expression of PLVAP, a marker for fenestrated endothelial cells and transcytosis.

reducing neuroinflammatory processes. Hedgehog signaling has a protective role in neuroinflammatory diseases such as multiple sclerosis (MS). MS patients display elevated Hedgehog signaling components in the CNS and pharmacological blockade of Hedgehog signaling in experimental autoimmune encephalomyelitis (EAE) models results in greater severity of the disease, with increases in proinflammatory cytokines, leukocyte accumulation in the CNS, and demyelination. It should be noted that the above studies focus on Hedgehog signaling as essential for BBB maintenance, not necessarily induction, as astrocytes are born and manifest in the NVU around birth. Although Shh is robustly expressed in the CNS during embryonic development and Shh knockout mice display reduced tight junction expression at E13.5, shortly before BBB maturation, the early roles of Hedgehog signaling during barriergenesis are still not well characterized. It will be interesting to explore the early inductive roles of Shh during BBB development before the onset of astrocyte-mediated Shh signaling to maintain BBB integrity.

Although it is well established that the neural microenvironment contains factors that induce CNS endothelial cells to manifest BBB properties, recent studies demonstrate that environmental cues and factors extrinsic to the CNS can also impact BBB development and integrity. Braniste *et al.* demonstrated that the gut microbiota influences the regulation of the BBB through epigenetic control of tight junction expression in CNS endothelial cells [57]. Emerging studies have demonstrated that an organism's microbiota influences many physiological functions, including behavior [58]. Furthermore, the gut microbiota has been reported to influence tissue barrier systems [59]. Comparing pathogen-free mice (control) and germ-free mice (altered microbiota), these authors discovered that germ-free mice display BBB dysfunction in both embryonic development and postnatal life, due to downregulation of tight junction protein expression. Unlike pathogen-free mice, germ-free mice display extravasation of Evans Blue dye. Consistent with low tight junction expression, ultrastructural analysis by EM revealed disruption of tight junction complexes. Remarkably, transplanting fecal matter from pathogen-free mice to recolonize the intestinal microbiota of germ-free mice can restore the dysfunctional BBB observed in germ-free adult mice. Germ-free mice with recolonized microbiota have restored tight junction protein expression in the CNS with accompanying restriction of dye tracers to CNS endothelium. The molecular determinants from microbiota impacting BBB integrity in these experiments were short-chain fatty acids such as butyrate, which has been reported to strengthen the integrity of the intestinal epithelial barrier. Treatment with butyrate was sufficient to elevate tight junction protein expression and restore BBB integrity in the germ-free mice. The authors suggest that butyrate epigenetically regulates tight junction expression in the CNS by increasing histone acetylation. This crosstalk between the microbiota and the BBB is intriguing and provocative. It will be interesting to explore whether short-chain fatty acids directly increase BBB integrity or cause secondary effects on other signaling pathways throughout the body. Furthermore, it will be of clinical interest to explore how the use of strong antibiotics that eliminate the gut microbiota influences the BBB.

Another recent study highlighted how foreign microbes impact the BBB. Acute bacterial meningitis is an infection in the CNS that causes neural damage and can result in mental impairment, seizures, paralysis, and death if untreated [60]. To induce meningitis, bacteria must first breach the BBB [61]. However, it is unclear how bacteria penetrate through the BBB. Kim *et al.* demonstrated that blood-borne bacteria such as group B *Streptococcus* (GBS) can weaken BBB integrity by upregulating the expression of *Snail1*, a zinc finger transcription factor, in host CNS endothelial cells, which subsequently suppresses tight junction protein expression [62]. Exposure of GBS to CNS endothelial cells *in vitro* and *in vivo* upregulates *Snail1* expression and downregulates tight junction protein expression, with accompanying increases in GBS counts in the brain. Furthermore, transgenic dominant-negative *Snai1* zebrafish are more resistant to GBS-mediated lethality. It is interesting that bacteria can manipulate the gene expression in host CNS endothelial cells to weaken the integrity of the BBB. It will be of clinical

interest for future studies to explore what bacterial molecules interact with host CNS endothelial cells to alter gene expression and to determine whether bacteria breach the BBB via weakened tight junctions.

