Structural Alterations to the Endothelial Tight Junction Complex During Stroke

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1 Introduction

Cerebral endothelial cells and their linking tight junctions (TJs) form the blood–brain barrier (BBB) [1]. That blood/brain interface regulates the movement of compounds and cells into and out of brain. The BBB controls nutrient supply, aids in the removal of potential neurotoxic compounds from brain, and is an essential component regulating the composition of brain extracellular environment which is vital for normal neuronal function [1].

Stroke, including ischemic and hemorrhagic forms, causes BBB dysfunction [2–4]. Thus, stroke causes increased BBB permeability to blood-borne molecules, cerebral edema formation, and leukocyte infiltration. Such changes may enhance stroke-induced brain injury and worsen stroke outcome. This chapter describes the effects of stroke on the cerebral endothelium and the impact of those changes on brain injury. It examines the underlying mechanisms and potential therapeutic

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approaches to reduce stroke-induced BBB dysfunction. It particularly focuses on alterations in endothelial TJs, but it also addresses the potential role of enhanced endothelial transcytosis after stroke.

2 Normal BBB Structure and Function.

In contrast to systemic capillaries, brain capillary endothelial cells are linked by TJs and have a low basal rate of transcytosis (Fig. 1; [1, 5]). These characteristics limit the para- and transcellular pathways across the endothelium resulting in a very low permeability to many compounds. Thus, for example, the transendothelial electrical resistance, a measure of ionic impermeability, is orders of magnitude greater in cerebral compared to systemic capillaries [6, 7]. Exceptions to such low permeability are compounds which can diffuse across the endothelial cell membrane (e.g., O_2, CO_2, H_2O and molecules with high lipophilicity) or those with specific BBB influx transporters, such as D-glucose [1]. There are also an array of efflux transporters, such as *p*-glycoprotein, that enhance brain to blood transport [1]. Such transporters are involved in preventing potential neurotoxins from entering the brain and clearing metabolites from brain. They are a significant obstacle to the delivery of therapeutic agents for neurological disorders [1]. The BBB also has an array of enzymes that can degrade neuroactive compounds preventing their entry from blood into brain (i.e., it is also a metabolic barrier [1, 5]).

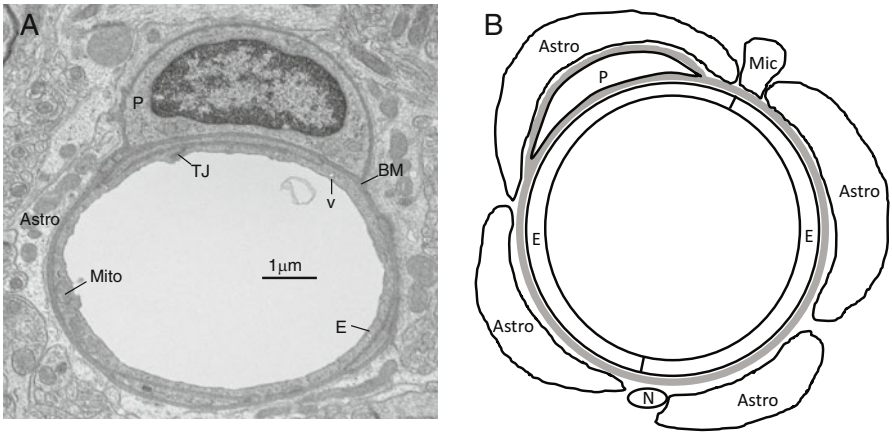

Fig. 1 (**a**) An electron micrograph showing the structure of a normal mouse cerebral capillary. (**b**) A schematic showing the relationship between the endothelium and other elements of the neurovascular unit. Brain capillary endothelial cells (E) are linked by tight junctions (TJ), show few vesicles (v) and have more mitochondria (mito) than systemic capillaries. Sharing the same basement membrane (bm) as the endothelium are pericytes (P). Capillaries are surrounded by astrocyte endfeet (astro), with occasional neuronal (N) and microglial processes (Mic). The endothelial cells and the ensheathing cells/basement membrane are called the neurovascular unit

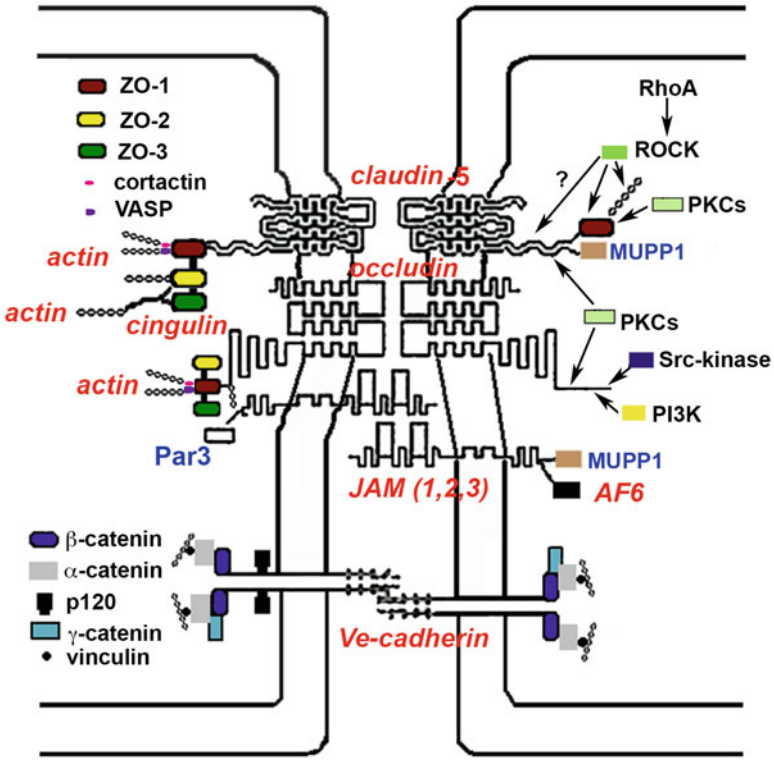

Fig. 2 Schematic representation of the basic components of the brain endothelial junctional complex. The *left* of the panel shows potential structural components of the TJ complex. Claudin-5, occludin, and the JAMs are transmembrane proteins that link adjacent cells. Claudin-5 is the major occlusive protein at the BBB while the cytoplasmic plaque protein, ZO-1, is important for clustering of claudin-5 and occludin and establishing connection with actin filaments. The roles of ZO-2, ZO-3, MUPP1, Par3, Af6 are less clear in brain endothelial cells. Brain endothelial cells also possess adherens junctions, containing the transmembrane protein, Ve-cadherin, and cytosolic accessory proteins (e.g., catenins). The *right* of the panel shows signaling molecules involved in modulating TJ complex assembly and disassembly

Endothelial TJs are integral for BBB function. They are comprised of transmembrane proteins, which form the links between adjacent endothelial cells, and cytoplasmic plaque proteins that form a physical scaffold for the TJs and regulate TJ function [1, 8–10]. In addition, there are links between TJs and the actin cytoskeleton and the adherens junctions that are important for TJ stability and formation (Fig. 2) [9, 10]. TJs are dynamic structures with, for example, claudin-5 and occludin having half-lives of 70–90 min and 6 h, respectively [8].

Transmembrane proteins: These include the claudins, occludin, and the junctional associated molecules (JAMs). In addition, tricellulin, a molecule with homology to occludin, is present at points of three cell contact. The transmembrane

proteins can form *trans*-interactions with proteins on other cells or *cis*-interactions within the same plasma membrane [8].

The claudins are a 27 gene family which are involved in closing the paracellular space between cells (e.g., claudin-5) and in forming ion pores (e.g., claudin-2) [8]. At the BBB, claudin-5 is by far the most predominant claudin although there is evidence of some other claudins (-3, -12, and possibly -1) [8]. Claudin-5 structure and function is regulated by phosphorylation (see below; [8]). It can undergo proteasomal degradation after ubiquitination as well as lysosomal degradation [11]. The claudin-5 knockout results in increased BBB permeability to small molecular weight compounds [12].

Although occludin is not directly involved limiting paracellular permeability, it is thought to be a central regulatory component of the TJ being involved in TJ formation and maintenance [8, 13]. It is a major link to cytoplasmic plaque proteins via binding to ZO-1. Like claudin-5, occludin structure and function is regulated by phosphorylation and it also undergoes ubiquitination and proteasomal degradation [8, 13].

Junctional adhesion molecules (JAM-A, -B, -C) are members of the immunoglobulin superfamily. Their role at the BBB has received much less attention than claudin-5 and occludin, but evidence indicates they are involved in regulating not only paracellular permeability but also leukocyte/endothelial interactions [14–16].

