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Blood-Brain Barrier Integrity and Glial Support: Mechanisms that can be targeted for Novel Therapeutic Approaches in Stroke

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Abstract

The blood-brain barrier (BBB) is a critical regulator of CNS homeostasis. Additionally, the BBB is the most significant obstacle to effective CNS drug delivery. It possesses specific charcteristics (i.e., tight junction protein complexes, influx and efflux transporters) that control permeation of circulating solutes including therapeutic agents. In order to form this "barrier," brain microvascular endothelial cells require support of adjacent astrocytes and microglia. This intricate relationship also occurs between endothelial cells and other cell types and structures of the CNS (i.e., pericytes, neurons, extracellular matrix), which implies existence of a "neurovascular unit." Ischemic stroke can disrupt the neurovascular unit at both the structural and functional level, which leads to an increase in leak across the BBB. Recent studies have identified several pathophysiological mechanisms (i.e., oxidative stress, activation of cytokine-mediated intracellular signaling systems) that mediate changes in the neurovascular unit during ischemic stroke. This review summarizes current knowledge in this area and emphasizes pathways (i.e., oxidative stress, cytokine-mediated intracellular signaling, glial-expressed receptors/targets) that can be manipulated pharmacologically for i) preservation of BBB and glial integrity during ischemic stroke and ii) control of drug permeation and/or transport across the BBB in an effort to identify novel targets for optimization of CNS delivery of therapeutics in the setting of ischemic stroke.

Keywords

Astrocyte; Blood-Brain Barrier; Drug Delivery; Endothelial Cell; Ischemic Stroke; Tight Junction; Transporters

Introduction

The blood-brain barrier (BBB) is an essential physical and metabolic barrier that separates the central nervous system (CNS) from the systemic circulation. It is formed by a monolayer of capillary endothelial cells that have evolved to greatly limit brain uptake of circulating substances in an effort to precisely maintain cerebral homeostasis. Additionally, the BBB is the most significant obstacle to CNS drug delivery. In fact, many existing drugs have limited or no efficacy in treatment of neurological diseases primarily due to an inability to permeate the BBB and attain therapeutic concentrations within the CNS (1). A critical concept in BBB

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biology is that brain microvascular endothelial cells are not intrinsically capable of forming a “barrier.” In fact, formation and function of the BBB requires support of adjacent glial cells (i.e., astrocytes, microglia) as well as neurons, pericytes, and extracellular matrix (2). Cell-cell interactions and signaling occur in a co-coordinated mannerbetweenthese multiple cell types, events required forphysiological and pathological functioning of the BBB. Such an intricate relationship implies existence of a “neurovascular unit (NVU).” During ischemic stroke, various NVU cell types are triggered by pathological stimuli that disrupt the BBB. Understanding the endothelial and glial cell responses that are involved in modifying the NVU/BBB in the context of ischemic stroke provides an opportunity not only to protect BBB integrity during pathological insult but also to target these mechanisms for optimization of CNS drug delivery. Multiple studies by our group have provided rigorous evidence on how such pathways alter NVU/BBB physiology and how they can be targeted for improvement of CNS pharmacotherapy (3–13). Here, we provide a summary of the BBB and interactions with associated cell types/structures (i.e., glial cells, pericytes, neurons, extracellular matrix) of the NVU. Additionally, we review pathophysiological mechanisms in endothelial cells and glial cells that contribute to BBB/NVU dysfunction in ischemic stroke. Finally, we provide insights on how such mechanisms can be targeted in an effort to protect BBB integrity and optimize CNS drug delivery for treatment of ischemic stroke.

The Neurovascular Unit

The NVU consists of all major cell types found in the CNS including brain microvascular endothelial cells, astrocytes, microglia, pericytes, and neurons (14–16). The concept of the NVU emphasizes that normal brain function as well as dysfunction occurs via the co-coordinated interaction between these various cell types (17;18). In fact, decreased BBB functional integrity is known to occur prior to neuronal injury in stroke, an observation that suggests that NVU dysfunction is directly linked to compromise of the BBB (19–21). Below we provide an outline of these NVU constituents with an emphasis on properties that are involved in the pathophysiological response to ischemic stroke.

Components of the Neurovascular Unit

Endothelial Cells and the Blood-Brain Barrier

The CNS is the most critical and sensitive organ system in the human body. Proper neuronal and glial function necessitates precise regulation of the brain extracellular milieu. Additionally, the metabolic demands of CNS tissue are considerable with the CNS accounting for approximately 20% of oxygen consumption in humans (22). Therefore, the interface between the CNS and the systemic circulation must possess highly selective and effective mechanisms that can facilitate nutrient transport, exactly regulate ion balance, and provide a barrier to toxic substances that may be present in the systemic circulation. The requirement for a physical and metabolic barrier is further emphasized by the extreme sensitivity of CNS tissues to exogenous solutes. Therefore, brain entry of some substances must be permitted while accumulation of other substances in brain parenchyma must be excluded. This homeostatic function of the cerebral microvasculature primarily at the level of the brain microvascular endothelium, the principal cell type of the BBB.

The current understanding of its basic structure is built primarily upon work by Reese, Karnovsky, and Brightman in the late 1960s (23;24). Anatomically, BBB endothelial cells are distinguished from those in the periphery by a lack of fenestrations, minimal pinocytotic activity, and presence of tight junctions (TJs) (25). Cerebral endothelial cells are demarcated by increased mitochondrial content as compared with other endothelium in the body (26). This increased content of mitochondria is required for transport of solutes into and out of the brain thereby contributing to maintenance of CNS homeostasis. Additionally, several

receptors, ion channels, and influx/efflux transport proteins are prominently expressed in brain microvascular endothelial cells. Functionally, these transport systems are similar to well-characterized systems in other tissues (i.e., D-glucose transporter, L-amino acid carrier systems, Na^+/K^+ ATPase), although the capacity and rate of transport can vary. Transporters involved in transcellular flux of drugs have also been identified and characterized at the BBB endothelium. Examples of such transporters known to be expressed in brain endothelial cells include efflux transporters such as P-glycoprotein (P-gp) (27–30), Multidrug Resistance Proteins 1–6 (MRP1-6 in humans; Mrp1-6 in rodents) (27;31–34), and Breast Cancer Resistance Protein (BCRP in humans; Bcrp in rodents) (29;35;36). Additionally, transporters that have been shown to facilitate drug permeation across the BBB include organic anion transporting polypeptides (OATPs in humans; Oatps in rodents) (13;30;37;38), organic anion transporters (32;39–41), monocarboxylate transporters (39;42), nucleoside transporters (43), and peptide transporters (44).

Molecular Characteristics of the BBB

a) TJ Protein Complexes—BBB endothelial cells are interconnected by TJs, large multiprotein complexes that are maintained by astrocytic trophic factors (figure 1). TJs form a continuous, almost impermeable barrier that limits paracellular flux of xenobiotics with the exception of small, lipid-soluble molecules (25). The high BBB transendothelial resistance (1,500 – 2,000 Ωcm^2) further restricts the free flow of water and solutes (45). BBB TJs are formed by junction adhesion molecules (JAMs), occludin, and claudins (i.e., claudin-1, -3, and -5), transmembrane proteins linked to the cytoskeleton through interactions with accessory proteins (i.e., zonula occluden (ZO)-1, -2, and -3) (14). ZO proteins act as a scaffold for multiple intracellular signaling pathways and are involved in regulation of TJ function (46). Additionally, other protein constituents (i.e., cingulin, AF6, 7H6, EMP-1) have been localized to the TJ but their exact role has yet to be elucidated. A brief description of the primary proteins that constitute TJ protein complexes at the BBB is described below. Although endothelial cells of the BBB do also possess adherens junctions, which are ubiquitous in the vasculature and mediate inter-endothelial cell adhesion, these will not be discussed in this review.

Several JAM isoforms have been identified at the mammalian BBB including JAM-1, JAM-2, and JAM-3 (14;47). JAM-1 is a 40-kDa member of the IgG superfamily and is believed to mediate the early attachment of adjacent endothelial cells during development of the BBB (48). In addition to their developmental roles, JAMs regulate the transendothelial migration of leukocytes (47). Although their function in mature BBB is largely unknown, loss of JAM protein expression is directly correlated with BBB breakdown (49;50). Studies in an immortalized human brain endothelial cell line (hCMEC/d3) showed that inflammatory stimulation led to increased paracellular permeability to dextran 3000 that correlated with movement of JAM away from the tight junction, further suggesting that JAMs play a critical role in maintaining BBB functional integrity (51). Interestingly, serum levels of JAM-A were unchanged over time in 13 patients with acute ischemic stroke, suggesting that this JAM isoform is not a suitable biomarker of BBB breakdown (51).