Future Directions of BBB Research

Although the BBB research community has recently made significant strides in identifying novel molecular regulators and inductive signals that mediate BBB function and integrity, the field remains in its infancy, with many fundamental questions waiting to be answered (see Outstanding Questions). Further refinements of cell-type purification techniques and next-generation sequencing technologies will unravel key molecular regulators and core pathways essential for BBB formation and function. Currently, only a single CNS endothelial cell RNAseq dataset from mouse exists, but it nevertheless has been fruitful to compare gene expression among multiple purified cell types in the CNS [63]. However, this dataset is limited because it is only from normally developing mice at age P7. The RNAseq approach to unravel the transcriptome of the BBB and other purified cell types in the NVU in different developmental, physiological, aging, and disease contexts will address many questions about BBB regulation. Next-generation sequencing will be invaluable to address the underappreciated heterogeneity of the BBB. For example, different brain regions use different Wnt/ β -catenin molecular components for BBB function. Norrin is the Wnt signal in the retina and cerebellum whereas Wnt 7a/7b are the signals in the forebrain. [33]. Furthermore, it would be interesting to explore how the molecular signatures of the CNS endothelial cells in circumventricular organs (the regions of the CNS that do not display a BBB, such as the median eminence) differ from those of CNS endothelial cells displaying the BBB [64–66].

Transcriptomics has proved invaluable in identifying genes that regulate BBB function and integrity [67]. However, the genes from these datasets remain merely candidates until validated as indeed mediating BBB regulation. Therefore, the field needs high-throughput screening not only to validate candidate genes but also to discover drugs that can modulate BBB permeability. The advent of genome editing methods such as CRISPR–Cas9 has facilitated the generation of *in vivo* loss-of-function transgenesis but this process remains arduous and too low throughput to validate a list of candidates from transcriptomics datasets [68]. Thus, the use of more tractable model organisms with a simpler BBB and robust loss-of-function genetic manipulations could accelerate the validation of candidates.

The ideal high-throughput screening method would be an *in vitro* BBB system that reproduces the properties of the BBB *in vivo* such as CNS endothelial cell polarity and restrictive paracellular and transcellular permeability. Because the functional BBB requires interaction among the multiple cell types of the NVU, CNS endothelial cells readily lose their BBB properties *ex vivo* [69]. Several studies demonstrated that co-culture with astrocytes and pericytes to mimic the NVU can enhance BBB properties in endothelial cells [70]. Furthermore, stimulation of cultured endothelial cells with Wnt and Shh is sufficient to induce BBB properties without the need to co-culture other cell types, suggesting that activating core BBB pathways is sufficient to elicit BBB properties and further emphasizing the need to determine the molecular regulators of BBB function activated by these signaling pathways [55,71]. Intriguingly, studies reported that human pluripotent stem cells treated with retinoic acid can differentiate to endothelial cells displaying BBB properties [72,73]. The development of these new technologies will accelerate the discoveries of key molecules and essential signaling pathways in BBB and neuroscience research.

Although the BBB field has made significant progress in identifying key molecules that mediate BBB function, the molecular and cellular mechanisms of how these molecules mediate BBB function and integrity remain poorly understood. This gap in knowledge is partly due to the limitations in the current technologies for BBB research. Currently, EM analyses in conjunction with dye tracers are the main techniques to monitor BBB properties [27], but these techniques

provide only a static snapshot of the BBB and do not provide essential information such as the kinetics of vesicular trafficking. There is a pressing need to develop an *in vivo*, high-resolution technique to monitor BBB properties such as tight junction complexes and transcytosis in real time.