Cytoplasmic plaque proteins: As well as the transmembrane proteins, there are an array of cytosolic proteins associated with the TJs that form the cytoplasmic plaque (Fig. 2). These proteins can be divided on the basis of whether they contain PDZ binding domains. Those which do include members of the membrane-associated guanylate-kinase (MAGUK) superfamily, ZO-1, -2 and -3, as well as Par3, Par6, and AF6 [9]. As many of these proteins contain multiple PDZ domains (e.g., the ZO family members contain three [17]), they can act as scaffolding proteins linking different elements of the TJ (e.g., transmembrane proteins, cytoplasmic plaque proteins, and the actin cytoskeleton).

Cytoplasmic plaque proteins that do not contain PDZ domains include cingulin, 7H6, Rab13, ZONAB, AP-1, PKCζ, and PKCλ, as well as several G proteins (Gαi, Gαs, Gα12, Gαo). These proteins have multiple functions. Thus, for example, it is thought that cingulin acts as a cross link between TJ transmembrane proteins and the actin-myosin cytoskeleton and that the PKC isoforms and the G proteins regulate TJ assembly and maintenance [9].

Adherens junctions: Cerebral endothelial cells possess adherens junctions (AdJs) as well TJs. AdJs are also a complex of transmembrane (Ve-cadherin) and cytosolic accessory proteins (α, β catenin, p120) closely associated with actin filaments [9, 10]. Crosstalk between AdJs and TJs has been proposed to regulate TJ function [9, 10]. For example, Ve-cadherin upregulates claudin-5 expression in brain endothelial cells [18].

Cell cytoskeleton: The cytoskeleton comprises of actin microfilaments, intermediate filaments, and microtubules. The actin cytoskeleton is linked to the TJs via cytoplasmic plaque proteins such as ZO-1 and to AdJs via catenins (Fig. 2 [9]). Increasing evidence indicates that the actin cytoskeleton is a major regulator of TJ

function [19]. Changes in that cytoskeleton may impact TJ function by altering the physical support (scaffold) for the junction and by transmitting physical forces to the junction proteins.

Neurovascular unit (NVU): Although the cerebral endothelial cells and their linking TJs are the ultimate determinants of BBB permeability, perivascular cells (pericytes, astrocytes, neurons, microglia) have a large role in regulating that permeability. In particular, pericytes, which share the same basement membrane as the endothelium, and astrocytes, whose endfeet almost completely surrounds cerebral capillaries (Fig. 1), both regulate barrier permeability [20–22]. These cells, together with smooth muscle cells in larger vessels, act in concert to regulate cerebrovascular function (blood flow and barrier function), forming the NVU.

3 Alterations in BBB Function After Stroke

The hallmarks of ischemic and hemorrhagic stroke include an increased influx of compounds, such as plasma proteins (Fig. 3), into brain from blood, brain swelling due to a net movement of fluid from blood to brain (brain edema) and brain leukocyte infiltration [4, 23–25]. BBB dysfunction participates in all three of these processes.

Ischemic and hemorrhagic stroke cause increased BBB permeability to both small and large molecules [4, 23–25]. The degree and time course BBB disruption varies depending on the severity of the injury, the location of the tissue being sampled relative to the injury and whether or not there is reperfusion. The BBB disruption can be biphasic [24]. In animal models, BBB barrier disruption reaches a peak

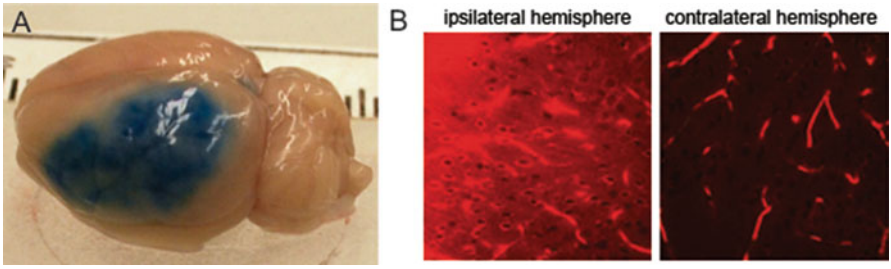

Fig. 3 (**a**) An example of BBB disruption after rat focal cerebral ischemia at the macroscopic level. A hyperglycemic rat underwent 2 h of middle cerebral artery (MCA) occlusion followed by 2 h of reperfusion. The rat was injected intravenously with Evans blue, which binds to albumin in the bloodstream and is excluded from normal brain by the BBB. In the ischemic MCA territory there was marked extravasation of the Evans blue. (**b**) An example of BBB disruption after mouse focal cerebral ischemia at the microscopic level. A mouse underwent 30 min of MCA occlusion followed by 24 h of reperfusion. Enhanced dextran-Texas red (40 kDa) leakage through the disrupted BBB was detected by confocal laser scanning micrographs of the brain ipsi- and contralateral to the occlusion. Note that the dextran was confined to the blood vessels in the contralateral tissue but had spread into the parenchyma in the ischemic tissue

~3–7 days after stroke and then gradually resolves [26]. However, there is evidence that there can be a chronic low level of BBB dysfunction after stroke [27–29]. BBB disruption may result in vasogenic edema and contribute to neuroinflammation. Thus, for example, an influx of prothrombin across the damaged BBB results in the production of thrombin in the brain which is pro-inflammatory and can cause brain edema [30]. Similarly, the entry of fibrinogen from blood to brain is a pro-inflammatory signal [31]. One potential beneficial effect of BBB dysfunction is that it may allow greater entry of therapeutics.

Brain edema is a major consequence of stroke which can result in increased intracranial pressure (ICP) and brain herniation. It is a major cause of morbidity and mortality in ischemic and hemorrhagic stroke [32, 33]. Brain edema is classically classified as cytotoxic or vasogenic dependent on whether the underlying cause is injury to parenchymal cells or the cerebrovasculature [33, 34]. It should be noted, however, that in stroke there is injury to both (mixed edema) and that in both cytotoxic and vasogenic there is a net influx of water from blood to brain across the BBB.

Stroke results in leukocyte (neutrophils, macrophages, and lymphocytes) infiltration into brain [35, 36]. That involves a stepwise process with leukocyte:endothelium interactions causing rolling, adhesion and then diapedesis of the leukocyte across the endothelium [35]. The expression of adhesion molecules on the endothelial luminal membrane plays a prominent role in that process. Leukocytes have been implicated in having a detrimental role in stroke, but they are also involved in tissue repair [35, 36].

The effects of stroke on other barrier functions, such as transport and enzyme activity, have received much less attention, although such changes might have important consequences (e.g., for edema formation and drug delivery) [37, 38]. In addition, although the cerebral and systemic vasculatures possess different properties, they share hemostatic properties that are very important in the occurrence and response to ischemia and hemorrhage [39].

4 Alterations to TJ Structure After Stroke

Alterations in TJ function may result from many different processes, including TJ protein modification (e.g., phosphorylation), altered TJ protein/protein interaction, TJ protein relocation, TJ protein degradation and alterations in transcription/translation (Figs. 4 and 5). There is evidence for each of these processes occurring in stroke and they are intimately linked (e.g., phosphorylation may lead to relocation and degradation). The relative importance of each of the processes likely depends on stroke severity and time point.

Protein modifications: Alterations in the phosphorylation state of TJs proteins are crucial factors influencing BBB permeability. Nevertheless, there is controversy due to the actions of different kinases on distinct residues on the same TJ proteins [40–43]. Most evidence on the effects of phosphorylation/dephosphorylation is on three TJ proteins, the transmembrane proteins occludin and claudin-5, and the scaffolding protein ZO-1.

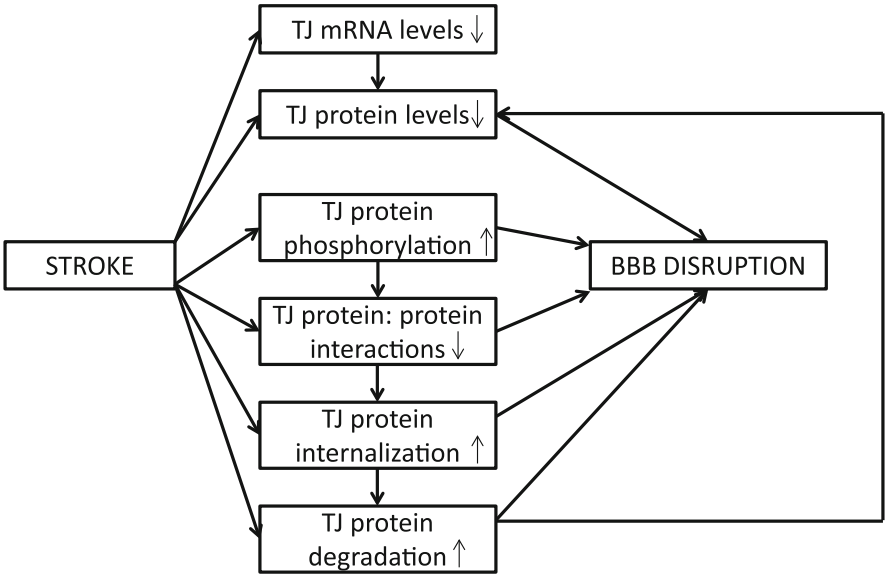

Fig. 4 Schematic showing changes occurring in TJ proteins after stroke that participate in BBB hyperpermeability. Although a potential link between stroke-induced TJ protein phosphorylation, loss of TJ protein interactions, TJ protein internalization and degradation is shown, stroke may affect those processes by several mechanisms, e.g., it may also affect TJ protein degradation by MMP activation

