Minireview

Transcellular routes of blood–brain barrier disruption

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Impact Statement

This is a review of the transcellular pathways by which the blood–brain barrier becomes disrupted.

Abstract

Disruption of the blood–brain barrier (BBB) can occur through different mechanisms and pathways. As these pathways result in increased permeability to different classes of substances, it is likely that the neurological insults that occur will also differ for these pathways. The major categories of BBB disruption are paracellular (between cells) and transcellular (across cells) with a subcategory of transcellular leakage involving vesicles (transcytotic). Older literature, as well as more recent studies, highlights the importance of the transcellular pathways in BBB disruption. Of the various transcytotic mechanisms that are thought to be active at the BBB, some are linked to receptor-mediated transcytosis, whereas others are likely involved in BBB disruption. For most capillary beds, transcytotic mechanisms are less clearly linked to permeability than are membrane spanning canaliculi and fenestrations. Disruption pathways share cellular mechanisms to some degree as exemplified by transcytotic caveolar and transcellular canaliculi formations. The discovery of some of the cellular components involved in transcellular mechanisms of BBB disruption and the ability to measure them are adding greatly to our classic knowledge, which is largely based on ultrastructural studies. Future work will likely address the conditions and diseases under which the various pathways of disruption are active, the different impacts that they have, and the cellular biology that underlies the different pathways to disruption.

Keywords: Blood–brain barrier, disruption, transcellular, transcytosis, paracellular, clathrin, caveolae, adsorptive transcytosis, fenestrations

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Introduction

The vascular blood–brain barrier (BBB) controls the exchange of substances and cells between the central nervous system (CNS) and the blood stream. It exerts this control in part through modifications of the capillary bed that prevent the unregulated leakage of blood-borne substances into the interstitial fluids of the brain. When the BBB fails and leakage occurs, CNS dysfunction accompanied by neuroinflammation and oxidative stress is often the result. An increasing number of diseases are associated with BBB disruption, including common diseases such as Alzheimer's disease, diabetes mellitus, and stroke.

However, the BBB is not disrupted in the same way by all diseases, or necessarily is it disrupted the same way during a disease's course. Instead, the loss of BBB integrity can occur through various mechanisms and pathways. Because these pathways differ in their characteristics, the resulting neurological insult resulting from BBB disruption potentially differs. The first clue to what these mechanisms and pathways are was provided by the early ultrastructural studies of Reese and Karnovsky.[1] They found that in comparison to other vascular beds, that of the BBB is neither fenestrated nor discontinuous/sinusoidal. Fenestrae, Latin for windows, are regions of the endothelial cell that are extremely thin (about 6 nm thick), with no cytoplasm and a fusion of the luminal and abluminal cell membranes (Figure 1). Brain capillaries also have a greatly reduced number of pinocytes and an absence of canalicular structures. In addition, the gaps between endothelial cells were closed by tight junctions. Thus, the loss of fenestrae, canaliculi, and pinocytosis reduces transcellular leakage and the presence of tight junctions reduces paracellular leakage across the BBB (Table 1).

It has been assumed that reversal of one or more of these ultrastructural characteristics is the basis for disruption of the BBB (Table 1). Thus, loss of tight junction function would allow substances to leak between cells (paracellular leakage), the reinstitution of the various routes across an individual cell are transcellular leakage, and those transcellular routes involving vesicles are transcytotic leakage. More recently,