Monomeric occludin is a 60- to 65-kDa protein that has four transmembrane domains with the carboxyl and amino terminals oriented to the cytoplasm and two extracellular loops that span the intercellular cleft (52). It is highly expressed and consistently stains in a distinct, continuous pattern along endothelial cell margins in the cerebral vasculature (10;53;54). In contrast, occludin distribution is considerably more diffuse in non-neural endothelia (55). In a previous literature review, it was suggested that while occludin is integrally localized to the TJ, it is not essential for TJ assembly and has no direct “tightening” function (56). We do not agree nor find any compelling data to support this statement. In contrast, recent studies

by our group have clearly shown that occludin is a critical regulator of BBB permeability *in vivo* (8;54;57). This essential role for occludin has also been shown to occur via interaction between two extracellular loops on occludin monomers and homologous segments on occludin molecules localized to adjacent endothelial cells (58;59). Feldman and colleagues described this interactionthat creates a tight seal and restricts paracellular solute diffusion (59). Occludin also assembles into dimers and higher order oligomers at the TJ (8;54;57). Such occludin oligomeric assemblies are required for physiological function at the TJ, particularly as a restrictor of paracellular permeability and supports the critical role for occludin's direct tightening function (54). Altered expression of occludin is associated with disrupted BBB function in various pathologies associated with ischemic stroke including hypoxia/aglycemia (60) and H/R stress (10).

Claudins have similar membrane topography to occludin but no sequence homology (52). Claudins are 20- to 24-kDa proteins, of which at least 24 have been identified in mammals (14). All claudins have similar sequence homology among themselves in the first and fourth transmembrane domains and extracellular loops (61). The extracellular loops of claudins interact via homophilic and heterophilic interactions between cells (62). Overexpression of claudin isoforms in fibroblasts can induce cell aggregation and formation of TJ-like strands. Conversely, occludin only localizes to TJs in cells that have already been transfected with claudins (63). Thus, it is hypothesized that claudins form the primary "seal" of the TJ. In the cerebral microvascular endothelium, various isoforms of claudins have been detected including claudin-1, -3, and -5 (3;53;64–67). In experimental models of ischemic stroke, reduced expression of claudin-5 (68;69) and disruption of interaction between claudin-5 and occludin (70) has been reported.

Proper physiological functioning of the BBB, particularly restriction of paracellular solute transport, requires association of transmembrane constituents of tight junction protein complexes with accessory proteins localized within the endothelial cell cytoplasm. These include members of the membrane-associated guanylate kinase-like (MAGUK) family. In brain microvascular endothelial cells, MAGUK proteins are involved in coordination and clustering of tight junction protein complexes to the cell membrane and in establishment of specialized domains within the membrane (71). Three MAGUK proteins have been identified at the TJ: ZO-1, -2. and -3. ZO-1 was the first protein that was shown to be directly associated with TJ complexes (72). It is a 220 kDa protein that links transmembrane proteins of the TJ (i.e., occludin, claudins) to the actin cytoskeleton (73). Previous studies have demonstrated that dissociation of ZO-1 from the junction complex is associated with increased permeability (74–76), which implies that the ZO-1-transmembrane protein interaction is critical to TJ stability and function. ZO-1 may also act as a signaling molecule that communicates the state of the TJ to the cellular interior, or vice versa. ZO-1 has been shown to localize to the nucleus under conditions of proliferation and injury (77), following Ca^{2+} depletion (78), and in response to nicotine (53). It has also been colocalized with transcription factors (79) and various G-proteins (80). ZO-2, a 160-kDa protein, has high sequence homology to ZO-1 (81) and is known to bind structural tight junction constituents, signaling molecules, and transcription factors (82). ZO-2 localizes to the nucleus during stress and proliferation, a property similar to ZO-1 (83;84). In cultured brain microvessel endothelial cells, ZO-2 is localized along the plasma membrane at points of cell-cell contact (76), although it may be distributed more diffusely in whole cerebral microvessels (53). Of particular note, ZO-2 may function somewhat redundantly with ZO-1 as it has been shown to facilitate formation of TJs that are morphologically intact in cultured epithelial cells lacking ZO-1 (85). More recently, ZO-3, a 130-kDa protein, has been identified at the BBB (86) but its exact role in TJ formation and/or function has not been elucidated. Using the cortical aspiration lesion experimental stroke model in rodents, Li and colleagues reported decreased ZO-1 expression in ipsilateral thalamus (87). Additionally, Jiao and colleagues

reported that redistribution of ZO-1 away from the TJ in cerebral microvessels correlated with an increase in Evans blue-albumin leak across the BBB (88).

In addition to MAGUK family members, other accessory proteins have been identified at the TJ. These include cingulin, AF-6, 7H6, and EMP-1 (14;89). Cingulin is a 140- to 160-kDa protein that associates with ZOs, JAM-1, and myosin (90) and is hypothesized to mediate interactions between the cytoskeleton and the TJ. AF-6, a 180-kDa protein, interacts directly with ZO-1. This interaction is inhibited by inactivation of Ras, suggesting that disruption of ZO-1/AF-6 complex may be critical in modulation of the TJ by Ras-dependent pathways (91). The function of 7H6 at the BBB is unknown (14) but it is known to reversibly dissociate from the TJ under conditions of ATP depletion (92) as may be observed during ischemic stroke. More recently, studies have identified a novel TJ protein known as epithelial membrane protein-1 (EMP-1) (89). Bangsow and colleagues showed that EMP-1 is enriched in porcine and murine brain microvessel endothelial cells as well as rat brain microvessels and colocalizes with occludin (89). Of particular note, EMP-1 is upregulated under ischemic conditions while occludin is downregulated (89), which suggests that this protein may play a role in maintenance of barrier function during ischemic stroke and/or H/R stress.

b) Transport Proteins—For many substances, uptake into the brain and extrusion from the brain is governed by transport proteins that are endogenously and selectively expressed at the BBB endothelium. Such transport proteins that have been shown to be involved in influx and/or efflux of circulating solutes include ATP-binding cassette (ABC) transporters and solute carrier (SLC) transporters (93;94). In order to target transporters for optimization of CNS drug delivery, it is critical to understand localization (i.e., luminal versus abluminal) and functional expression of transport proteins at the BBB endothelium. Below, we summarize current knowledge on such membrane transporters that are known to be involved in determining CNS delivery of therapeutic agents. Localization of specific transport proteins known to be involved in CNS drug delivery is depicted in figure 2.

ABC Transporters: The ABC superfamily is among the largest and most ubiquitously expressed protein families known to date. ABC transporters are involved in translocation of opioids and their metabolites against their concentration gradient. The energy to transport xenobiotics is provided by binding and subsequent hydrolysis of ATP. In humans, 48 ABC genes have been identified and are classified according to seven subfamilies (95). ABC drug transporters, specifically P-glycoprotein (P-gp), Multidrug Resistance Proteins (MRPs in humans; Mrps in rodents) and Breast Cancer Resistance Protein (BCRP; also known as ABCG2) are known to be involved in cellular extrusion of therapeutic agents and thus constitute a considerable barrier to effective delivery of drugs to the brain. In general, P-gp transports cationic or basic and neutral compounds, whereas MRPs/Mrps are involved in cellular efflux of anionic drugs as well as their glucuronidated, sulfated, and glutathione-conjugated metabolites (96). BCRP/Bcrp has significant overlap in substrate specificity profile with P-gp and has been shown to recognize a vast array of sulfoconjugated organic anions, hydrophobic, and amphiphilic compounds (97).

P-gp is a 170-kDa ATP-dependent integral membrane protein that was originally identified in colchicine-resistant Chinese hamster ovary cells (98). It was designated as “P-glycoprotein” because of its inherent ability to affect membrane permeability of biological substances that may be potentially toxic (98). Physiologically, P-gp is believed to function as a biological defense mechanism against entry of toxic substances from the gut into the blood and for protection of vital organ systems such as the brain (99). The majority of P-gp transport substrates are weakly amphipathic and relatively hydrophobic. Additionally, many (but not all) substrates contain an aromatic ring and a positively charged tertiary nitrogen

atom in their chemical structure (100). P-gp orthologues from different species have greater than 70% sequence identity (99) and are encoded by closely related genes (i.e., multidrug resistance (MDR) genes), which have two isoforms in humans (MDR1, MDR2) and three isoforms in both mice (i.e., mdr1, mdr2, mdr3) and rats (i.e., mdr1a, mdr1b, mdr2). The human MDR2 gene and the murine/rodent mdr2 gene products are exclusively involved in hepatic transport of phosphatidylcholine and will not be further discussed. In contrast, human MDR1, murine mdr1/mdr3, and rodent mdr1a/mdr1b are involved in transport of therapeutic agents in several tissues including BBB endothelium. Specifically, P-gp expression has been identified at both the luminal (101–103) and abluminal membrane (103–105) of brain microvascular endothelial cells. Abluminal localization of P-gp has been identified on perivascular astrocyte foot processes (104;105) and on the abluminal plasma membrane of the endothelial cell itself (103). Increased expression of P-gp has been reported in hippocampal microvessels isolated from stroke-prone spontaneously hypertensive rats (106), which suggests that alterations in P-gp expression and/or activity may be a critical component of the BBB response to ischemic stroke. Furthermore, this increase of an efflux transporter in an *in vivo* experimental stroke model may also represent a significant impediment to drug delivery to the ischemic brain.