Occludin has several Ser, Thr, and Tyr phosphorylation sites on the C-terminus [40, 44, 45]. Elevated Ser/Thr phosphorylation of occludin is associated with BBB dysfunction during inflammation in the encephalitic human brain [46]. Particularly important findings on the role of Ser/Thr phosphorylation status and barrier function have come from the analysis of vascular endothelial growth factor (VEGF)-induced barrier disruption in retinal endothelial cells, a mechanism underlying diabetic retinopathy [47]. VEGF was shown to induce occludin phosphorylation at sites Thr-168, Thr-404, Sr-408, Ser-471, and Ser-490 via PKCβ, with Ser-490 impacting occludin/ZO-1 interaction and leading to occludin ubiquitination and degradation [47, 48]. In stroke, direct evidence regarding occludin phosphorylation on these Ser/Thr residues is still lacking. There is though evidence that protein kinase C (PKC) isozymes (nPKC-θ and aPKC-ζ) are activated during hypoxia/reoxygenation injury and that cPKCα and Rho-kinase activation is induced by the chemokine CCL2, a major driver of leukocyte entry during stroke-induced brain injury [49, 50].

In epithelial cells, occludin Tyr residues are normally minimally phosphorylated and their phosphorylation is associated with barrier disruption [51]. Tyr-398 and Tyr-402 on occludin are involved in the interaction with ZO-1 and their phosphorylation destabilizes barrier function [44]. At the cerebral endothelium, focal cerebral ischemia, and glutamate treatment in vitro induces occludin Tyr phosphorylation. A Src-kinase inhibitor, PP2, reduces the occludin Tyr phosphorylation and BBB dysfunction found during ischemia/reperfusion (I/R) injury [52–54].

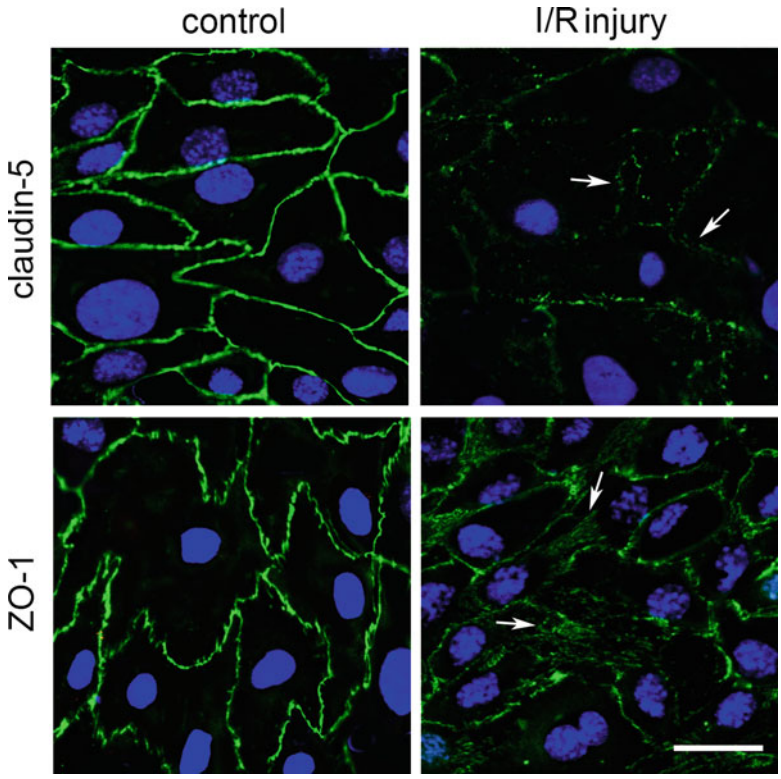

Fig. 5 Mouse brain endothelial cells were exposed to oxygen–glucose deprivation (OGD/ischemia) for 5 h followed by normal oxygen and glucose condition (reperfusion) for 1 h as a model of ischemia/reperfusion (I/R) injury. Control represents cells exposed to normal oxygen and glucose. Immunofluorescent staining was used to visualize the localization of claudin-5 and ZO-1 under the control and I/R conditions. Notice the alteration in claudin-5 and ZO-1 localization/expression on the lateral border of the brain endothelial cells after I/R injury (*arrows*). Scale bar = 20 μm

Phosphorylation of claudin-5 on the Thr-207 residue in the C-terminal domain by PKA or Rho kinase generally affects TJ integrity in brain endothelial cells and increases barrier permeability [46, 55]. However, direct evidence on the pattern of claudin-5 phosphorylation in stroke is still lacking. The activation of nPKC-θ, aPKC-ζ, cPKC-α cPKC-β, PI3Kγ, p38MAPK in different phases of stroke injury and correlation with alterations in claudin-5 localization and expression indirectly point to an essential role of Ser/Thr phosphorylation of claudin-5 in TJ complex disassembly [49, 50, 56, 57].

Evidence indicates that ZO-1 phosphorylation regulates barrier permeability in different systems [58–60]. VEGF increase pTyr-ZO-1 levels in retinal endothelial cells and causes barrier dysfunction [61]. In inflammation, ZO-1 phosphorylation by PKC-ε on threonine 770/772 causes endothelial barrier disruption [62]. Several recent studies also pinpoint that in brain endothelial cell models, cytokine-induced

injury (TNF-α, IL-6 or CCL2) significantly induces Tyr, Thr, and Ser phosphorylation of ZO-1 [43, 49].

The phosphorylation of TJ proteins is tightly associated with the "status" of protein/protein interactions in the junctional complex. Claudin-5/ZO-1, occludin/ZO-1, ZO-1/JAM-A, and ZO-1/actin cytoskeleton interactions are considered essential for the localization and stability of the TJ complex as well as for establishing the trans-interactions and adhesion properties of claudin-5 between adjacent cells [8, 9]. Thus, occludin phosphorylation on Thr 424/Thr438 by PKCζ is required for TJ assembly in epithelial cells, while phosphorylation on Tyr (Tyr398 and Tyr402) and Ser residues (Ser490) attenuates interaction with ZO-1 and promotes dislocation from the lateral membrane in oxidative stress-induced barrier alterations [45, 46, 48, 63, 64]. A similar effect is also described for occludin phosphorylation on Ser408, with dissociation from ZO-1 and increased paracellular permeability [45]. In transient cerebral ischemia, occludin interaction with ZO-1 is diminished due to increased Tyr phosphorylation of occludin and inhibiting Src-kinase (inhibitor PP2) reduces BBB dysfunction [54].

The C-terminus of claudin-5 contains a PDZ binding motif for direct binding to ZO-1, ZO-2, and ZO-3 [65, 66]. Direct evidence regarding diminished interaction between claudin-5 and ZO-1 in the TJ complex during pathological conditions such as ischemia/reoxygenation is lacking although the disappearance of claudin-5 from the cell boundary and increased Ser and Thr phosphorylation suggest diminished interaction with ZO-1 in TJ complex disassembly [50, 56, 57, 67].

Relocation: Besides phosphorylation and diminished protein/protein interactions, disassembly of TJ complex is associated with redistribution of transmembrane TJ proteins. Both occludin and claudin-5 are internalized in inflammatory as well as ischemic conditions [68, 69]. Redistribution of claudin-5 in early brain ischemic conditions is mediated by caveolae [68]. CCL2, a chemokine increased in brain after I/R injury, also causes claudin-5 and occludin by caveolae-mediated internalization [69]. On the other hand, occludin may also be redistributed by clathrin-dependent pathways described in retinal endothelial cells [47]. In contrast, another transmembrane TJ protein, JAM-A, shows macropinocytotic-dependent redistribution under inflammatory conditions [16]. It is relocated from the lateral to the apical side of brain endothelial cells during I/R injury and BBB disruption, as well as during inflammation [15, 16]. JAM-A contains LFA binding sites in the C2 domain and has a role in leukocyte adhesion and diapedesis. Thus, the pattern of JAM-A relocation during TJ disassembly is closely associated with its role in leukocyte infiltration [15, 16].

Degradation: Many studies have described a loss of TJ immunostaining after stroke (e.g., [70–75]). This may result from in situ degradation or internalization of the proteins followed by degradation by the proteasome or lysosomes. The most extensively studied in situ degradation is via matrix metalloproteinases (MMPs). MMP-2 and -9 have been shown to degrade occludin and claudin-5 after cerebral ischemia [68, 75]. Intracellular proteasomal and lysosomal degradation of TJ proteins can occur after ubiquitination, with polyubiquitination generally favoring proteasomal degradation and monoubiquitination lysosomal [11]. With cerebral

ischemia there is evidence of ubiquitination of occludin at the BBB by an E3 ubiquitin ligase, Itch, followed by degradation [76]. Similarly, with retinal ischemia, occludin undergoes polyubiquitination after phosphorylation at the blood retinal barrier [77].