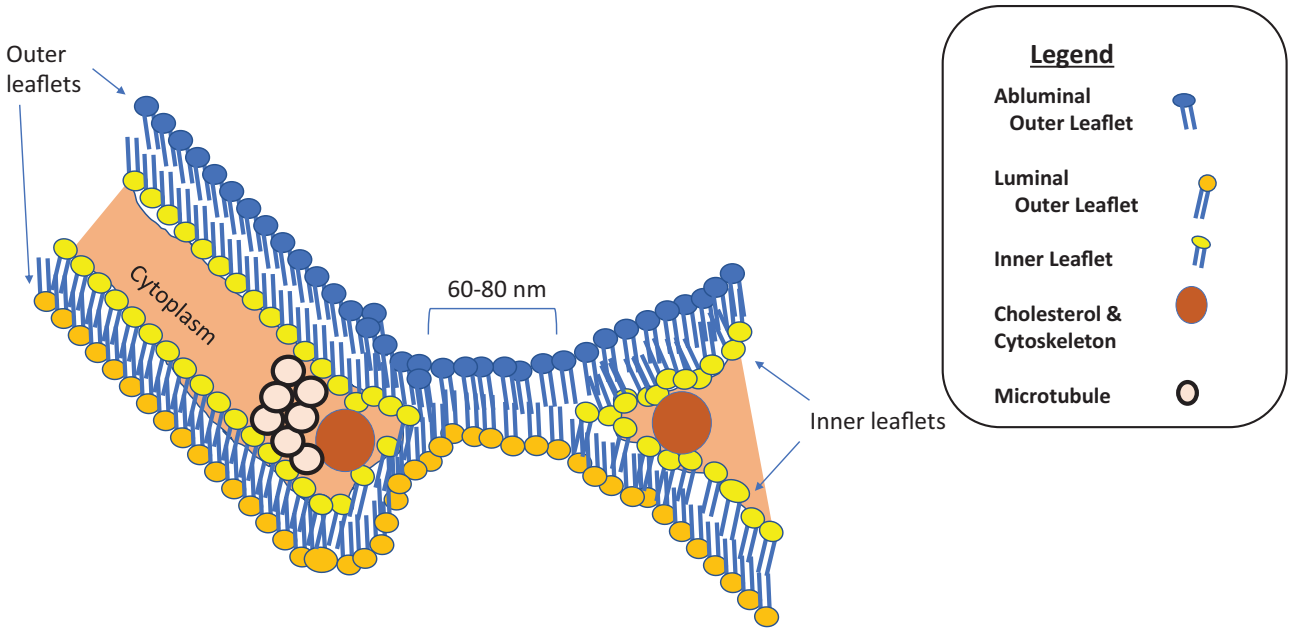

Figure 1. Fenestral construction. Conceptual illustration of the fusion of cell membranes forming the fenestral pore. Diaphragm is not illustrated, but would be a concentric/octagonal opening up to 15 nm in diameter associated with PLVAP. Fenestrae are ringed by cholesterol and cytoskeleton and tend to be clustered into sieve plates that in turn are delimited by microtubules. (A color version of this figure is available in the online journal.)

Table 1. Pathways of blood–brain barrier disruption.

Paracellular
Transcellular
Fenestrations
Diaphragm
No diaphragm
Canaliculi
Diaphragm
No diaphragm
Transcytotic
Epicellular
Basement membrane
Glycocalyx

Table 2. Classification of endocytosis.

Phagocytosis
Pinocytosis
Macropinocytosis
Induced
Constitutive
Micropinocytosis
Clathrin-dependent endocytosis
Clathrin-dependent receptor-mediated endocytosis
Caveolin-mediated endocytosis
Podocytosis (caveolin-mediated receptor-mediated endocytosis)
Adsorptive endocytosis
Other non-clathrin/caveolin-mediated endocytosis

the roles of the basement membrane on the abluminal capillary surface and the glycocalyx on the luminal surface of the capillary have been proposed to be important in BBB impermeability,[2,3] which we have termed in Table 1 as epicellular. Interestingly, Palade and co-workers noted the importance of the basement membrane in vascular permeability in the early 1960s.[4] It should be noted that the very leaky sinusoidal capillary beds have an attenuated or absent basement membrane and that at least one model of a leaky BBB, that of the diabetic BTBR mouse, has an attenuated glycocalyx with no obvious attenuation of tight junctions or increased transcytosis.[5] Each of these possible ways in which the BBB can be disrupted have their own special features with regards to mechanisms of disruption and the characteristics of the substance that is allowed to leak into the brain.