The mammalian MRP family belongs to the ABCC group of proteins, which contains 13 members including one ion channel (i.e., CFTR), two surface receptors (i.e., SUR1 and 2) and a truncated protein that does not mediate transport (i.e., ABCC13) (31). These proteins are not involved in drug transport and will not be further discussed. Many of the functionally characterized MRP isoforms that are known to be involved in drug transport have been localized to the mammalian BBB. These include MRP1/Mrp1, MRP2/Mrp2, MRP4/Mrp4, Mrp5 and Mrp6 (33;36;107–110). The presence of multiple MRP homologues at the BBB may be a vital determinant in controlling the delivery of therapeutic agentsto the brain. Additionally, the ability of Mrp isoforms to actively efflux the endogenous antioxidant glutathione (GSH) may have significant implications in ischemic stroke. GSH is a critical factor responsible for maintenance of cellular redox balance and antioxidant defense in the brain during ischemic stroke. It has been previously demonstrated that functional expression of Mrps is upregulated in response to oxidative stress conditions, which leads to enhanced cellular efflux of GSH (111). In the ischemic brain, an upregulation of Mrp isoforms at the BBB could potentially cause reduced brain concentrations of GSH, an alteration in CNS redox status, and increased potential for neuronal and glial cell injury and death. Therefore, changes in functional expression of Mrps at the BBB in response to ischemic stroke warrants further investigation.

A third ABC superfamily member that may be involved in xenobiotic efflux is BCRP. Several recent studies have demonstrated localization of BCRP at the brain microvasculature, particularly along the luminal side of the BBB (112–114). In terms of transport activity, data from recent *in vitro* and *in vivo* studies are controversial. Although some studies have suggested that BCRP is not functional at the BBB (35;114;115) or plays a minimal role in xenobiotic efflux from the brain (116), more detailed analyses have confirmed that BCRP is a critical determinant of drug permeation across the BBB (29;117–119). The effect of ischemic stroke and/or cerebral hypoxia on BBB functional expression of BCRP is a critical point for future study.

Solute Carrier (SLC) Transporters: The second major group of drug transport proteins at the BBB endothelium is the SLC transporters. In contrast to ABC transporters, membrane transport of SLC family members is governed by either an electrochemical gradient utilizing an inorganic or organic solute as a driving force or the transmembrane concentration gradient of the substance actually being transported. To date, 319 SLC genes (i.e., SLC1 – SLC43 families) have been identified in humans (120). Of the 43 known families of SLC

transporters, members of SLC21A/SLCO and SLC22 are known to be expressed at the BBB and play a critical role in determining xenobiotic permeation across the brain microvascular endothelium (121).

Of the SLC transporters known to transport drugs at the BBB, perhaps the most viable candidates for transporter targeting are members of the SLC21A/SLCO family that includes organic anion transporting polypeptides (OATPs in humans; Oatps in rodents). OATPs/Oatps have distinct substrate preferences for amphipathic solutes (122). OATPs/Oatps are well-known to transport 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (i.e., statins), which have been recently shown to exhibit considerable neuroprotective and antioxidant activity in the CNS (123–126). For example, studies in *Xenopus laevis* oocytes have shown Oatp1a4-mediated uptake of pravastatin (127). More recently, studies in Oatp1a4(−/−) mice demonstrated reduced blood-to-brain transport of pitavastatin and rosuvastatin as compared to wild-type controls, which suggests that Oatp1a4 is involved in statin transport across the BBB (30). Oatp isoforms are also involved in uptake transport of opioid peptides, compounds that have recently been shown to have neuroprotective efficacy in experimental stroke models (128;129). Studies in *Xenopus laevis* oocytes have shown OATP1A2, the human homologue of rodent Oatp1a4, mediated uptake of opioid peptides such as [D-penicillamine^{2,5}]-enkephalin (DPDPE) (130). Although OATP isoforms are expressed in several tissues, not all exist at the BBB. Immunofluorescence staining of human brain frontal cortex demonstrated OATP1A2 (previously known as OATP-A) localization at the level of the brain microvascular endothelium (130). In rodent brain, expression of Oatp1a4 and Oatp1c1 has been reported in capillary enriched fractions, capillary endothelial cells and/or isolated brain microvessels (13;37;38;131–133). Oatp1c1 is selectively expressed at the BBB (133) and has relatively narrow substrate specificity and primarily transports thyroxine and conjugated sterols (37;38). It has proposed that Oatp1a4, a rodent homologue of OATP1A2, is the primary drug transporting Oatp isoform expressed at the rat BBB (122). Recently, our laboratory demonstratedthat Oatp1a4 is a BBB transporter that can be effectively targeted for facilitation of effective CNS drug delivery (13).

Astrocytes

Astrocytes are the most abundant cell type in the brain. Previous studies have shown that astrocytes, localized between neuronal cell bodies and endothelial cells and ensheath over 99% of cerebral capillaries with their end-feet (14;21), are critical in the development and/or maintenance of BBB characteristics (134–139). For example, studies using human umbilical vein endothelial cells showed that these cells could develop BBB properties when co-cultured with astrocytes, which implies that astrocytes secrete trophic factors critical to maintenance of the BBB phenotype (137). Astrocytes may be involved in transient regulation of cerebral microvascular permeability (140), in particular via dynamic Ca^{2+} signaling between astrocytes and the endothelium via gap junctions and purinergic transmission (141;142). Recent evidence also suggests that astrocytes may play a critical role in regulating water and ion exchange across the brain microvascular endothelium (143;144). Astrocytes possess two high-affinity transporters for uptake of glutamate, termed excitatory amino-acid transporter 1 and 2 (i.e., EAAT1 (i..e, GLAST) and EAAT2 (i.e., GLT-1) (145). These transporters are criticalin removal of excess glutamate from the synapse and contribute to maintenance of excitatory neurotransmitter concentrations in the brain. Elevated brain levels of glutamate may lead to a pathological condition known as excitotoxicity, which has been implicated in neuronal damage in ischemic stroke (146). Additionally, astrocytes are known to express volume-regulated anion channels (VRACs). These channels are involved in Ca^{2+}-independent release of anionic amino acids (i.e., glutamate, aspartate, taurine) during conditions that cause astrocyte swelling such as

cerebral hypoxia (147). Astrocytes are also known to express transport proteins including P-gp (103;148;149), MRP/Mrp isoforms (111;150–153), and Bcrp (150). Studies in human glioma tissue have detected mRNA expression of various MRP and OATP isoforms (154). The expression of multiple drug transporters in astrocytes suggests that these glial cells may act as a secondary barrier to CNS drug permeation. That is, the balance of transporters in astrocytes may either sequester drugs within the astrocyte cytoplasm, thereby preventing these compounds from reaching their site of action in the brain, or concentrate drugs in brain extracellular fluid. Pharmacological agents within brain extracellular space can be effluxed by active transport mechanisms at brain barrier sites or via “sink” effects of the CSF (96). For more detailed information on transport mechanisms in astrocytes, readers are referred to a recent review (96).