TJ mRNA expression: As well as degradation, loss of TJ proteins after stroke may result from decreased transcription or translation. Reductions in claudin-5, occludin, and ZO-1 mRNA levels have been described after ischemic stroke [67, 75, 78].

Oligomerization and the redox response of TJ proteins: Occludin and claudin-5 have cysteine residues in their cytosolic C-termini that are involved in oligomerization through disulfide bridge formation [40–42]. By building disulfide bridge formation occludin, for example, may become increasingly oligomerized [79]. Such bridges are redox-dependent with normoxic conditions supporting occludin oligomerization and TJ assembly, while hypoxia-reoxygenation results in TJ disruption [79].

5 Signaling Mechanisms Underlying Alterations in TJ Structure in Stroke

Direct and indirect effects of stroke on the endothelium. The effects of ischemic stroke on the endothelial TJs, and thus BBB disruption, may be directly on the endothelium or indirectly via effects on other cell types within the NVU or infiltrating leukocytes which then signal to the endothelium. It has been difficult to assess the relative importance of direct endothelial cell effects in vivo, but the generation of endothelial-specific knockout and transgenic mice will help to examine this question. That the endothelium can be directly affected by ischemia is demonstrated by in vitro experiments on brain endothelial monocultures using oxygen glucose deprivation (OGD) to partially mimic ischemia (Fig. 5). OGD with and without reoxygenation causes disruption of the barrier formed by brain endothelial cell monolayers [68, 80, 81] and this is associated with marked changes in endothelial cell TJs (e.g., relocation and/or loss of TJ proteins [68, 81]).

One of the major components of ischemic and hemorrhagic injury is inflammation with an infiltration of leukocytes into brain, the activation of resident microglia and the production of inflammatory mediators. Inflammation and inflammatory mediators have a major impact on the brain endothelial TJ complex. Thus, different cytokines (IL-1β, TNF-α), chemokines (IL-8, CCL2), MMPs (MMP-2, MMP-9), adhesion molecules (ICAM-1), transcription factors (NfκB), Poly(ADP-ribose) polymerase-1 (PARP) affect TJ structure and function either directly or indirectly (by attracting leukocytes which bind to the endothelium and also produce inflammatory mediators) (e.g., [81–84]). Inflammation and inflammatory mediators are strong modulators of TJ complex disassembly and BBB disruption after cerebral ischemia (e.g., [81, 83, 85, 86]). Long-term changes in the TJ complex and BBB permeability after stroke may fuel alterations in the NVU towards development of chronic inflammatory foci which may lead to further disruption of BBB and progression of inflammation and injury[27–29].

Apart from direct effects of stroke on the cerebral endothelium and indirect effects due to inflammation, ischemic and hemorrhagic stroke may impact the endothelium by other effects on the NVU (e.g., altered trophic support). The reader is referred to other chapters in this book concerning the relationships between cells of the NVU.

Protein kinase C: Hypoxic, ischemic, and I/R injury trigger a variety of signaling pathways involved in regulating TJs and barrier permeability. Among the most extensively studied TJ regulators is the PKC family of serine/threonine kinases. They regulate a variety of cell functions including proliferation, gene expression, cell cycle, differentiation, cytoskeletal organization cell migration, and apoptosis [87–89]. The PKC family includes many different isozymes that are involved in signal transduction from membrane receptors to the nucleus. The activation of cPKC-βII, nPKC-θ, PKC-ζ, PKC-α, and PKC-μ during hypoxia and post-hypoxic reoxygenation or under influence of endothelin-1 or CCL2 regulate TJ disassembly [50, 90–95].

Protein tyrosine kinases: The protein tyrosine kinases (PTKs) are another set of signaling molecules essential for regulating BBB integrity. There are two main classes of PTKs: receptor PTKs and cellular, or non-receptor, PTKs [96–98]. The receptor PTKs have extracellular domains comprised of one or more identifiable structural motifs. They are involved in the BBB effects of growth factors such as VEGF and PDGF [99, 100]. For example, Flt-1 activation and activation of signaling molecules associated with intracellular domain of receptor tyrosine kinases (phosphatidylinositol 3-kinase/Akt (PI3-K/Akt) nitric oxide syntheses (NOS) and protein kinase G (PKG)) mediate hypoxia/VEGF-induced brain endothelial hyperpermeability and brain edema formation [101, 102].

The cellular PTKs (SRC, JAK, ABL, FAK, FPS, CSK, SYK, and BTK) are involved in some of critical events in TJ assembly and disassembly. For example, the Src-family kinases can modulate tyrosine phosphorylation on occludin attenuating its interaction with ZO-1, ZO-2, and ZO-3. They also regulate MMP activity and occludin degradation as well as caveolin-1 phosphorylation and activation and, thus, relocalization of TJ proteins, particularly during I/R-induced BBB disruption [52–54, 68, 103]. Some cellular PTKs, the JAKs, phosphorylate members of the signal transducer and activator of transcription family (the JAK/STAT pathway). That pathway is important in regulating claudin-5, ZO-1 and inflammation in the brain endothelial cells exposed to HIV [104]. PTKs are a major target of reactive oxygen species (ROS) [105]. Thus, for example, ROS generated during ischemia, brain injury, monocyte/neutrophil activation or alcohol exposure, can activate MMPs (-1, -2, and -9) and decrease tissue inhibitors of MMPs (TIMP-1 and -2) in a PTK-dependent manner [68, 106, 107].

MAP kinases. Besides tyrosine kinases, mediators of oxidative stress can also activate a complex system of MAP kinases (p38, Erk1/2) and regulate brain endothelial permeability [57, 108–110]. MAP kinases may be a nodal point in, for example, PTK signaling but they may also directly phosphorylate TJ proteins as occurs during exposure to 4-hydroxy-2-nonenal (4-HNE), one of the major biologically active aldehydes formed during inflammation and oxidative stress [110].

Rho GTPases: Another important regulator of TJs and paracellular permeability is the Rho family of GTPases. That family regulate TJs both indirectly, via effects on the actin cytoskeleton, and directly. Rho, GEF-H1 a guanine nucleotide exchange factor for Rho, Rac, and ROCK regulate cytoskeletal contractile responses via myosin ATPase activity [111]. ROCK, for example, promotes phosphorylation of the regulatory light-chain of myosin (MLC) on Ser19 and Thr18 through phosphorylation of the myosin light-chain phosphatase (MLCP) and, thus, blocks MLC dephosphorylation [112, 113]. This site-specific phosphorylation of MLC in turn elevates myosin ATPase activity, leading to actin-myosin contraction [111, 113]. Besides the specific actin filament polymerization (stress fiber formation), which generate in turn contractile intraendothelial forces impacting the TJs, all of these factors may also induce phosphorylation of TJ and AdJ proteins and their redistribution [112]. These data indicate that Rho/ROCK not only causes remodeling of actin cytoskeleton but also directly or indirectly via activation other signal pathways induces phosphorylation and remodeling of the TJ and AdJ complexes [70, 114]. In addition, ROCK is involved in regulating the expression of the proteolytic enzymes, such as MMP-9 and urokinase-type plasminogen activator (uPA), that impact BBB permeability [115]. Recent evidence has highlighted the beneficial effect of ROCK inhibition, with fasudil or Y2763, in treating BBB disruption in stroke and post-stroke conditions [70, 116–118].

Cerebral hemorrhage. In contrast to the signaling events occurring in the cerebral endothelium during cerebral ischemia, little is known about the events after cerebral hemorrhage [2]. A component of ICH-induced brain injury is related to clot-derived factors such as thrombin, hemoglobin, and iron [23]. The direct impact of such factors on brain endothelial cell signaling and TJ function in relation to ICH is generally understudied. However, there is evidence that thrombin can disassemble claudin-5 from endothelial TJs [119], that hemoglobin exposure can reduce claudin-5 and ZO-1 levels at the BBB [120] and that iron participates in the loss of BBB occludin and ZO-1 after cerebral ischemia [121]. Thus, it is very likely that clot-derived factors impact BBB TJs after hemorrhagic stroke.