Here, we will concentrate on the transcellular mechanisms of BBB disruption. Much has been written about BBB disruption resulting from loss of tight junctions, whereas there are far fewer reviews on transcellular and transcytotic disruption.[6,7] Yet early studies found that transcellular pathways were dominant over paracellular pathways in many kinds of brain injury.[8–10] More recent work strongly supports that transcellular routes of leakage play a major role in BBB disruption postinjury.[7,11,12] As examples, in a rat stroke model, there is substantial leakage of fluorescein isothiocyanate conjugate (FITC)–albumin tracer into the brain in the first 24-h postinjury, but ultrastructural evidence for paracellular leakage routes through dysfunctional tight junctions was lacking. Instead, the leakage appeared to be mediated through increased vesicles and endothelial degeneration.[13] Shifts of relative permeability over time from smaller substances to larger substances can be explained by an increased role for transcellular pathways.[14,15]

Endocytosis versus transcytosis

Much of what we assume we know about endocytosis/transcytosis and the BBB is derived from three related fields: the general topic of cellular endocytosis, transcytosis in epithelial cells, and transcytosis in other capillary beds. Table 2 shows a standard classification of endocytotic pathways. Phagocytes engulf large particles, including organisms,

and are generally 1–2 microns in diameter. Macropinocytes are usually stated to be larger than 250 nm in diameter and with the role of nutrient internalization. Recent work has divided macropinocytes into induced (classic) and constitutive, the latter with a role in antigen presentation. Clathrin-dependent endocytosis is the most common form of receptor-mediated transcytosis and engages in classic endocytic–exocytic cycling. The interior diameter of clathrin vesicles is estimated at 30–40 nm.[16] Clathrin vesicles often produce buds that return the receptor to the cell membrane with the remainder of the ligand-containing vesicle routing to phagosomes.[17] Caveolin-dependent endocytes are flask-shaped invaginations in the cell membrane with diameters of 60–80 nm,[18] arise in lipid rafts, and may also engage in receptor-mediated transcytosis. Adsorptive endocytosis or adsorptive-mediated endocytosis is characterized by interactions between highly charged ligands with regions of the cell surface.[19] A classic example is the lectin wheat germ agglutinin binding to cell membrane glycoproteins, inducing trafficking of cell membrane to lysosomes.[20,21] It is generally assumed that there are other endocytic systems yet to be described.

Tight junctions polarize the brain endothelial cell, dividing its cell membrane into luminal and abluminal regions. This gives the endocytic vesicle a choice of destinations, with transcytosis occurring when an endocyte arising from one membrane surface (e.g. the luminal membrane) exocytoses at the other membrane surface (e.g. abluminal). However, the destination of an endosome is not likely to be randomly chosen. Vesicles endocytosed on the apical surface tend to exocytose there as well and those arising from the basolateral surface to exocytose there.[17] This shows that transcytosis is not simply a random event that occurs after endocytosis. In addition, endocytosis and exocytosis in a non-polarized cell are directed at serving the needs of that cell, whereas transcytosis is directed at serving the needs of the adjacent cells. Therefore, transcytosis is likely to be a cell's response to signals from other cells and not in response to its own needs.

Of the various pinocytes, those related to fluid-phase transcytosis are assumed to be more related to BBB disruption than those related to the transport of specific ligands, such as receptor-mediated transcytosis. However, evidence suggests that the vesicular systems share to some degree cellular machinery and it is possible that invoking one pathway affects the activation of others. As discussed below, caveolae and canaliculi have cellular machinery that is particularly linked. At the least, all transcellular and paracellular disruption pathways involve rearrangement of the cytoskeleton.

Vesicles are appealing candidates for explaining transcellular leakage at the BBB. Their diameter of 30–100 nm would allow virtually any molecule and many viruses (but not bacteria or cells) free passage into brain. The possibility that they coalesce to form channels or tubes that span the approximate 150 nm width of a brain capillary is also appealing.[22,23] However, studies with various sized molecules in non-BBB capillary beds show that most leakage can be explained by a pore size of 4 nm[24,25] with a second class of pores at an estimated size of 11–35 nm,[25,26] although this estimate increases to about 60 nm in the case of a few tissues.[27] Sinusoidal capillary beds, such as that of liver, are characterized by open fenestrae, lack of basement membranes and glycocalyx, and high levels of phagocytosis and have estimated diameters of 180–280 nm.[27] A study by Stewart found a poor correlation between the vesicular content of the endothelial beds for various tissues and their degree of leakage. For example, vesicles in brain capillary endothelial cells occur at a frequency of about 14/μm^3, whereas in the much leakier capillary bed of the testis, the frequency is about 9/μm^3.[28] Stewart concluded that most substances crossing capillary beds did so by way of selective processes rather than leakage. This suggests that only a minority of the vesicles seen in a non-disrupted barrier tissue's capillary bed are involved in leakage.