Microglia

The Spanish neuroanatomist del Rio-Hortega first described microglia (155), a cell type from the monocyte lineage that represents approximately 20% of the total glial cell population within the CNS (156). Microglia are ubiquitously distributed within the CNS, with the basal ganglia and cerebellum possessing considerably greater numbers than the cerebral cortex (157). Under normal physiological conditions, microglia exist in a quiescent state lacking endocytotic and phagocytotic activity. These microglia possess a ramified morphology characterized by a small (5–10 μm) cell body and many radial cell processes extending from the cell body. Ramified microglia are thought to contribute to maintenance of homeostasis by participating in extracellular fluid cleansing and neurotransmitter deactivation (96). During disease or trauma, microglia may become activated and the degree of this activation is directly correlated to the type and severity of brain injury (158). Activated microglia are identified morphologically by their larger cell body and relatively short cytoplasmic processes. Biochemically, activated microglia are identified by upregulation of cell surface receptors such as CD14 and toll-like receptors (TLRs) (159;160). The degree of microglial activation appears to be correlated to the type and severity of brain injury (156;158). During an immune response, activated microglia may be further converted to a reactive state, which is characterized by a spheroid or rod-like morphology and presence of phagocytotic activity. Microglia activation and proliferation has been implicated in development of neuronal death in various CNS pathological states including ischemic stroke and cerebral hypoxia (161–163). Furthermore, activation of microglia is directly associated with dysfunction of the BBB characterized by changes in TJ protein expression and enhanced paracellular permeability (16). When activated, microglia produce high levels of neurotoxic mediators such as nitric oxide and peroxide as well as inflammatory cytokines (i.e., TNF-α), proteases, and complement components (158;164). Excessive production of these substances may further lead to cell injury in the CNS characterized by astrocyte activation, further microglia activation, and neuronal cell death.

Microglia express several ion channels including multiple potassium, calcium, sodium, and chloride channels (165). The expression patterns of these ion channels depend on the microglial functional state and are involved in a variety of physiological functions including proliferation, ramification, and maintenance of membrane potential, intracellular pH regulation and cell volume regulation (166–169). Glutamate receptors (170) and nutrient carrier systems such as GLUT-1 (171) are expressed in microglia. These cells also express membrane proteins involved in drug transport. Studies in a continuous rat microglia cell line (i.e., MLS-9) demonstrated functional expression of P-gp (172;173), Mrp1 (174) and Mrp4/Mrp5 (175); however, the ability of microglia to contribute to drug permeation and/or distribution in the CNS requires further study.

Pericytes

In addition to glia, pericytes also play a crucial role in maintenance of BBB homeostasis (176). Pericytes are flat, undifferentiated, contractile cells that attach at irregular intervals along capillary walls and communicate with other cell types of the neurovascular unit (176). These cells, via secretion of pericyte-derived angiopoetin, induce expression of occludin at the BBB, which suggests that pericytes are directly involved in induction and/or maintenance of barrier properties (177). Involvement of pericytes in induction of BBB properties is also exemplified by *in vitro* demonstration thatproper localization of endothelial proteins (i.e., P-gp, utrophin) requires co-culture with pericytes (178). Further evidence for the role of pericytes in maintenance of BBB phenotype comes from the observation that pericytes migrate away from the endothelium during hypoxia (179) or brain trauma (180), conditions that are associated with increased brain microvascular permeability. More recently, studies using adult-viable pericyte-deficient mouse mutants demonstrated that pericytes are critical in maintaining expression of BBB-specific genes in endothelial cells (i.e., transferrin receptor) and by inducing polarization of astrocyte end-feet adjacent to the cerebral microvasculature (181). Additionally, MRP isoforms (MRP1, MRP4, MRP5) have been identified in pericytes *in vitro*, which implies that pericytes may contribute to regulation of BBB xenobiotic permeability (182).

Neurons

There is considerable evidence for direct innervation of both brain microvascular endothelium and associated astrocyte processes. Noradrenergic (183;184), serotonergic (185), cholinergic (186;187), and GABAergic (188) neurons have been shown to make distinct connections with other cell types of the neurovascular unit. The need for direct innervation of brain microvasculature comes from the dynamic nature of neural activity and the metabolic requirements of nervous tissue, implying that the cerebral microcirculation must be highly responsive to the needs of CNS tissue. Indeed, “metabolic coupling” of regional brain activity to blood flow is the basis of functional neuroimaging (189), although the cellular mechanisms of this process are not well understood (190). Interestingly, disruption of BBB integrity induced by pathophysiological factors (i.e., inflammation, hypertension, ischemia) often accompanies changes in cerebral blood flow and perfusion pressure (191–193) and there is evidence that such BBB opening may be a selective, compensatory event rather than a simple anatomical disruption. This implies that communication between neurons and the brain microvasculature may not simply regulate blood flow, but BBB permeability as well.

Extracellular Matrix

In addition to cellular components of the neurovascular unit, the extracellular matrix of the basal lamina also interacts with the BBB endothelium. Disruption of extracellular matrix is strongly associated with increased BBB permeability in pathological states including stroke (194;195). The extracellular matrix seems to serve as an anchor for the endothelium via interaction of laminin and other matrix proteins with endothelial integrin receptors (196). Such cell-matrix interactions can stimulate a multitude of intracellular signaling pathways (197). Matrix proteins can influence expression of TJ proteins (69;198;199), indicating that although the TJ protein complexes constitute the primary impediment to paracellular diffusion, the proteins of the basal lamina are likely involved in their maintenance.

Ischemic Stroke

Overview of Ischemic Stroke

Stroke is the third most common cause of death in the United States and is the number one cause of long-term morbidity (200). On a global scale, these statistics are even more staggering as stroke is now the second leading cause of death worldwide (18). Stroke is one of the leading causes of functional disability and currently affects approximately 45 million people living in the United States (201). Of all strokes, 87% are ischemic (201). Each year, approximately 795,000 people experience either a new or recurrent stroke, which averages one incidence of stroke every 40 seconds (201). Following an ischemic stroke, the mean lifetime cost of medical and rehabilitation is estimated at $140,048 per patient, a figure that is considerably higher for people over 45 years of age (202). In the United States, the total cost of stroke therapy was $73.7 billion (201). Several factors have been identified that increase risk of stroke including history of transient ischemic attacks, hypertension, impaired glucose tolerance and diabetes mellitus, atrial fibrillation, cigarette smoking, and low serum concentrations of HDL cholesterol (201).

Stroke is characterized by a heterogeneous spectrum of conditions caused by interruption of blood flow supplying the brain (18;203;204). Such a deficit in cerebral blood flow causes an irreversibly damaged ischemic core and salvageable surrounding neural tissue known as the penumbra (205). Physiologically, energy requirements of the CNS are met by brain uptake of both glucose and oxygen, which are metabolized to enable phosphorylation of ADP to ATP. Most ATP generated within the brain is utilized to maintain intracellular homeostasis and transmembrane gradients for monovalent and divalent ions (i.e., sodium, potassium, calcium) (206). When blood flow to the brain is interrupted in a stroke, the ischemic core is rapidly deprived of oxygen and glucose. Inability to provide sufficient quantities of ATP causes collapse of ion gradients and subsequent release of neurotransmitters (i.e., dopamine, glutamate). This uncontrolled increase in extracellular dopamine/glutamate concentrations is highly toxic to neurons, an event that causes neuronal cell death and development of an infarction (206). Excess release of glutamate is particularly deleterious to the CNS due to overstimulation of glutamate receptors, activation of phospholipases/sphingomyelinases, phospholipid hydrolysis, release of arachidonic acid and ceramide, and disruption of CNS calcium homeostasis (18;206–208). Oxidative stress is also observed in the CNS at early time points following ischemic injury and is well known to contribute to cell death in the ischemic core (209). As neuronal cell damage extends to the ischemic penumbra, neuroinflammation and apoptosis become more prevalent and dramatically affect viability of salvageable brain tissue within the penumbra (209).

Cell death processes in the ischemic core occur extremely rapidly (i.e., within minutes) thereby rendering this region difficult to protect using pharmacological approaches (18). In contrast, cells within the ischemic penumbra die more slowly by active cell death mechanisms thus rendering therapeutic interventions theoretically possible (18). Therefore, the primary goal of drug therapy for acute ischemic stroke is to salvage the penumbra as much as possible and as early as possible (205). Currently, there is only one therapeutic agent approved by the FDA for acute ischemic stroke treatment, recombinant tissue plasminogen activator (r-tPA) (210). The primary goal of r-tPA therapy is to restore blood flow and oxygen supply to ischemic brain tissue; however, most cellular damage to the brain occurs when cerebral perfusion is re-established (i.e., reoxygenation) (211). Such hypoxia/reoxygenation (H/R) stress/injury is directly associated with neuronal apoptosis characterized by cytochrome c release, caspase-3 activation and internucleosomal DNA fragmentation (212). Pathophysiological mechanisms that can cause neuronal apoptosis during H/R include oxidative stress secondary to increased production of reactive oxygen species (ROS) (213). ROS contribute to brain injury by interacting with proteins, lipids, and

nucleic acids as well as via activation of redox-sensitive signaling pathways. Such responses are characterized by increased CNS production of hydrogen peroxide (214), upregulation of the cellular stress marker heat shock protein-70 (10), and increased nuclear expression of hypoxia-sensitive transcription factors such as hypoxia-inducible factor-1 and nuclear factor-κB (10;215). The H/R component of stroke is also associated with decreased brain concentrations of the endogenous antioxidant GSH (214), an effect that is further indicative of oxidative stress. These molecular events associated with H/R injury emphasize a critical need in stroke therapy for discovery of new therapeutic agents that can be administered alone or in conjunction with r-tPA for "rescue" of salvageable neural tissue.