6 Non-TJ Pathways Involved in BBB Hyperpermeability After Stroke

Although there is evidence for a role of TJ alterations in BBB disruption after stroke, two other pathways have also been implicated, increased transcytosis at the cerebral endothelium and endothelial cell death [122, 123] (Fig. 6). While cerebral endothelial cells have very few vesicles compared to systemic capillaries, there is some transcytosis under basal conditions. Such receptor-mediated transcytosis, via clathrin- and caveolin-1-coated vesicles, is important for the uptake of a number of macromolecules into brain (e.g., transferrin and leptin, [1, 124, 125]). In addition, some heavily cationized proteins undergo adsorptive-mediated transcytosis [1]. After ischemic stroke there is a marked increase in the number of vesicles in the

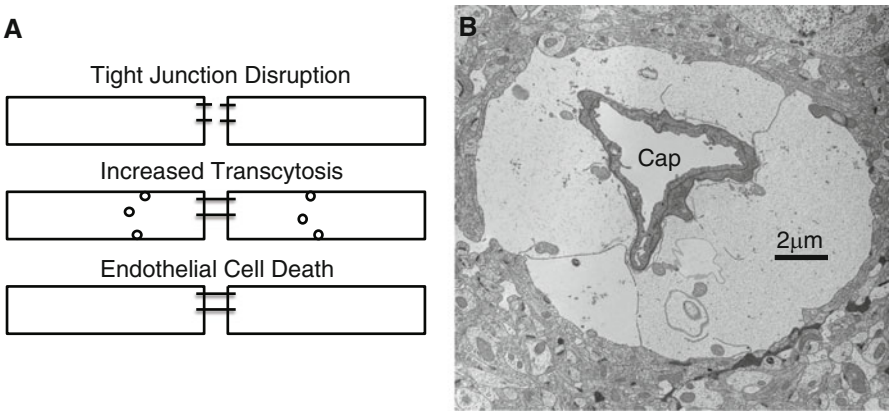

Fig. 6 (**a**) Routes proposed to participate in BBB disruption after stroke. Note that these routes are not mutually exclusive. (**b**) Electron micrograph of a cerebral capillary (cap) from the ischemic penumbra in a mouse that underwent 3 h of middle cerebral artery occlusion. Note the marked perivascular edema in the absence of marked changes in the rest of the parenchyma (compare to Fig. 1a)

cerebral endothelium [122, 126, 127]. If the increase in vesicles reflects increased transcytosis, this may contribute to increased BBB permeability after stroke. This may be particularly important for large molecular weight compounds as this pathway would be relatively size independent. Knowland et al. [122] recently proposed that such an increase in transcytosis is responsible for BBB disruption to albumin (but not a smaller tracer, Biocytin-TMR) observed 6 h after ischemic stroke, whereas profound TJ changes are responsible for later disruption at 2 days. Mice without caveolin-1, an essential component of caveolae, had reduced levels of endothelial- and parenchymal-associated albumin after stroke. It should be noted that caveolae are also involved in internalization of claudin-5 and occludin [69] and, therefore, the caveolin-1 knockout may have also affected TJ function after stroke although Knowland et al. did not identify such an effect [122].

One method to elucidate the relative importance of the transcellular (vesicular) and paracellular routes involved in BBB hyperpermeability is examining size selectivity. Preston and Webster [128] examined the relative permeability of two tracers (sucrose and inulin) of different molecular weights after cerebral ischemia and found that the absolute increase in permeability was greater for the smaller compound. They concluded that the results fitted with diffusion through a pore (with some steric hindrance) rather than vesicular transport. However, it should be noted that those experiments were 24–72 h after the ischemic event.

It has also recently been suggested that endothelial cell death is the primary cause of BBB disruption after cerebral ischemia [123]. Such endothelial cell death has been noted by other investigators after cerebral ischemia [129, 130]. A question that arises with a loss of endothelial cells being the route of BBB disruption is the extent to which such vessels continue to remain patent. Endothelial damage results in the formation of a platelet plug and activation of the coagulation cascade.

7 Therapeutic Interventions

Despite decades of work on developing neuroprotective agents for stroke [131] only reperfusion-based therapy has so far shown clinical efficacy. Tissue plasminogen activator [132] and now mechanical thrombectomy [133] improve outcomes in ischemic stroke. Those approaches do not directly target BBB changes after stroke. Indeed, tPA is associated with increased risk of cerebral hemorrhage after ischemic stroke [132]. There have been preclinical [134, 135] and now clinical trials (NCT02222714 and the European trial, Imatinib Treatment in Acute Ischemic Stroke) of agents that might protect the cerebral vasculature if given in combination with tPA. Reducing the occurrence of ICH would alleviate one impediment to its use in stroke patients. It might also increase the time window over which tPA can be given.

In preclinical models, many agents have been shown to decrease BBB hyperpermeability and alterations in TJ structure after ischemic stroke (e.g., [70–75]). A question arises as to whether those BBB effects are direct or indirect (e.g., via reducing parenchymal damage)? Endothelial-specific genetic manipulations should address this question and also whether BBB damage may contribute to parenchymal damage. It should be noted that if the BBB is a therapeutic target, drug delivery to the BBB avoids many of the problems with delivery to the brain parenchyma (e.g., brain penetration).

8 Conclusions

BBB disruption occurs in both ischemic and hemorrhagic stroke. It may contribute to brain injury by enhancing edema formation, leukocyte influx, and entry of neurotoxic compounds from blood to brain. While endothelial transcytosis and cell death have been proposed as being important in BBB dysfunction after stroke, considerable evidence indicates a major role for TJ modification. The extent to which the BBB is a primary therapeutic target for limiting brain injury, with and without reperfusion therapy, still needs to be fully clarified, but the tools necessary for such studies are becoming available.

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References

1. Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ. Structure and function of the blood-brain barrier. Neurobiol Dis. 2010;37(1):13–25.
2. Keep RF, Zhou N, Xiang J, Andjelkovic AV, Hua Y, Xi G. Vascular disruption and blood-brain barrier dysfunction in intracerebral hemorrhage. Fluids Barriers CNS. 2014;11:18.

3. Rosenberg GA. Neurological diseases in relation to the blood-brain barrier. J Cereb Blood Flow Metab. 2012;32(7):1139–51.
4. Yang Y, Rosenberg GA. Blood-brain barrier breakdown in acute and chronic cerebrovascular disease. Stroke. 2011;42(11):3323–8.
5. Strazielle N, Ghersi-Egea JF. Physiology of blood-brain interfaces in relation to brain disposition of small compounds and macromolecules. Mol Pharm. 2013;10(5):1473–91.
6. Butt AM, Jones HC, Abbott NJ. Electrical resistance across the blood-brain barrier in anaesthetized rats: a developmental study. J Physiol. 1990;429:47–62.
7. Crone C, Christensen O. Electrical resistance of a capillary endothelium. J Gen Physiol. 1981;77(4):349–71.
8. Haseloff RF, Dithmer S, Winkler L, Wolburg H, Blasig IE. Transmembrane proteins of the tight junctions at the blood-brain barrier: structural and functional aspects. Semin Cell Dev Biol. 2015;38:16–25.
9. Stamatovic SM, Keep RF, Andjelkovic AV. Brain endothelial cell-cell junctions: how to "open" the blood brain barrier. Curr Neuropharmacol. 2008;6(3):179–92.
10. Weiss N, Miller F, Cazaubon S, Couraud PO. The blood-brain barrier in brain homeostasis and neurological diseases. Biochim Biophys Acta. 2009;1788(4):842–57.
11. Mandel I, Paperna T, Volkowich A, Merhav M, Glass-Marmor L, Miller A. The ubiquitin-proteasome pathway regulates claudin 5 degradation. J Cell Biochem. 2012;113(7): 2415–23.
12. Nitta T, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, et al. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol. 2003;161(3):653–60.
13. Blasig IE, Bellmann C, Cording J, Del Vecchio G, Zwanziger D, Huber O, et al. Occludin protein family: oxidative stress and reducing conditions. Antioxid Redox Signal. 2011;15(5):1195–219.
14. Lamagna C, Meda P, Mandicourt G, Brown J, Gilbert RJ, Jones EY, et al. Dual interaction of JAM-C with JAM-B and alpha(M)beta2 integrin: function in junctional complexes and leukocyte adhesion. Mol Biol Cell. 2005;16(10):4992–5003.
15. Sladojevic N, Stamatovic SM, Keep RF, Grailer JJ, Sarma JV, Ward PA, et al. Inhibition of junctional adhesion molecule-A/LFA interaction attenuates leukocyte trafficking and inflammation in brain ischemia/reperfusion injury. Neurobiol Dis. 2014;67:57–70.
16. Stamatovic SM, Sladojevic N, Keep RF, Andjelkovic AV. Relocalization of junctional adhesion molecule A during inflammatory stimulation of brain endothelial cells. Mol Cell Biol. 2012;32(17):3414–27.
17. Itoh M, Furuse M, Morita K, Kubota K, Saitou M, Tsukita S. Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins. J Cell Biol. 1999;147(6):1351–63.
18. Taddei A, Giampietro C, Conti A, Orsenigo F, Breviario F, Pirazzoli V, et al. Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. Nat Cell Biol. 2008;10(8):923–34.
19. Lai CH, Kuo KH, Leo JM. Critical role of actin in modulating BBB permeability. Brain Res Rev. 2005;50(1):7–13.
20. Abbott NJ, Ronnback L, Hansson E. Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci. 2006;7(1):41–53.
21. Armulik A, Genove G, Mae M, Nisancioglu MH, Wallgard E, Niaudet C, et al. Pericytes regulate the blood-brain barrier. Nature. 2010;468(7323):557–61.
22. Daneman R, Zhou L, Kebede AA, Barres BA. Pericytes are required for blood-brain barrier integrity during embryogenesis. Nature. 2010;468(7323):562–6.
23. Keep RF, Hua Y, Xi G. Intracerebral haemorrhage: mechanisms of injury and therapeutic targets. Lancet Neurol. 2012;11(8):720–31.
24. Sandoval KE, Witt KA. Blood-brain barrier tight junction permeability and ischemic stroke. Neurobiol Dis. 2008;32(2):200–19.
25. Xi G, Keep RF, Hoff JT. Mechanisms of brain injury after intracerebral haemorrhage. Lancet Neurol. 2006;5(1):53–63.