A comprehensive understanding of the molecular constituents and mechanisms of BBB function and integrity would offer novel strategies for CNS therapeutics. Although the functional BBB is essential for proper neuronal function, the restrictive BBB is an impediment to the delivery of therapeutics, including recombinant proteins, antibodies, and even small molecules, to the brain parenchyma [74]. Thus, a major focus of BBB research is identifying strategies to enhance the delivery of therapeutics across the BBB. Here we highlight three promising methods to manipulate BBB properties to deliver drugs. First, several groups have demonstrated that hijacking receptor-mediated transcytosis pathways could deliver large genetically engineered proteins across barrier endothelium [74]. The transferrin receptor (TfR), which binds to its ligand transferrin-bound iron and undergoes clathrin-mediated transcytosis to facilitate iron delivery to the brain, has been the main target of this work [75,76]. For example, chimeric monoclonal antibodies with α -Tfn fused to α -A β antibodies has been successful in hijacking the TfR pathway to reduce A β in an Alzheimer's disease mouse model [77]. Second, scanning ultrasound (SUS), in which systemically injected circulating microbubbles cause transient opening of tight junctions when activated with ultrasound, has been reported to safely and transiently permeabilize the BBB [78,79]. Third, as we identify mediators of BBB function and better understand the molecular and cellular pathway underlying BBB regulation, we could target and manipulate these genes to enhance therapeutic delivery. For example, functional blocking of Frizzled4 antibodies has been shown to permeabilize the blood–retina barrier, offering a temporal opportunity for enhanced drug delivery [80]. As we further understand the cellular pathways and molecular mechanisms that regulate BBB function and integrity, we can develop creative strategies to manipulate these molecules to enhance drug delivery. Answering fundamental questions in BBB research and identifying the molecular constituents of barrier regulation will enhance the development of therapeutics to modulate the BBB for drug delivery and neurological disorders.

Concluding Remarks

The BBB comprises specialized CNS endothelial cells that regulate CNS homeostasis to ensure proper neuronal function. In this review we have highlighted that improvements in experimental tools have facilitated the recent finding of molecular constituents that mediate BBB function and integrity. These discoveries have greatly expanded our molecular and cellular understanding of this specialized vasculature that has fascinated physiologists for more than a century. Nevertheless, these discoveries open many more fundamental questions waiting to be resolved. We have emphasized the pressing demand for refinements of experimental technologies that will certainly accelerate our discoveries of novel molecules and our understanding of their cellular mechanisms that mediate BBB function. We believe that these findings will directly benefit therapeutics for neurological disorders in both drug delivery and repairing the dysfunctional barrier in certain neurological diseases.

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Outstanding Questions

What are the repertoires of the molecules and genetic programs that mediate BBB formation and function? Cell type-specific purification methods and gene profiling have facilitated recent strides in elucidating the molecular mediators and cellular pathways that confer fundamental BBB properties on CNS endothelial cells. Therefore, improvement in these experimental techniques such as using highly sensitive, unbiased, and high-throughput next-generation sequencing technologies will unravel key molecular regulators and core pathways essential for the BBB.

What are the minimal core components necessary for endothelial cells *in vitro* to recapitulate the BBB properties displayed *in vivo*? Recent studies have demonstrated that activation of Wnt or Shh signaling is sufficient to elevate certain BBB properties in endothelial cells *in vitro*. A robust BBB *in vitro* system would be essential for high-throughput screening of drugs that can modulate the permeability of the BBB.

What are the molecular and cellular mechanisms of how these key molecules mediate BBB properties?

How tightly coupled is the relationship between CNS angiogenesis and barrierogenesis? These two developmental processes may be more independent than once appreciated, especially with emerging studies demonstrating: (i) that CNS angiogenesis continues well after barrierogenesis and throughout postnatal life and (ii) the identity of genes that specifically regulate barrierogenesis independent of angiogenesis, such as *Mfsd2a*.

How can we target the molecular regulators of BBB function to manipulate BBB properties for delivery of therapeutics? A major focus in BBB research has targeted the transferrin receptor to hijack clathrin-mediated transcytosis for the delivery of therapeutics.

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