The Neurovascular Unit in Ischemic Stroke

Disruption of the Blood-Brain Barrier

A critical facet of early neurovascular damage is manifested as significant perturbations in BBB function. BBB homeostasis is remarkably dependent on interactions between endothelial cells, astrocytes, and extracellular matrix (216;217). Perturbation of the extracellular matrix (i.e., type IV collagen, heparan sulfate proteoglycan, laminin, fibronectin, perlecan) disrupts cell-matrix and cell-cell signaling mechanisms critical to proper functioning of the NVU (195). Many proteinases, in particular the matrix metalloproteinases (MMPs), contribute to breakdown of the extracellular matrix and disruption of the BBB in stroke (218). This includes MMPs that are activated by a HIF-1α-dependent mechanism (i.e., MMP2) and MMPs whose activation is associated with upregulation of pro-inflammatory cytokines (i.e., TNF-α, IL-1β) such as MMP3 and MMP9 (217). Involvement of MMPs in BBB disruption following ischemic stroke has been demonstrated in many experimental stroke models (219–223). Furthermore, a recent clinical study demonstrated that MMP9 levels were significantly elevated in patients with acute ischemic stroke (224). MMPs degrade the extracellular matrix that comprises the basal lamina and thereby directly compromise the BBB. Additionally, activity of MMPs opens the BBB by directly degrading TJ constituent proteins such as claudin-5 and occludin (69). MMP-mediated opening of the BBB in ischemic stroke may be regulated by nitric oxide (NO) signaling. Specifically, pharmacological inhibition of nitric oxide synthase (NOS) reduced activity of MMP2/MMP9 and prevented disruption of the BBB in a rodent model of focal cerebral ischemia (225). Damage and subsequent opening of the BBB is a key event in development of intracerebral hemorrhage and brain edema following an ischemic stroke.

Experimental models of focal cerebral ischemia have provided considerable information on solute leak across the BBB following an ischemic stroke. Using the transient middle cerebral artery occlusion (MCAO) rodent model, Pfefferkorn and Rosenberg demonstrated increased leak of sucrose, a vascular marker that does not typically cross the BBB (226), in the ischemic hemisphere (227). However, BBB disruption following an ischemic insult is much more profound than to allow leak of small molecules only. Recently, it was shown that BBB disruption following focal cerebral ischemia was sufficient to allow blood-to-brain leak of Evan's blue dye (88). Evan's blue dye, when unconjugated to plasma proteins, is a relatively small molecule with a molecular weight of 960.8 Da. It is well established that Evan's blue dye irreversibly binds to serum albumin *in vivo*. This leads to the formation of a very large, solute-protein complex (i.e., in excess of 60,000 Da) that can only traverse the BBB under considerable pathological stress such as that observed during an ischemic stroke (228;229). Of particular note, Jiao and colleagues observed redistribution of critical TJ proteins occludin, claudin-5, and ZO-1 following 2 h of focal cerebral ischemia, an event that directly correlated with increased blood-to-brain flux of Evan's blue-albumin (88). Of particular note, these researchers observed that changes in BBB TJ constituent proteins follow a distinct biphasic pattern, which they observed at 3 h and 72 h following ischemic

insult (88). Reorganization of TJ proteins following focal cerebral ischemia is mediated by vascular endothelial growth factor (VEGF) (230–232) and NO (5;233).

Reorganization of TJ protein complexes and associated leak across the BBB following focal ischemiaenables considerable movement of vascular fluid across the microvascular endothelium and development of vasogenic edema (234–236). Recent studies using the MCAO model have shown that water movement across the BBB is exacerbated by enhanced blood-to-brain movement of sodium. This phenomenon dramatically alters oncotic pressure across the brain microvascular leading to enhanced movement of water into brain parenchyma. Alterations in sodium gradients across the microvascular endothelium is facilitated by increased functional expression of Na-K-Cl cotransporter, which is typically expressed at the luminal aspect of the BBB (237), as well as Na-H exchangers NHE1 and/or NHE2 (238). MCAO studies in spontaneously hypertensive rats demonstrated that the NHE1 transporter is a critical regulator of ischemic-induced infarct volume (6). Disruption of sodium gradients across the BBB during ischemic stroke may also involve upregulation of sodium-dependent glucose transporters such as sodium-glucose cotransporter (SGLT). Specifically, pharmacological inhibition of SGLT in MCAO rats significantly reduced infarct and edema ratios, which implies that this transporter may be a critical determinant of stroke outcome (239).

Functional BBB integrity can also be disrupted by production of reactive oxygen species (ROS) and subsequent oxidative stress. Production of highly potent ROS such as superoxide anion is a well-established component of global and focal ischemia (234;240). Biological activity of superoxide anion, a by-product of normal physiological processes, is tightly controlled by superoxide dismutase (SOD) enzymes. During cerebral ischemia, superoxide is produced at such high levels that the ability of SODs to metabolize it is overwhelmed. This phenomenon is supported by the observation that pharmacological inhibition of SOD markedly reduced cerebral edema and infarct size in transgenic mice engineered to overexpress SOD (240). Increased levels of superoxide also contribute to ischemic injury to the BBB endothelium (56;241;242). BBB damage can be further intensified by conjugation of superoxide and NO to form peroxynitrite, a potent cytotoxic and proinflammatory molecule. Peroxynitrite is well-known to induce cellular damage by its ability to nitrosylate tyrosine, leading to functional modifications of critical proteins (243). Breakdown of peroxynitrite into nitrogen dioxide and hydroxyl radicals is also known to contribute to endothelial cell dysfunction and BBB disruption in cerebral ischemia (234). Overall, oxidative stress contributes to endothelial dysfunction and BBB disruption by promoting redistribution and/or disappearance of critical TJ proteins such as claudin-5 and occludin (244).

Inflammatory stimuli are also critical mediators of BBB dysfunction in the setting of ischemic stroke. Previous research has demonstrated that inflammatory responses in focal cerebral ischemia are primarily mediated through pro-inflammatory cytokines TNF-α and IL-1β, which appear within 2 -6 h following ischemic insult (245). Signaling mediated by these cytokines induces an upregulation of adhesion molecules and subsequent transmigration of activated neutrophils, lymphocytes or monocytes into brain parenchyma (246). Under normal conditions, expression of vascular adhesion molecules such as ICAM-1 and VCAM-1 are barely detectable in brain microvessel endothelial cells; however, their expression is dramatically increased in response to pathological stressors such as ischemic stroke (246–249). Pro-inflammatory mediators are well known to alter functional expression of endogenous BBB transporters. For example, TNF-α increased expression of P-gp but decreased BCRP expression in hCMEC/d3 cells (250). Similarly, TNF-α was shown to increase mdr1b promoter activity in an immortalized rat brain endothelial cell line (RBE4) (251). Pro-inflammatory stimuli (i.e., lipopolysachharide-induced production and secretion

of TNF-α and IL-1β) was also shown to alter expression of critical TJ proteins occludin and ZO-1 (252), suggesting that inflammation may play a role in exacerbating paracellular leak in the context of ischemic stroke.

Ischemic Stroke and Glial Support of the BBB

It is well established in the literature that the glial response to an ischemic insult is highly complex and multifaceted; however, it is known that injury and/or activation of astrocytes at the NVU leads to compromise of the BBB. Studies using male Fisher F344 rats injected with 3-chloropropanediol, an astrocyte-selective toxin, has provided evidence that astrocyte injury and deathis a critical component ofBBB disruption. Specifically, focal astrocyte loss leads to disassembly of BBB TJ protein complexes characterized by decreased expression of TJ constituent proteins and a corresponding increase in paracellular solute leak (138;139). In the inferior colliculus, focal astrocyte loss demarcated by reduced GFAP immunoreactivity directly corresponded with decreased paracellular localization of critical TJ proteins claudin-5, occludin, and ZO-1 (139). At the same time points that TJ proteins were downregulated, an increase in leak of dextran (10 kDa) and fibrinogen was observed (139), which suggests significant disruption of the BBB due to astrocyte cell death. The results of this study and others (134;253;254) illustrate the requirement of intercellular interactions/communication between astrocytes and endothelial cells for maintenance of BBB integrity. Additionally, astrocytes have many other features that contribute to BBB physiology. For example, astrocytes are well known to express the water channel aquaporin 4 (AQP4) at end-feet localized adjacent to brain microvascular endothelium, which contributes to endothelial cell polarity and brain water volume (255). Astrocytes secrete VEGF and fibroblast growth factor-2 (FGF-2), which promote angiogenesis and regulate biological transport at the BBB (21;256).