26. Menzies SA, Betz AL, Hoff JT. Contributions of ions and albumin to the formation and resolution of ischemic brain edema. J Neurosurg. 1993;78(2):257–66.
27. Doyle KP, Quach LN, Sole M, Axtell RC, Nguyen TV, Soler-Llavina GJ, et al. B-lymphocyte-mediated delayed cognitive impairment following stroke. J Neurosci. 2015;35(5):2133–45.
28. Garbuzova-Davis S, Haller E, Williams SN, Haim ED, Tajiri N, Hernandez-Ontiveros DG, et al. Compromised blood-brain barrier competence in remote brain areas in ischemic stroke rats at the chronic stage. J Comp Neurol. 2014;522(13):3120–37.
29. Liu Y, Tang G, Li Y, Wang Y, Chen X, Gu X, et al. Metformin attenuates blood-brain barrier disruption in mice following middle cerebral artery occlusion. J Neuroinflammation. 2014;11:177.
30. Xi G, Reiser G, Keep RF. The role of thrombin and thrombin receptors in ischemic, hemorrhagic and traumatic brain injury: deleterious or protective? J Neurochem. 2003;84(1):3–9.
31. Davalos D, Akassoglou K. Fibrinogen as a key regulator of inflammation in disease. Semin Immunopathol. 2012;34(1):43–62.
32. Balami JS, Buchan AM. Complications of intracerebral haemorrhage. Lancet Neurol. 2012;11(1):101–18.
33. Khanna A, Kahle KT, Walcott BP, Gerzanich V, Simard JM. Disruption of ion homeostasis in the neurogliovascular unit underlies the pathogenesis of ischemic cerebral edema. Transl Stroke Res. 2014;5(1):3–16.
34. Marmarou A. A review of progress in understanding the pathophysiology and treatment of brain edema. Neurosurg Focus. 2007;22(5):E1.
35. Iadecola C, Anrather J. The immunology of stroke: from mechanisms to translation. Nat Med. 2011;17(7):796–808.
36. Liesz A, Hu X, Kleinschnitz C, Offner H. Functional role of regulatory lymphocytes in stroke: facts and controversies. Stroke. 2015;46(5):1422–30.
37. O'Donnell ME. Blood-brain barrier Na transporters in ischemic stroke. Adv Pharmacol. 2014;71:113–46.
38. Shah K, Abbruscato T. The role of blood-brain barrier transporters in pathophysiology and pharmacotherapy of stroke. Curr Pharm Des. 2014;20(10):1510–22.
39. del Zoppo GJ, Izawa Y, Hawkins BT. Hemostasis and alterations of the central nervous system. Semin Thromb Hemost. 2013;39(8):856–75.
40. Cummins PM. Occludin: one protein, many forms. Mol Cell Biol. 2012;32(2):242–50.
41. Dorfel MJ, Huber O. Modulation of tight junction structure and function by kinases and phosphatases targeting occludin. J Biomed Biotechnol. 2012;2012:807356.
42. Gonzalez-Mariscal L, Tapia R, Chamorro D. Crosstalk of tight junction components with signaling pathways. Biochim Biophys Acta. 2008;1778(3):729–56.
43. Rochfort KD, Cummins PM. Cytokine-mediated dysregulation of zonula occludens-1 properties in human brain microvascular endothelium. Microvasc Res. 2015;100:48–53.
44. Elias BC, Suzuki T, Seth A, Giorgianni F, Kale G, Shen L, et al. Phosphorylation of Tyr-398 and Tyr-402 in occludin prevents its interaction with ZO-1 and destabilizes its assembly at the tight junctions. J Biol Chem. 2009;284(3):1559–69.
45. Raleigh DR, Boe DM, Yu D, Weber CR, Marchiando AM, Bradford EM, et al. Occludin S408 phosphorylation regulates tight junction protein interactions and barrier function. J Cell Biol. 2011;193(3):565–82.
46. Yamamoto M, Ramirez SH, Sato S, Kiyota T, Cerny RL, Kaibuchi K, et al. Phosphorylation of claudin-5 and occludin by rho kinase in brain endothelial cells. Am J Pathol. 2008;172(2):521–33.
47. Murakami T, Felinski EA, Antonetti DA. Occludin phosphorylation and ubiquitination regulate tight junction trafficking and vascular endothelial growth factor-induced permeability. J Biol Chem. 2009;284(31):21036–46.
48. Murakami T, Frey T, Lin C, Antonetti DA. Protein kinase cbeta phosphorylates occludin regulating tight junction trafficking in vascular endothelial growth factor-induced permeability in vivo. Diabetes. 2012;61(6):1573–83.

49. Stamatovic SM, Dimitrijevic OB, Keep RF, Andjelkovic AV. Protein kinase Calpha-RhoA cross-talk in CCL2-induced alterations in brain endothelial permeability. J Biol Chem. 2006;281(13):8379–88.
50. Willis CL, Meske DS, Davis TP. Protein kinase C activation modulates reversible increase in cortical blood-brain barrier permeability and tight junction protein expression during hypoxia and posthypoxic reoxygenation. J Cereb Blood Flow Metab. 2010;30(11):1847–59.
51. Rao R. Occludin phosphorylation in regulation of epithelial tight junctions. Ann N Y Acad Sci. 2009;1165:62–8.
52. Kago T, Takagi N, Date I, Takenaga Y, Takagi K, Takeo S. Cerebral ischemia enhances tyrosine phosphorylation of occludin in brain capillaries. Biochem Biophys Res Commun. 2006;339(4):1197–203.
53. Mao XW, Pan CS, Huang P, Liu YY, Wang CS, Yan L, et al. Levo-tetrahydropalmatine attenuates mouse blood-brain barrier injury induced by focal cerebral ischemia and reperfusion: involvement of Src kinase. Sci Rep. 2015;5:11155.
54. Takenaga Y, Takagi N, Murotomi K, Tanonaka K, Takeo S. Inhibition of Src activity decreases tyrosine phosphorylation of occludin in brain capillaries and attenuates increase in permeability of the blood-brain barrier after transient focal cerebral ischemia. J Cereb Blood Flow Metab. 2009;29(6):1099–108.
55. Soma T, Chiba H, Kato-Mori Y, Wada T, Yamashita T, Kojima T, et al. Thr(207) of claudin-5 is involved in size-selective loosening of the endothelial barrier by cyclic AMP. Exp Cell Res. 2004;300(1):202–12.
56. Camire RB, Beaulac HJ, Brule SA, McGregor AI, Lauria EE, Willis CL. Biphasic modulation of paracellular claudin-5 expression in mouse brain endothelial cells is mediated through the phosphoinositide-3-kinase/AKT pathway. J Pharmacol Exp Ther. 2014;351(3):654–62.
57. Dong L, Qiao H, Zhang X, Zhang X, Wang C, Wang L, et al. Parthenolide is neuroprotective in rat experimental stroke model: downregulating NF-kappaB, phospho-p38MAPK, and caspase-1 and ameliorating BBB permeability. Mediators Inflamm. 2013;2013:370804.
58. Howarth AG, Singer KL, Stevenson BR. Analysis of the distribution and phosphorylation state of ZO-1 in MDCK and nonepithelial cells. J Membr Biol. 1994;137(3):261–70.
59. Kurihara H, Anderson JM, Farquhar MG. Increased Tyr phosphorylation of ZO-1 during modification of tight junctions between glomerular foot processes. Am J Physiol. 1995;268(3 Pt 2):F514–24.
60. Van Itallie CM, Balda MS, Anderson JM. Epidermal growth factor induces tyrosine phosphorylation and reorganization of the tight junction protein ZO-1 in A431 cells. J Cell Sci. 1995;108(Pt 4):1735–42.
61. Antonetti DA, Barber AJ, Hollinger LA, Wolpert EB, Gardner TW. Vascular endothelial growth factor induces rapid phosphorylation of tight junction proteins occludin and zonula occluden 1. A potential mechanism for vascular permeability in diabetic retinopathy and tumors. J Biol Chem. 1999;274(33):23463–7.
62. Ivey NS, Renner NA, Moroney-Rasmussen T, Mohan M, Redmann RK, Didier PJ, et al. Association of FAK activation with lentivirus-induced disruption of blood-brain barrier tight junction-associated ZO-1 protein organization. J Neurovirol. 2009;15(4):312–23.
63. Dorfel MJ, Westphal JK, Bellmann C, Krug SM, Cording J, Mittag S, et al. CK2-dependent phosphorylation of occludin regulates the interaction with ZO-proteins and tight junction integrity. Cell Commun Signal. 2013;11(1):40.
64. Jain S, Suzuki T, Seth A, Samak G, Rao R. Protein kinase Czeta phosphorylates occludin and promotes assembly of epithelial tight junctions. Biochem J. 2011;437(2):289–99.
65. Poliak S, Matlis S, Ullmer C, Scherer SS, Peles E. Distinct claudins and associated PDZ proteins form different autotypic tight junctions in myelinating Schwann cells. J Cell Biol. 2002;159(2):361–72.
66. Wu J, Peng D, Zhang Y, Lu Z, Voehler M, Sanders CR, et al. Biophysical characterization of interactions between the C-termini of peripheral nerve claudins and the PDZ(1) domain of zonula occludens. Biochem Biophys Res Commun. 2015;459(1):87–93.