For the BBB, these considerations highlight the need for further studies on the role of transcytosis in the various diseases that involve disruption. The work of Broadwell *et al*.[21,29] with wheat germ agglutinin showed that adsorptive endocytosis induced vesicles that tended to be routed to lysosomes, but also involved routing to other membrane structures, including the abluminal membrane (i.e. transcytosis). Viruses such as HIV and SARS-CoV-2 likely cross the BBB by inducing adsorptive transcytosis, which is consistent with their routing to and need to survive the lysosomal compartment.[30] It is also likely that many of the Trojan horse delivery systems, particularly those that bind to off-target sites or depend on a greatly altered endogenous ligand, induce adsorptive transcytosis rather than receptor-mediated transcytosis. This would explain, in part, the surprisingly disappointing results of the various Trojan horse approaches.

Non-vesicular transcellular leakage

The main two categories of structures involved in non-vesicular transcellular leakage are canalicular structures and fenestrae (Figure 2). Canaliculi or similar structures have also been termed vesiculo-canaliculi, vesiculo-tubules, transendothelial channels, conduits, and vesiculo-vesicular organelles.[31,32] As defined by Lossinsky and Shivers,[31] canaliculi only occur in brain endothelial cells in the presence of injury and span the membrane forming transcellular channels, although others have noted they often dead-end in lysosomes. Lossinsky and Shivers note similarities and differences of this system with other transcellular channel-like networks, such as that involved in the diapedesis of immune cell trafficking. However, since diapedesis is a highly regulated and selective process, it is not considered in our review except to note that when extremely active, enough fluid-phase passage can accompany the immune cell trafficking as to give the appearance of BBB disruption.[33,34]

Early studies noted that canaliculi formation was affected by cholesterol altering drugs such as nystatin and cyclodextrins, and so classic canaliculi were assumed to be related to caveolae. Thus, caveolar mechanisms have been related to both a specific transcytotic pathway[35] and a more non-specific transcellular leakage.[36,37] The major proteins that facilitate formation of caveolae are caveolins and cavins. Caveolins are integral membrane proteins synthesized from CAV1, 2, and 3 genes.[38] In endothelial cells, including those of the BBB, caveolae are comprised of Cav-1 and 2, whereas Cav-3 expression is not detected.[39] Cav-3 expression is more