Astrocytes are critical contributors in the brain immunological response during ischemic stroke. In the normal non-pathological brain, astrocytes maintain focal contacts with neighboring microglia and maintain these cells in a dormant, ramified state (158). This property of astrocytes has been shown *in vitro* when microglia are cultured on a monolayer of astrocytes (257;258) or exposed to astrocyte-conditioned medium (259). Regulation of microglia by astrocytes is prevented by inflammatory signaling thus enabling microglia to elicit an immune response (158). Astrocytes can also directly contribute to brain immunological responses via upregulation of adhesion molecules (i.e., ICAM-1, VCAM) at their cell surface, an event that contributes to CNS targeting of leukocytes (260;261). This enhancement is also facilitated by astrocytic secretion of chemokines such as macrophage inflammatory protein-1α/β (MIP-1α/β), monocyte chemoattractant protein-1 (MCP-1) and regulated upon activation normal T-cell expressed and secreted (RANTES). Production and secretion of these chemokines by astrocytes is well known to occur in response to ischemic stroke (262–264) and to increased brain parenchymal concentrations of tPA (265). Of particular note, MCP-1 secretion by astrocytes was shown to coincide with a significant increase in FITC-albumin leak in an *in vitro* co-culture of endothelial cells and astrocytes subjected to 5 h oxygen-glucose deprivation, suggesting that MCP-1 may be a critical factor involved in BBB opening following an ischemic stroke (263). Astrocytes also synthesize several pro- and anti-inflammatory cytokines including interleukins (i.e., IL-1α, IL-1β, IL-4–8, IL-10), TNF-α, and interferon-γ (158;202;266–268) as well as transforming growth factor-β (TGF-β) (269;270). Cytokines such as TNF-α, IL-1α, IL-1β, and interferon-γ can directly trigger the endothelium and activate processes involved in BBB disruption (202;268;271;272). Although secretion of TGF-β1 may play a neuroprotective role in brain parenchyma (270), its effects on the endothelium are much more deleterious. Specifically, excessive endothelial stimulation by TGF-β1 affects the angiogenic response to ischemic stroke by causing formation of defective capillaries (140). Histologically, these capillaries

differ considerably from normal brain microvasculature because they lack pericytes, consist of fewer endothelial cells, and are shorter in length (140). This observation suggests that pharmacological inhibition/targeting of TGF-β signaling at the level of the brain microvascular endothelium may be an efficacious approach for protection of BBB integrity and/or preservation ofthe angiogenic response to ischemic stroke.

Inflammatory signaling by astrocytes is a critical event in exacerbation of CNS oxidative stress during ischemic stroke. Previous studies have shown that astrocytic production of proinflammatory cytokines can induce deleterious processes in astrocytes themselves via upregulation of inducible nitric oxide synthase (iNOS) (273). Upregulation of iNOS leads to a significant enhancement in NO production, which can react with superoxide to produce peroxynitrite. Increased exposure of astrocytes to peroxynitrite can lead to rapid astrocyte proliferation and hypertrophy (i.e., reactive astrocytosis) and astrocyte apoptosis (274–277). This has been demonstrated in the rodent MCAO model where increased expression of iNOS in brain parenchyma correlated with enhanced astrocytic expression of GFAP, a hallmark of reactive astrocytosis (276). Reactive astrocytosis is associated with disruption of TJs between adjacent brain microvessel endothelial cells and increased BBB permeability (278).

Although astrocyte injury is a critical determinant of BBB dysfunction in the setting of ischemic stroke, endothelial damage may also be induced via immune stimulated microglia (279). This is supported by the observation that minocycline, a pharmacological inhibitor of activated microglia, dramatically reduced cell death in cultures of murine endothelial cells exposed to activated microglia *in vitro* (279). Activated microglia produce proinflammatory cytokines (i.e., TNF-α, IL-1β, IL-6) in response to cerebral ischemia (280–282), all of which can trigger BBB disruption. In the setting of ischemic stroke, cytokine production in microglia is mediated by NF-kB signaling (283). Inflammatory signaling in microglia may also involve cyclooxygenase-2 (COX2), which is inducible in response to ischemic injury and contributes to opening of the BBB (217). In neuroinflammation, COX2 activates sphingomyelinases leading to release of ceramides, an event that leads to activation of p38 mitogen activated protein kinase (MAPK) (284). Activation of the p38 MAPK pathway is also associated with production and secretion of proinflammatory cytokines in microglia (285;286). This is supported by the observation that pharmacological treatment with SB203580, a specific p38 MAPK inhibitor, attenuates production and secretion of TNF-α in primary cultures of rat microglia (285). More recently, administration of SB203580 following MCAO was shown to suppress microglia secretion of TNF-α and IL-1β as well as decreased microglial expression of COX2, results that point to a critical role for microglia in the inflammatory response to ischemic stroke (286).

Targeting the Neurovascular Unit in Ischemic Stroke

Targeting the Tight Junction

The studies reviewed above clearly demonstrate that the BBB and associated glial support network of the NVU may be compromised in response to ischemic stroke. A critical “component” of ischemic stroke is cerebral hypoxia and subsequent brain injury resulting from reoxygenation/reperfusion (i.e., H/R stress). Over the past several years, our laboratory has studied BBB changes associated with H/R stress in an *in vivo* rodent model (3;7;8;10;11;215;287). This *in vivo* system employs an acute, moderate hypoxic insult of inhaled 6% O_2 for 1 h followed by reoxygenation under normal atmospheric conditions (i.e., 21% O_2) for various time points. This approach offers several advantages for the study of NVU biology in the context of cerebral ischemia/hypoxia. Firstly, the *in vivo* nature of the model allows interaction of all cellular components of the NVU (i.e., endothelial cells, astrocytes, microglia, pericytes, neurons) and systemic circulation mediators, which *in vitro*

systems are unable to accurately represent (7). Secondly, the degree of hypoxic stress does not induce necrotic damage of the endothelium, often associated with other *in vivo* focal ischemia models, allowing us to study a dynamically regulated and recoverable BBB (7). Changes in BBB integrity under H/R conditions were demarcated by enhanced brain accumulation of ^{14}C-sucrose (3;7;10;215), a vascular marker that does not typically cross the brain microvascular endothelium. Additionally, H/R stress also increased vascular leak to dextrans (molecular weight range 4-kDa to 10 kDa) in hippocampal and cortical microvessels (11). These alterations in BBB permeability in animals subjected to H/R stress were directly associated with an increase in expression of HIF-1α and NF-kB in nuclear fractions isolated from intact microvessels (215). In our studies, changes in brain solute uptake is not likely attributed to altered cerebral blood flow because we have previously shown that blood flow changes are negligible in our *in vivo* H/R model (3). Changes in BBB permeability to ^{14}C-sucrose and dextrans were directly correlated with altered organization and/or expression of constituent TJ proteins including occludin (3;10;11), claudin-5 (11), and ZO-1 (11). Of paramount significance was the observation that H/R stress disrupted disulfide-bonded occludin oligomeric assemblies, thereby preventing monomeric occludin from forming an impermeable physical barrier to paracellular transport (8). TJ protein organization and expression were found to be regulated by protein kinase C (PKC) signaling involving nPKC-θ and aPKC-ζ isoforms (11). These changes in TJ organization and expression and BBB solute leak also correlated with a significant increase in brain water content following H/R, providing further evidence that disruption of the BBB under conditions of cerebral ischemia contributes to vasogenic edema (7).

Production of ROS and subsequent oxidative stress has been previously shown to alter BBB expression of claudin-5 and occludin leading to increased paracellular solute leak (244). Therefore, we hypothesized that oxidative stress associated changes in BBB permeability and occludin expression could be attenuated with the use of an antioxidant drug. In order to conduct these studies, we utilized 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPOL), a stable, membrane-permeable, water-soluble nitroxide antioxidant. TEMPOL shows SOD-like activity towards the superoxide anion as well as reactivity with hydroxyl radicals (288), nitrogen dioxide, and the carbonate radical (289). TEMPOL readily crosses the BBB (290) and has been previously shown to provide neuroprotection as a free radical scavenger in several models of brain injury and ischemia (291–294). Using the dual artery *in situ* brain perfusion technique, we demonstrated that administration of TEMPOL 10 min before H/R treatment significantly attenuated CNS uptake of ^{14}C-sucrose as compared to animals subjected to H/R only (10). This reduction in ^{14}C-sucrose leak was associated with a preservation of occludin localization and occludin oligomerization at the TJ (10). Specifically, TEMPOL inhibits breakage of disulfide bonds on occludin monomers and thus prevents breakdown of occludin oligomeric assemblies and subsequent blood-to-brain leak of circulating solutes (figure 3) (10). Restoration of BBB functional integrity coincided with a decrease in nuclear translocation of HIF-1α and a decrease in microvascular expression of the cellular stress marker heat shock protein 70 (hsp70) in rats subjected to H/R stress and administered TEMPOL (10). Taken together, these observations provide evidence that the TJ can be targeted pharmacologically during ischemic stroke for the purpose of reducing both oxidative stress associated injury to the brain microvascular endothelium and blood-to-brain solute leak. Future studies will focus on how TEMPOL can be utilized to modulate CNS drug delivery in the setting of ischemic stroke and H/R injury.