67. Jiao H, Wang Z, Liu Y, Wang P, Xue Y. Specific role of tight junction proteins claudin-5, occludin, and ZO-1 of the blood-brain barrier in a focal cerebral ischemic insult. J Mol Neurosci. 2011;44(2):130–9.
68. Liu J, Jin X, Liu KJ, Liu W. Matrix metalloproteinase-2-mediated occludin degradation and caveolin-1-mediated claudin-5 redistribution contribute to blood-brain barrier damage in early ischemic stroke stage. J Neurosci. 2012;32(9):3044–57.
69. Stamatovic SM, Keep RF, Wang MM, Jankovic I, Andjelkovic AV. Caveolae-mediated internalization of occludin and claudin-5 during CCL2-induced tight junction remodeling in brain endothelial cells. J Biol Chem. 2009;284(28):19053–66.
70. Gibson CL, Srivastava K, Sprigg N, Bath PM, Bayraktutan U. Inhibition of Rho-kinase protects cerebral barrier from ischaemia-evoked injury through modulations of endothelial cell oxidative stress and tight junctions. J Neurochem. 2014;129(5):816–26.
71. Jin G, Arai K, Murata Y, Wang S, Stins MF, Lo EH, et al. Protecting against cerebrovascular injury: contributions of 12/15-lipoxygenase to edema formation after transient focal ischemia. Stroke. 2008;39(9):2538–43.
72. Kraft P, Schwarz T, Gob E, Heydenreich N, Brede M, Meuth SG, et al. The phosphodiesterase-4 inhibitor rolipram protects from ischemic stroke in mice by reducing blood-brain-barrier damage, inflammation and thrombosis. Exp Neurol. 2013;247:80–90.
73. Reischl S, Li L, Walkinshaw G, Flippin LA, Marti HH, Kunze R. Inhibition of HIF prolyl-4-hydroxylases by FG-4497 reduces brain tissue injury and edema formation during ischemic stroke. PLoS One. 2014;9(1):e84767.
74. Wang Y, Jia J, Ao G, Hu L, Liu H, Xiao Y, et al. Hydrogen sulfide protects blood-brain barrier integrity following cerebral ischemia. J Neurochem. 2014;129(5):827–38.
75. Yang Y, Estrada EY, Thompson JF, Liu W, Rosenberg GA. Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat. J Cereb Blood Flow Metab. 2007;27(4):697–709.
76. Zhang GS, Tian Y, Huang JY, Tao RR, Liao MH, Lu YM, et al. The gamma-secretase blocker DAPT reduces the permeability of the blood-brain barrier by decreasing the ubiquitination and degradation of occludin during permanent brain ischemia. CNS Neurosci Ther. 2013;19(1):53–60.
77. Muthusamy A, Lin CM, Shanmugam S, Lindner HM, Abcouwer SF, Antonetti DA. Ischemia-reperfusion injury induces occludin phosphorylation/ubiquitination and retinal vascular permeability in a VEGFR-2-dependent manner. J Cereb Blood Flow Metab. 2014;34(3):522–31.
78. Wang Z, Xue Y, Jiao H, Liu Y, Wang P. Doxycycline-mediated protective effect against focal cerebral ischemia-reperfusion injury through the modulation of tight junctions and PKCdelta signaling in rats. J Mol Neurosci. 2012;47(1):89–100.
79. McCaffrey G, Willis CL, Staatz WD, Nametz N, Quigley CA, Hom S, et al. Occludin oligomeric assemblies at tight junctions of the blood-brain barrier are altered by hypoxia and reoxygenation stress. J Neurochem. 2009;110(1):58–71.
80. Culot M, Mysiorek C, Renftel M, Roussel BD, Hommet Y, Vivien D, et al. Cerebrovascular protection as a possible mechanism for the protective effects of NXY-059 in preclinical models: an in vitro study. Brain Res. 2009;1294:144–52.
81. Dimitrijevic OB, Stamatovic SM, Keep RF, Andjelkovic AV. Effects of the chemokine CCL2 on blood-brain barrier permeability during ischemia-reperfusion injury. J Cereb Blood Flow Metab. 2006;26(6):797–810.
82. Argaw AT, Zhang Y, Snyder BJ, Zhao ML, Kopp N, Lee SC, et al. IL-1beta regulates blood-brain barrier permeability via reactivation of the hypoxia-angiogenesis program. J Immunol. 2006;177(8):5574–84.
83. Orion D, Schwammenthal Y, Reshef T, Schwartz R, Tsabari R, Merzeliak O, et al. Interleukin-6 and soluble intercellular adhesion molecule-1 in acute brain ischaemia. Eur J Neurol. 2008;15(4):323–8.

84. Rom S, Zuluaga-Ramirez V, Dykstra H, Reichenbach NL, Ramirez SH, Persidsky Y. Poly(ADP-ribose) polymerase-1 inhibition in brain endothelium protects the blood-brain barrier under physiologic and neuroinflammatory conditions. J Cereb Blood Flow Metab. 2015;35(1):28–36.
85. Matsumoto T, Ikeda K, Mukaida N, Harada A, Matsumoto Y, Yamashita J, et al. Prevention of cerebral edema and infarct in cerebral reperfusion injury by an antibody to interleukin-8. Lab Invest. 1997;77(2):119–25.
86. Strecker JK, Minnerup J, Schutte-Nutgen K, Gess B, Schabitz WR, Schilling M. Monocyte chemoattractant protein-1-deficiency results in altered blood-brain barrier breakdown after experimental stroke. Stroke. 2013;44(9):2536–44.
87. Andreeva AY, Krause E, Muller EC, Blasig IE, Utepbergenov DI. Protein kinase C regulates the phosphorylation and cellular localization of occludin. J Biol Chem. 2001;276(42): 38480–6.
88. Rosse C, Linch M, Kermorgant S, Cameron AJ, Boeckeler K, Parker PJ. PKC and the control of localized signal dynamics. Nat Rev Mol Cell Biol. 2010;11(2):103–12.
89. Xiao H, Liu M. Atypical protein kinase C in cell motility. Cell Mol Life Sci. 2013;70(17):3057–66.
90. Bright R, Raval AP, Dembner JM, Perez-Pinzon MA, Steinberg GK, Yenari MA, et al. Protein kinase C delta mediates cerebral reperfusion injury in vivo. J Neurosci. 2004; 24(31):6880–8.
91. Miettinen S, Roivainen R, Keinanen R, Hokfelt T, Koistinaho J. Specific induction of protein kinase C delta subspecies after transient middle cerebral artery occlusion in the rat brain: inhibition by MK-801. J Neurosci. 1996;16(19):6236–45.
92. Selvatici R, Marino S, Piubello C, Rodi D, Beani L, Gandini E, et al. Protein kinase C activity, translocation, and selective isoform subcellular redistribution in the rat cerebral cortex after in vitro ischemia. J Neurosci Res. 2003;71(1):64–71.
93. Srivastava K, Shao B, Bayraktutan U. PKC-beta exacerbates in vitro brain barrier damage in hyperglycemic settings via regulation of RhoA/Rho-kinase/MLC2 pathway. J Cereb Blood Flow Metab. 2013;33(12):1928–36.
94. Sun X, Budas GR, Xu L, Barreto GE, Mochly-Rosen D, Giffard RG. Selective activation of protein kinase C in mitochondria is neuroprotective in vitro and reduces focal ischemic brain injury in mice. J Neurosci Res. 2013;91(6):799–807.
95. Wang LF, Li X, Gao YB, Wang SM, Zhao L, Dong J, et al. Activation of VEGF/Flk-1-ERK pathway induced blood-brain barrier injury after microwave exposure. Mol Neurobiol. 2015;52(1):478–91.
96. al-Obeidi FA, Wu JJ, Lam KS. Protein tyrosine kinases: structure, substrate specificity, and drug discovery. Biopolymers. 1998;47(3):197–223.
97. Bannerman DD, Goldblum SE. Direct effects of endotoxin on the endothelium: barrier function and injury. Lab Invest. 1999;79(10):1181–99.
98. Carbajal JM, Schaeffer Jr RC. H2O2 and genistein differentially modulate protein tyrosine phosphorylation, endothelial morphology, and monolayer barrier function. Biochem Biophys Res Commun. 1998;249(2):461–6.
99. Ma Q, Huang B, Khatibi N, Rolland 2nd W, Suzuki H, Zhang JH, et al. PDGFR-alpha inhibition preserves blood-brain barrier after intracerebral hemorrhage. Ann Neurol. 2011;70(6):920–31.
100. Ma Y, Zechariah A, Qu Y, Hermann DM. Effects of vascular endothelial growth factor in ischemic stroke. J Neurosci Res. 2012;90(10):1873–82.
101. Leung JW, Chung SS, Chung SK. Endothelial endothelin-1 over-expression using receptor tyrosine kinase tie-1 promoter leads to more severe vascular permeability and blood brain barrier breakdown after transient middle cerebral artery occlusion. Brain Res. 2009; 1266:121–9.
102. Vogel C, Bauer A, Wiesnet M, Preissner KT, Schaper W, Marti HH, et al. Flt-1, but not Flk-1 mediates hyperpermeability through activation of the PI3-K/Akt pathway. J Cell Physiol. 2007;212(1):236–43.