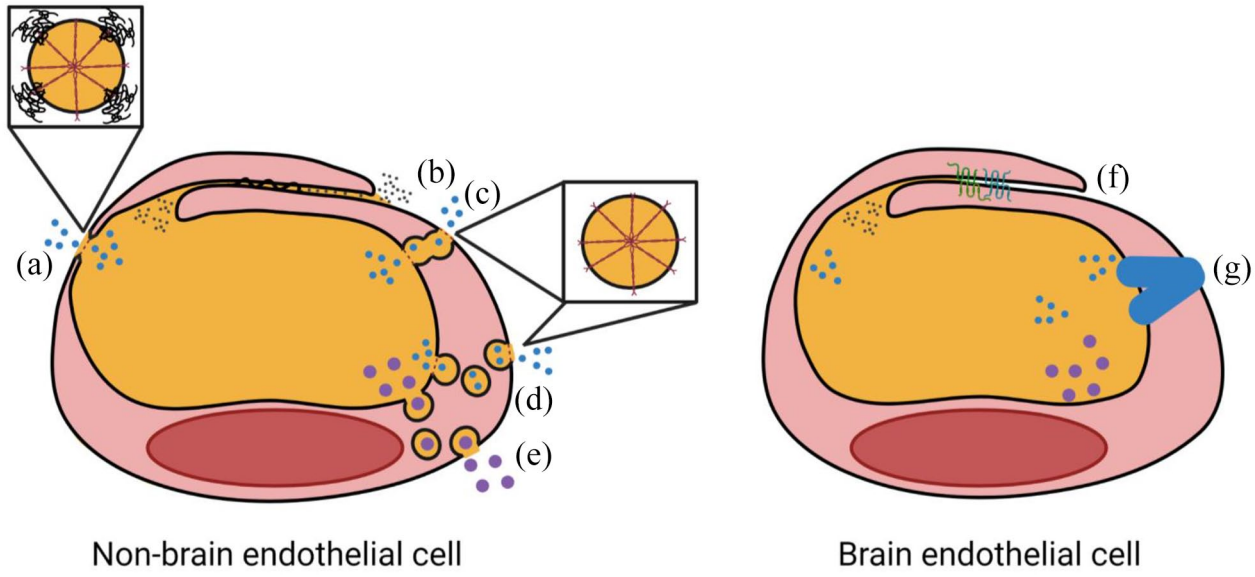

Figure 2. Transcellular processes: caveolae and fenestrae. In fenestrated non-brain endothelial cells (a), fenestrae permit leakage of medium-sized molecules. Fenestrae typically express fenestral diaphragms comprised of PLVAP (purple strands) and tufts of heparan sulfate proteoglycan (black), shown in inset. Paracellular junctions of most non-brain endothelial cells are leaky, and permit leakage of small solutes between cells (b). Caveolae contribute to leakage either through formation of transendothelial channels (c), or transcellular vesicular transport (d and e). Caveolar vesicles and transendothelial cell channels can express stomatal diaphragms comprised of PLVAP (purple strands) but that lack heparan tufts (inset, c and d). Caveolae that lack stomatal diaphragms (e) may permit leakage of larger molecules into tissues. In brain endothelial cells, tight junction proteins limit paracellular leakage of substances (f). Fenestrae and caveolar formation is suppressed, in part, by expression of Mfsd2a (blue, g), which transports DHA to the inner leaflet of the endothelial cell plasma membrane which inhibits association of caveolin-1 with the membrane. (A color version of this figure is available in the online journal.)

limited and is typically observed in smooth and striated muscle.[40] In the brain, Cav-3 has been detected in astrocytes.[39] Caveolin proteins bind cholesterol and are essential for the formation of caveolae; genetic ablation of Cav-1 causes loss of caveolae,[41,42] although the mice remain viable. Cavins are cytoplasmic proteins that are important for the formation of caveolae, and include cavins 1–4.[43] Cavins are thought to facilitate caveolae formation by stabilizing caveolin oligomers, contributing to the membrane curvature, and inhibiting caveolin degradation.[43] More recent works have elucidated the protein major facilitator super family domain containing 2a (Mfsd2a) as a BBB-specific suppressor of caveolar vesicles.[44,45] Mfsd2a is selectively upregulated during a critical window of BBB formation during embryonic development, and its expression is dependent on pericyte associations with the BBB. Importantly, Mfsd2a controls BBB leakiness independently of TJPs.[44]

Leakier vascular beds have fenestrations, and endothelial cell gaps which form larger pores of 60–100 nm or more,[46] and are not comprised of caveolae.[47] An area of fused cellular membrane forms the fenestrae and is about 60–80 nm in diameter (Figure 1). In some capillary beds, fenestrae have diaphragms, which consist of radial protein fibrils with tufts of heparan sulfate bound on the luminal side of the endothelial cell.[48–50] The fenestral diaphragm affects the size of molecules that can pass through the pore, with larger molecules such as ferritin (11 nm) having very limited passage across fenestrated vascular beds versus horseradish peroxidase (4.5 nm) which can cross freely.[48] It has been estimated that the diaphragms reduce the functional permeability (pore size) of a fenestrae from a diameter of 15 nm to one of 6–11 nm.[27]