Targeting Endogenous BBB Transporters

The ability of a pharmacological agent to cross the BBB endothelium and achieve efficacious concentrations within the CNS is dependent upon multiple mechanisms of transport. Such mechanisms include uptake into the brain via an influx transporter and/or

extrusion from the CNS mediated by an efflux transporter. For many drugs, it is this discrete balance between influx and efflux that determines if a pharmacological agent will accumulate within the brain extracellular milieu and be able to elicit a therapeutic effect. The complexity of drug transport biology at the BBB is further underscored by the observation that functional expression of such transport proteins may be dramatically altered by pathophysiological stressors (13;295–298). A thorough understanding of the regulation and functional expression of endogenous BBB transporters in both health and disease is critical for optimization of pharmacotherapy. Furthermore, such information will enable efficient targeting of transporters and/or transporter regulatory mechanisms, thus allowing endogenous BBB transport systems to be exploited for purposes of improving CNS drug delivery.

Perhaps the most critical transporter determinant of solute permeation to the CNS is P-gp. Recently, it has been reported that P-gp plays a critical functional role in regulating brain injury associated with focal cerebral ischemia. Using MCAO, Murozono and colleagues showed a significant reduction in infarction size in mdr1a-knockout mice as compared to wild type controls (299). Similarly, pharmacological inhibition of P-gp with cyclosporine A (1 mg/kg) dramatically reduced infarctionvolume in ischemic brain tissue as compared to vehicle controls (300). Taken together, these studies indicate that P-gp actively controls CNS concentrations of neuronal modulatory substances such as cytokines and neuronal peptides (299). In terms of drug delivery, much effort has focused on blocking P-gp activity as a means of enhancing brain delivery of therapeutic compounds. In fact, several clinical trials have attempted to incorporate pharmacological inhibitors of P-gp into therapeutic regimens; however, these trials have not been encouraging because of systemic toxicity associated with high inhibitor doses necessary for effective transporter inhibitor (301–303). Therefore, pharmacological approaches that involve inhibition of P-gp mediated transport should be employed with extreme caution to avoid an unwanted elevation in CNS drug concentrations and subsequent toxicity as well as unexpected adverse drug reactions.

An alternative approach for optimizing delivery of drugs to the CNS is to focus on influx processes and target endogenous BBB transporters known to be involved in blood-to-brain transport of drugs. An intriguing candidate is Oatp1a4, which is known to transportboth HMG-CoA reductase inhibitors (i.e., statins) and opioids peptides. In the context of ischemic stroke pharmacotherapy, there is considerable interest in neuroprotective/ antioxidant properties of statins. Recent evidence suggests that statins can act as free-radical scavengers independent of their well-documented effects on cholesterol biosynthesis (125;304). For example, studies in dogs demonstrated that high-dose atorvastatin (80 mg/ day) reduced markers of oxidative and nitrosative stress (i.e., protein carbonyls, 4-hydroxy-2-nonenal, 3-nitrotyrosine) and increased the GSH:GSSG ratio in the brain but not in the periphery, suggesting efficacy as a neuroprotectant and CNS antioxidant (125;126). Although statins are associated with neurotoxic effects, these drugs generally do not compromise neuronal cell viability at concentrations below 1 μM (124). Additionally, there is evidence that opioid receptor agonists such as opioid peptide analgesics may have efficacy in treatment of ischemic stroke. For example, opioid peptides that selectively bind to the mu-opioid receptor (i.e., Tyr-D-Ala', N-CH, -Phe4, Glyol-Enkephalin (DAMGO)), delta-opioid receptor (i.e., [D-penicillamine^{2,5}]-enkephalin (DPDPE)), and kappa-opioid receptor (i.e., U50 488) all reduced water uptake in rat hippocampal slices *in situ* (129), suggesting that these drugs may be effective as stroke therapeutics.

Recently, we reported for the first time increased functional expression of Oatp1a4 at the BBB in rats subjected to a pathological stressor (i.e., peripheral inflammatory pain) (13). Evidence for increased Oatp1a4 transport at the BBB included i) increased brain accumulation of taurocholate, a selective Oatp substrate (305); ii) attenuation of taurocholate

uptake by Oatp transport inhibitors (i.e., digoxin, estrone-3-sulfate, fexofenadine); iii) increased in K_{IN} for taurocholate in response to pathological stress, which implies increased blood-to-brain transport; and iv) an increase in taurocholate accumulation within brain interstitial fluid but no change in taurocholate sequestration within the BBB endothelium itself (13). In order to determine if Oatp1a4 could effectively facilitate CNS drug delivery, we studied BBB transport of the opioid peptide DPDPE. Brain uptake of DPDPE is governed by multiple mechanisms in addition to Oatp1a4-mediated transport including transcytosis (306) and P-gp-mediated efflux (307). Although we showed increased Oatp1a4 functional expression at the BBB in animals subjected to peripheral inflammatory pain, we did not see any change in blood-to-brain DPDPE transport (13). In light of our previous work with P-gp (298), we proposed that Oatp1a4 influx transport was negated by P-gp efflux. This implies that the relative contribution of Oatp1a4 to overall brain uptake of DPDPE could only be determined in the absence of P-gp mediated transport activity. When we inhibited P-gp efflux transport using reversin 205, a selective P-gp inhibitory peptide (308), we observed that the relative contribution of Oatp1a4 to brain uptake of DPDPE increased from 56% in saline controls to 71% in animals subjected to peripheral inflammatory pain (13). These data are particularly critical because they showed, for the first time, that Oatp1a4 can be targeted for delivering drugs such as opioid peptides to the brain.

In order to successfully target a transporter system for optimization of CNS drug delivery, it is crucial to determine how a transport of interest is regulated at the molecular level. In the context of ischemic stroke, this includes identification and characterization of biological mechanisms that enable peripheral pain to “transmit” signals upstream and alter BBB drug transporters. Of particular interest is the TGF-β signaling pathway. TGF-β signaling regulates multiple cellular processes including vascular remodeling (309). The TGF-βs are a family of pleiotropic cytokines that modulate cellular function by binding to a heterotetrameric complex of type I and type II serine/threonine kinase receptors (310). The type I receptors, also known as activin receptor-like kinases (ALKs), propagate intracellular signals through phosphorylation of specific Smad proteins (i.e., receptor-regulated (R)-Smads) (figure 4). Phosphorylated (R)-Smads form complexes with the common Smad (i.e., Smad4) enabling them to be translocated to the nucleus and regulate transcription of target genes (310).

In the majority of tissues, TGF-β signaling is mediated by ALK5 (311); however, studies in cultured human endothelial cells (312) and in isolated arterial endothelium from ALK1-deficient mice (313) have indicated that ALK1 is also involved in vascular TGF-β signaling. TGF-β regulates the endothelial cell activation state through a precise balance between ALK1 and ALK5 signaling processes (312;314). Whereas the ALK1 pathway leads to endothelial activation characterized by increased permeability, ALK5-mediated signaling promotes vascular resolution that is demarcated by decreased permeability (311;312). Such effects on vascular permeability may be due to the ability of TGF-β signaling to alter expression of tight junction constituent proteins. For example, claudin-5 expression was increased by pharmacological ALK5 inhibition in embryonic stem cells, suggesting the involvement of TGF-β/ALK5 signaling in the regulation of tight junction constituent proteins (315). Similarly, we demonstrated that ALK5 inhibition using SB431542 increased expression of claudin-3, claudin-5, occludin monomers, and ZO-1 *in vivo* (9). Additionally, studies using human glioblastoma cells cocultured with human brain endothelial cells showed that activation of TGF-β-mediated signaling decreased endothelial expression of occludin, claudin-1, and claudin-5 (316).