103. Bai Y, Xu G, Xu M, Li Q, Qin X. Inhibition of Src phosphorylation reduces damage to the blood-brain barrier following transient focal cerebral ischemia in rats. Int J Mol Med. 2014;34(6):1473–82.
104. Chaudhuri A, Yang B, Gendelman HE, Persidsky Y, Kanmogne GD. STAT1 signaling modulates HIV-1-induced inflammatory responses and leukocyte transmigration across the blood-brain barrier. Blood. 2008;111(4):2062–72.
105. Knock GA, Ward JP. Redox regulation of protein kinases as a modulator of vascular function. Antioxid Redox Signal. 2011;15(6):1531–47.
106. Haorah J, Knipe B, Leibhart J, Ghorpade A, Persidsky Y. Alcohol-induced oxidative stress in brain endothelial cells causes blood-brain barrier dysfunction. J Leukoc Biol. 2005;78(6):1223–32.
107. Haorah J, Ramirez SH, Schall K, Smith D, Pandya R, Persidsky Y. Oxidative stress activates protein tyrosine kinase and matrix metalloproteinases leading to blood-brain barrier dysfunction. J Neurochem. 2007;101(2):566–76.
108. Fischer S, Wiesnet M, Renz D, Schaper W. H2O2 induces paracellular permeability of porcine brain-derived microvascular endothelial cells by activation of the p44/42 MAP kinase pathway. Eur J Cell Biol. 2005;84(7):687–97.
109. Qin LH, Huang W, Mo XA, Chen YL, Wu XH. LPS induces occludin dysregulation in cerebral microvascular endothelial cells via MAPK signaling and augmenting MMP-2 levels. Oxid Med Cell Longev. 2015;2015:120641.
110. Usatyuk PV, Parinandi NL, Natarajan V. Redox regulation of 4-hydroxy-2-nonenal-mediated endothelial barrier dysfunction by focal adhesion, adherens, and tight junction proteins. J Biol Chem. 2006;281(46):35554–66.
111. Jaffe AB, Hall A. Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol. 2005;21:247–69.
112. Nelson CM, Pirone DM, Tan JL, Chen CS. Vascular endothelial-cadherin regulates cytoskeletal tension, cell spreading, and focal adhesions by stimulating RhoA. Mol Biol Cell. 2004;15(6):2943–53.
113. Noma K, Oyama N, Liao JK. Physiological role of ROCKs in the cardiovascular system. Am J Physiol. 2006;290(3):C661–8.
114. Persidsky Y, Heilman D, Haorah J, Zelivyanskaya M, Persidsky R, Weber GA, et al. Rho-mediated regulation of tight junctions during monocyte migration across the blood-brain barrier in HIV-1 encephalitis (HIVE). Blood. 2006;107(12):4770–80.
115. Yu Y, Qin J, Liu M, Ruan Q, Li Y, Zhang Z. Role of Rho kinase in lysophosphatidic acid-induced altering of blood-brain barrier permeability. Int J Mol Med. 2014;33(3):661–9.
116. Liu K, Li Z, Wu T, Ding S. Role of rho kinase in microvascular damage following cerebral ischemia reperfusion in rats. Int J Mol Sci. 2011;12(2):1222–31.
117. Posada-Duque RA, Barreto GE, Cardona-Gomez GP. Protection after stroke: cellular effectors of neurovascular unit integrity. Front Cell Neurosci. 2014;8:231.
118. Rikitake Y, Kim HH, Huang Z, Seto M, Yano K, Asano T, et al. Inhibition of Rho kinase (ROCK) leads to increased cerebral blood flow and stroke protection. Stroke. 2005;36(10):2251–7.
119. Kondo N, Ogawa M, Wada H, Nishikawa S. Thrombin induces rapid disassembly of claudin-5 from the tight junction of endothelial cells. Exp Cell Res. 2009;315(17):2879–87.
120. Yang S, Chen Y, Deng X, Jiang W, Li B, Fu Z, et al. Hemoglobin-induced nitric oxide synthase overexpression and nitric oxide production contribute to blood-brain barrier disruption in the rat. J Mol Neurosci. 2013;51(2):352–63.
121. Won SM, Lee JH, Park UJ, Gwag J, Gwag BJ, Lee YB. Iron mediates endothelial cell damage and blood-brain barrier opening in the hippocampus after transient forebrain ischemia in rats. Exp Mol Med. 2011;43(2):121–8.
122. Knowland D, Arac A, Sekiguchi KJ, Hsu M, Lutz SE, Perrino J, et al. Stepwise recruitment of transcellular and paracellular pathways underlies blood-brain barrier breakdown in stroke. Neuron. 2014;82(3):603–17.

123. Krueger M, Bechmann I, Immig K, Reichenbach A, Hartig W, Michalski D. Blood-brain barrier breakdown involves four distinct stages of vascular damage in various models of experimental focal cerebral ischemia. J Cereb Blood Flow Metab. 2015;35(2):292–303.
124. de Lange EC. The physiological characteristics and transcytosis mechanisms of the blood-brain barrier (BBB). Curr Pharm Biotechnol. 2012;13(12):2319–27.
125. Smith MW, Gumbleton M. Endocytosis at the blood-brain barrier: from basic understanding to drug delivery strategies. J Drug Target. 2006;14(4):191–214.
126. Cole DJ, Matsumura JS, Drummond JC, Schultz RL, Wong MH. Time- and pressure-dependent changes in blood-brain barrier permeability after temporary middle cerebral artery occlusion in rats. Acta Neuropathol. 1991;82(4):266–73.
127. Dietrich WD, Nakayama H, Watson BD, Kanemitsu H. Morphological consequences of early reperfusion following thrombotic or mechanical occlusion of the rat middle cerebral artery. Acta Neuropathol. 1989;78(6):605–14.
128. Preston E, Webster J. Differential passage of [14C]sucrose and [3H]inulin across rat blood-brain barrier after cerebral ischemia. Acta Neuropathol. 2002;103(3):237–42.
129. Dietrich WD, Busto R, Watson BD, Scheinberg P, Ginsberg MD. Photochemically induced cerebral infarction. II. Edema and blood-brain barrier disruption. Acta Neuropathol. 1987;72(4):326–34.
130. Ito U, Ohno K, Yamaguchi T, Takei H, Tomita H, Inaba Y. Effect of hypertension on blood-brain barrier. Change after restoration of blood flow in post-ischemic gerbil brains. An electronmicroscopic study. Stroke. 1980;11(6):606–11.
131. Ginsberg MD. Neuroprotection for ischemic stroke: past, present and future. Neuropharmacology. 2008;55(3):363–89.
132. Emberson J, Lees KR, Lyden P, Blackwell L, Albers G, Bluhmki E, et al. Effect of treatment delay, age, and stroke severity on the effects of intravenous thrombolysis with alteplase for acute ischaemic stroke: a meta-analysis of individual patient data from randomised trials. Lancet. 2014;384(9958):1929–35.
133. Campbell BC, Donnan GA, Lees KR, Hacke W, Khatri P, Hill MD, et al. Endovascular stent thrombectomy: the new standard of care for large vessel ischaemic stroke. Lancet Neurol. 2015;14(8):846–54.
134. Su EJ, Fredriksson L, Geyer M, Folestad E, Cale J, Andrae J, et al. Activation of PDGF-CC by tissue plasminogen activator impairs blood-brain barrier integrity during ischemic stroke. Nat Med. 2008;14(7):731–7.
135. Wang Y, Zhao Z, Chow N, Rajput PS, Griffin JH, Lyden PD, et al. Activated protein C analog protects from ischemic stroke and extends the therapeutic window of tissue-type plasminogen activator in aged female mice and hypertensive rats. Stroke. 2013;44(12):3529–36.