The plasmalemma vesicle-associated protein (PLVAP) is the main constituent comprising fenestral diaphragms, and also forms structurally and biochemically distinct stomatal diaphragms on caveolae.[32,51] PLVAP is expressed by nascent blood vessels in the developing brain and retina, but its expression is suppressed by Wnt signaling that occurs with BBB maturation.[52,53] Fenestrated capillaries are also present in circumventricular organs of the brain, which lack a functional BBB.[54] Genetic ablation of PLVAP in mice results in an absence of diaphragms on fenestrae and caveolae, but fenestrae and caveolae remain present and otherwise indistinct from those of wild-type mice.[55] Mice lacking PLVAP show rapid decreases in plasma proteins <200 kDa and plasma protein accumulation into the interstitial space of fenestrated vessels, followed by hypertriglyceridemia, non-inflammatory edema of organs with fenestrated vascular beds (intestine, kidney, and pancreas), and eventually died in the prenatal or early postnatal periods.[55] PLVAP is also a positive regulator of leukocyte trafficking,[51] highlighting the previously noted interconnection among the various paracellular and transcellular pathways. Other than PLVAP, little is known about the identity and functions of other structural/regulatory components of endothelial cell fenestrae.

BBB disruption by transcellular routes: diseases and molecular machinery

The importance of understanding how the BBB is disrupted resides in part because the characteristics of the various pathways of disruption vary and this variation likely affects the resulting neurological sequelae. For example, disruption to larger molecules will require pathways other than

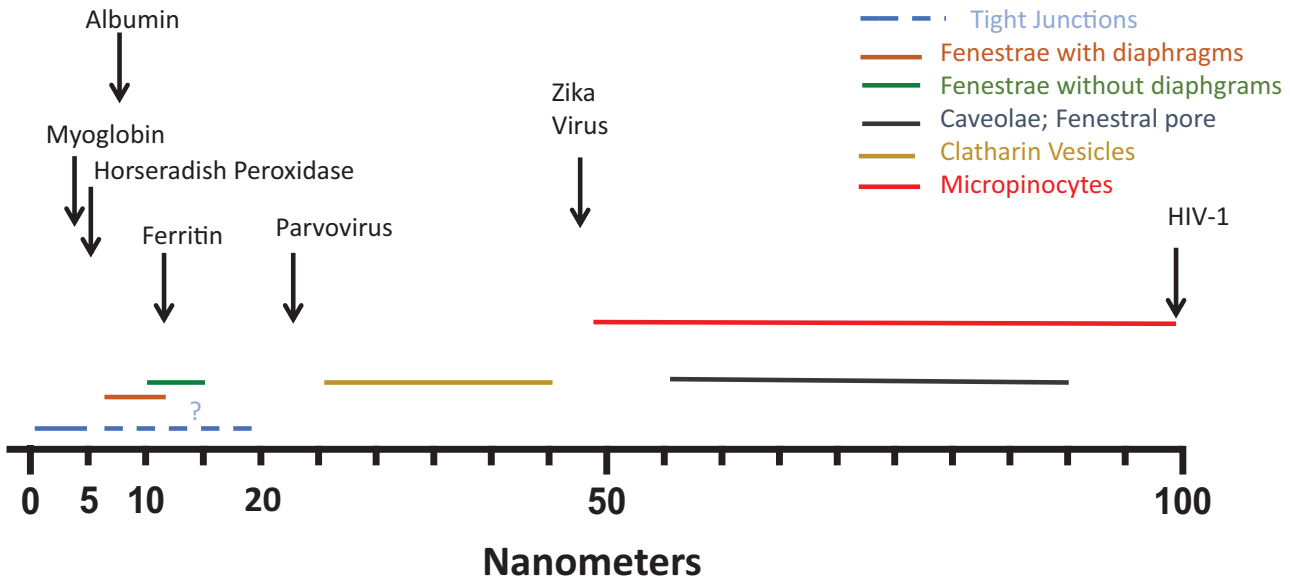

Figure 3. Size ranges of various pathways of BBB disruption. Open tight junctions are likely less than 5 nm diameter, but may be up to 20 nm, especially in sinusoidal capillary beds. The functional leakage size (fenestrae with diaphragms and fenestrae without diaphragms) and physical diameter of the entire fenestrae (fenestral pore) are both indicated. Arrows indicate the approximate diameter of several molecules and viruses, illustrating that leakage pathways vary in the size of substance they would permit to cross the BBB. (A color version of this figure is available in the online journal.)

paracellular leakage and entry of moderate-large viruses will require vesicular pathways (Figure 3). The presence or absence of heparan sulfate at the diaphragms of fenestrae may affect the permeability of substances on the basis of charge or glycosylation; this is clearly the case for adsorptive transcytosis. Vesicular mechanisms, required for the entry of the largest substances, cannot be detected by transendothelial electrical resistance, which depends on transfer of not only the smallest entity, electrons, but also on a patent channel.

Classically, much of the knowledge about the transcellular pathways of BBB disruption comes from ultrastructural studies, but increasingly proteins key to the transcellular processes are being studied. For example, biochemical data support that caveolae are involved in BBB leakage postinjury. Caveolins are the major structural components of caveolar vesicles and transendothelial channels.[56] Caveolin-1 upregulation has been reported to occur prior to changes in tight junction proteins in stroke and cortical cold injury models, with tight junction remodeling occurring in later phases.[57,58] Cav-1 knockout mice were also shown to have less BBB leakage in the early phases of postinjury;[58] however, Cav-1 knockout mice also have larger infarct volumes which may be due to impaired angiogenesis and apoptosis.[58,59] Cav-1 knockout does not appear to influence the expression or architecture of tight junctions,[58] although Cav-1 does interact with claudins and occludin,[60] and has been shown to regulate tight junction protein redistribution in cells following inflammatory and ischemic insults.[61–63] Caveolin-1 was also shown to regulate focused-ultrasound-mediated BBB disruption to large (500 kDa), but not mid-size (70 kDa) tracers,[64] further supporting its selective contribution to transcytotic routes of BBB disruption.

Caveolae expression, which is suppressed by Mfsd2a by incorporating DHA into the brain endothelial cell membrane,[45] has also been implicated in BBB disruption associated with brain injury. Mfsd2a is downregulated in intracranial hemorrhage, sepsis, and brain tumor models concurrently with increased BBB leakage, and overexpression of Mfsd2a rescues BBB leakage in these models,[65] supporting its involvement in upregulating transcellular leakage pathways in pathological conditions. Recent work has implicated phosphatase and tensin homolog (PTEN)/AKT signaling through the E3 ubiquitin ligase NEDD4-2 as an important regulatory pathway for Mfsd2a expression and caveolae suppression.[66] PTEN is a phosphatase that inhibits AKT activity, thus reducing the phosphorylation and ubiquitin ligase activity of NEDD4-2 and increasing Mfsd2a stability. Brain endothelial cell-specific deletion of PTEN results in decreased Mfsd2a and increased formation of caveolar vesicles.[66] However, another group has shown that brain injury causes the upregulation of PTEN, and that pharmacological PTEN inhibition reduces BBB disruption and improves functional outcomes postinjury.[67] It is possible that these apparently conflicting results could be due to differences in cell-type specific functions, or harmful functions of PTEN in an injury context.

The ultrastructural demonstration of fenestrae postinjury, to our knowledge, has not been described for brain endothelial cells. However, chemical induction of brain endothelial cell fenestrations has been shown before in rats, which was done by a 28-day continuous infusion of phorbol 12-myristate 13-acetate (PMA) into the cerebral cortex.[68] Although ultrastructural insight on fenestrae is lacking, PLVAP has been implicated in BBB leakage following pathological insults.[53] In mature vascular beds of the brain or retina, PLVAP expression is absent, but can be induced with diabetic retinopathy, brain ischemia, and in brain tumors.[53] In the retina, whose endothelial cells form a blood–retinal barrier (BRB), vascular endothelial growth factor (VEGF)-induced BRB leakage was inhibited by knocking down PLVAP expression.[69] The same study also found that PLVAP knockdown inhibited

VEGF-induced caveolae formation, which is interesting because PLVAP knockout mice do not have overt changes in caveolae of peripheral vascular beds, except for missing diaphragms.[55] Furthermore, the protective effect of PLVAP knockdown supports that fenestral structures are not involved in VEGF-induced BRB disruption, as it would be expected that PLVAP knockdown would exacerbate leakage if that were the case.[55] Therefore, PLVAP may have a broader regulatory role at brain barriers in context of injury. The mechanisms by which PLVAP contributes to leakage of the BBB and BRB remain unclear, but would be important to understand given its role in development and disease.

Although older literature has suggested an absence of BBB disruption with healthy aging (reviewed in Banks *et al*.[70]), newer studies suggest that BBB disruption occurs in aging humans with cognitive decline and predicts progression to Alzheimer's disease,[71–73] although the leakage is modest and highly variable with age.[70] Yang *et al*. have recently showed using proteomic methods that plasma protein uptake into the brain decreases with age, which is associated with a loss of pericyte coverage and a shift from clathrin-dependent receptor-mediated transcytosis to caveolae-mediated transcytosis. In parallel, Mfsd2a was downregulated and there was decreased incorporation of DHA in the cell membrane and increased caveolar vesicles.[11] One additional putative regulator of transcellular leakage was identified in the same study, which is ALPL, an alkaline phosphatase gene that is upregulated with aging. Pharmacological inhibition of ALPL had an apparently restorative effect on receptor-mediated transcytosis; however, it remains unclear how ALPL expression regulates transcytotic pathways of BBB leakage.

Toward treating the disrupted BBB

With BBB disruption being increasingly recognized as a component of so many diseases, the question arises as to whether BBB disruption can be treated. It seems likely that the BBB has a high degree of repair after injuries such as stroke, trauma to the CNS, or multiple sclerosis, although there is evidence in the latter two conditions that waves of repair and dysfunction occur.[74,75] Whereas most of our present knowledge focuses on mechanisms that cause BBB disruption, an improved understanding of endogenous pathways of BBB protection and repair may offer insight on mitigating the extent and/or duration of BBB disruption in diseases. While a comprehensive discussion of the existing literature is beyond the scope of this review, we highlight annexin A1 as one example of an endogenous BBB-protective molecule that facilitates BBB repair.[76] Recent three-dimensional (3D) *in vitro* modeling of the BBB derived from human iPSCs has been used to study BBB disruption and repair mechansims at high temporal and spatial resolution, and has identified bFGF as another example of BBB-preserving factor.[77] In these cases, the primary mode of BBB protection/restoration was against paracellular leakage mechanisms. Much less information is available on the mechanisms that restore suppression of transcellular leakage pathways after they are induced at the BBB.

As for therapeutic interventions, they will likely vary as to the nature of injury and, therefore, the type of disruption. Preclinical data show that BBB disruption caused by inflammation induced by lipopolysaccharide or by tumor necrosis factor (TNF) can be blocked by indomethacin, indicating that BBB disruption is mediated in this case by prostaglandins.[78] Traumatic brain injury induced by blast injury can be blocked by nitric oxide synthase inhibitors,[15] but not by indomethacin.[15] Deletion of eNOS protects the BBB from disruption in thiamine deficient mice.[79] Hyperglycemia-associated BBB disruption has been blocked by glucagon-like peptide-1 (GLP-1) agonists, fibroblast growth factor 21 (FGF21) treatment, epoxide hydrolase inhibition, pitavastatin, candesartan, and metabolic carbonic anhydrase inhibitors.[80–84] Mfsd2a attenuates BBB disruption accompanying intracerebral hemorrhage.[85] A type IV phosphodiesterase inhibitor or antibodies to interleukin-1 beta protects the BBB after focal cerebral ischemia.[86,87] BBB disruption accompanying obesity and diabetes in the BTBR mouse strain, which has deficient of leptin, can be normalized with leptin treatment.[5] BBB disruption can be induced by bradykinins, histamine, and TNF, and so antagonists of these agents should be effective when these agents are the underlying cause of BBB disruption.[88–91] These examples are not exhaustive, but illustrate that treatments are diverse, likely being dependent on the cause of injury and the characteristics of the BBB disruption.

Conclusions

In conclusion, there is an abundance of literature that emphasizes the importance of transcellular pathways of BBB disruption in different disease models. Yet, relatively little is known about the mechanisms and molecular components that regulate transcellular opening of the BBB. Advances in molecular tools that allow for cell biological and proteomic studies of the BBB have and will continue to improve our understanding of these intriguing transcellular routes of BBB leakage that emerge following injury and disease.

AUTHORS' CONTRIBUTIONS

Both authors contributed equally to review of the literature, writing, editing, and figure production.

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