The TGF-β1 isoform is dramatically induced in the CNS in response to pathological conditions that cause acute and chronic brain injury such as ischemic stroke (269;270). Recently, our laboratory demonstrated that pharmacological inhibition of TGF-β signaling

led to increased microvascular expression and activity of Oatp1a4 at the BBB (13). Of particular interest was the observation that this blockade of TGF-β/ALK5 signaling using SB431542 increased Oatp1a4 functional expression in saline control animals (13). A crucial consideration in interpretation of our data is contribution of paracellular diffusion to brain uptake of taurocholate. Our laboratory has previously reported that pharmacological inhibition of TGF-β/ALK5 signaling also increased BBB permeability via the paracellular route for solutes such as sucrose (9). The molecular weight of taurocholate (537.7 Da) is greater than sucrose (342 Da), suggesting a lesser degree of paracellular diffusion. Since our data showed no statistical difference in taurocholate uptake in the presence and absence of Oatp1a4 inhibitors in animals subjected to a pathological stressor known to open the BBB (i.e., pain/inflammation), we conclude that paracellular diffusion was not a significant factor in our study. This is not to say that paracellular drug transport is not a significant factor in determining blood-to-brain delivery of other drugs in the setting of a pathophysiological response. Therefore, it is critical to correct for paracellular transport in any study on the effect of targeting TGF-β signaling for optimization of CNS drug delivery. Although studies in immortalized mouse brain endothelial cells (MBE4) have shown involvement of ALK5-mediated signaling in P-gp regulation (317), we are the first to report TGF-β/ALK5 signaling regulation of an endogenous BBB drug uptake transporter. Our work on TGF-β/ALK5 signaling demonstrated that this pathway can regulate permeability at the BBB by increasing functional expression of an influx transporter. Furthermore, these studies highlight the potential of the TGF-β/ALK5 pathway as a pharmacological target that can be utilized for optimization of drug delivery to the CNS, particularly for treatment of ischemic stroke.

Targeting Glial Support of the BBB

In addition to the BBB endothelium, glial cells (i.e., astrocytes, microglia) also have considerable potential as a therapeutic target for treatment of ischemic stroke. As described above, glia play a crucial role in regulating BBB functional integrity in health and diseasethrough release of trophic factors that maintain TJ protein complexes, release of factors that promote angiogenesis, pro-inflammatory signaling, and production of ROS. Pharmacological manipulation of glial cell biology represents a therapeutic approach that may enable control of BBB/NVU pathophysiological mechanisms during ischemic stroke and/or H/R injury. A brief description of such promising glial-based strategies for treatment of ischemic stroke is discussed below.

An opportunity for cellular protection of glia in ischemic stroke involves targeting the proteinase-activated receptor (PAR) pathway. To date, four members of the PAR family (i.e., PAR-1, PAR-2, PAR-3, PAR-4) have been cloned and characterized (318). Both PAR-1 and PAR-2 are expressed on the cell surface of astrocytes (319–322) and microglia (321;323) as well as on the endothelial cell surface (324–326). PAR-1 has been implicated in cytoprotective mechanisms (327;328) while PAR-2 is involved in regulation of inflammatory responses (321;329). Recent research has focused on pharmaceutical development of agonists targeted to the PAR-1 receptor such as activated protein C (APC) (328;330–332). In a mouse model of transient cerebral ischemia, APC was shown to reduce ischemic brain damage and promote neovasacularization and neurogenesis, suggesting that pharmacological targeting of the PAR-1 receptor may be an efficacious approach for treatment of ischemic stroke (330). Using a murine model of cerebral venous sinus thrombosis, Nagai and colleagues demonstrated that neutralization of APC resulted in BBB dysfunction and brain edema, which suggests that the protein C pathway via the PAR-1 receptor is involved in cytoprotection against deleterious pathological responses (331). Brain vascular perfusion studies demonstrated that brain accumulation of APC was reduced by 64% in mice lacking the endothelial protein-C receptor (EPCR), suggesting that CNS

delivery of APC is dependent upon saturable EPCR-mediated transport at the BBB (333). Although native APC exhibits cytoprotection in stroke models, its use is limited by bleeding complications (334); however, a mutant form of APC termed 3K3A-APC has been discovered that exhibits considerable cytoprotective efficacy without complications of bleeding (328). Specifically, studies in human brain endothelial cells *in vitro* showed that 3K3A-APC protected these cells from oxygen-glucose deprivation to a significantly greater degree than APC (328). Furthermore, 3K3A-APC improved functional outcome and reduced infarction size at a level that was significantly better than APC in the *in vivo* murine distal MCAO model (328), which implies that 3K3A-APC offers a safer and more efficacious alternative to APC in pharmacological targeting of the PAR-1 receptor. In the case of the PAR-2 receptor, a small molecule PAR-2 antagonist (i.e., N1-3-methylbutyryl-N4-6-aminohexanoyl-piperazine; ENMD-1068) has been shown to attenuate inflammatory responses in a dose-dependent manner (335). More recently, this same PAR-2 antagonist reduced synovitis in rheumatoid and osteoarthritis patients (336). Currently, there is no report in the literature on the effect of PAR-1 activation or PAR-2 inhibition on regulating glial cell biology in the setting of stroke; however, this receptor may represent a novel target that can be exploited for protection of BBB/NVU glial support networks in the ischemic brain.

Minocycline is a tetracycline with anti-inflammatory properties that directly inhibit microglial activation. Minocycline easily crosses the BBB, has a good safety profile, and a delayed therapeutic window thus rendering it an ideal candidate drug for treatment of ischemic stroke (337). Blocking microglial activation may limit BBB disruption and reduce vasogenic edema in the context of ischemic stroke. For example, Yenari and colleagues reported that, *in vivo*, minocycline reduced infarction volume and neurological deficits as well as prevented BBB disruption and hemorrhage in a murine experimental stroke model (279). *In vitro*, inhibition of microglial activation with minocycline limited ischemic damage in cultured endothelial cells and reduced superoxide release following oxygen-glucose deprivation (279). Currently, minocycline has been incorporated into two clinical trials involving stroke patients. Results of these studies demonstrated that minocycline administration, both alone and in combination with tPA, improved functional neurological outcome following as ischemic stroke (337).

Toll-like receptors (TLRs) are widely expressed in the human CNS, particularly by astrocytes and microglia (338). Targeting these receptors has emerged as a promising goal for therapeutic control of ischemic stroke, primarily because TLRs are involved in all aspects of BBB dysfunction and NVU ischemic injury (339). While mRNA for TLRs 1 through 10 have been detected in murine microglia (340), all except TLR10 have been reported in human microglia (341). Astrocytes possess a much more limited complement of TLRs since mRNA for TLRs 2, 4, 5, and 9 have been detected in murine astrocytes (342) and only TLR3 mRNA in human astrocytes (343). While the large number of TLR receptors expressed on glial cells suggests a plethora of potential therapeutic targets for modification of glial pathology in the ischemic brain, much work needs to be done on understanding pharmacokinetics of TLR ligand binding and interactions between the TLR and the Toll/IL-1 receptor (TIR) before TLR based stroke therapeutics can reach development (339).

Conclusions

The field of BBB biology, particularly the study of TJ protein complexes and endogenous transport systems, has rapidly advanced over the past two decades. For example, it is now well-established that TJ protein complexes are dynamic in nature and can organize and reorganize in response to ischemic stroke. These changes in TJ protein complexes can lead to increased BBB permeability to small molecule drugs via the paracellular route.

Additionally, many previous studies reported on the controversial ability of transporters (i.e., Oatp1a4) to act as facilitators of brain drug uptake (figure 5). Now, it is beginning to be appreciated that endogenous BBB transporters can facilitate uptake of therapeutics from blood to the brain, thereby rendering these transport proteins potential targets for optimizing CNS drug delivery. Furthermore, molecular machinery involved in regulating endogenous BBB transport systems (i.e., TGF-β/ALK5 signaling, nuclear receptor systems) are just now being identified and characterized. These critical discoveries have identified multiple molecular targets that can be exploited for optimization of CNS delivery of therapeutic agents. Perhaps targeting of novel drugs to influx transporters such as Oatp1a4will lead to significant advancements in the treatment of ischemic stroke. Identification and characterization of intracellular signaling pathways that can regulate functional expression of uptake transporters provides yet another approach for pharmacological control of transporter systems in an effort to deliver therapeutics to the CNS. Additionally, identification and characterization of novel targets on glial cells (i.e., astrocytes, microglia) provide yet another opportunity for the design and development of therapeutics aimed at protecting the BBB/NVU during ischemic injury and, by extension, controlling CNS drug delivery. Future work will continue to provide more insight on the interplay of TJ protein complexes, transporters, and intracellular signaling pathways at the BBB/NVU and how these systems can be effectively targeted. Ultimately, data derived from these studies will enable achievement of more precise and more effective drug concentrations within the CNS and improved treatment for ischemic stroke.

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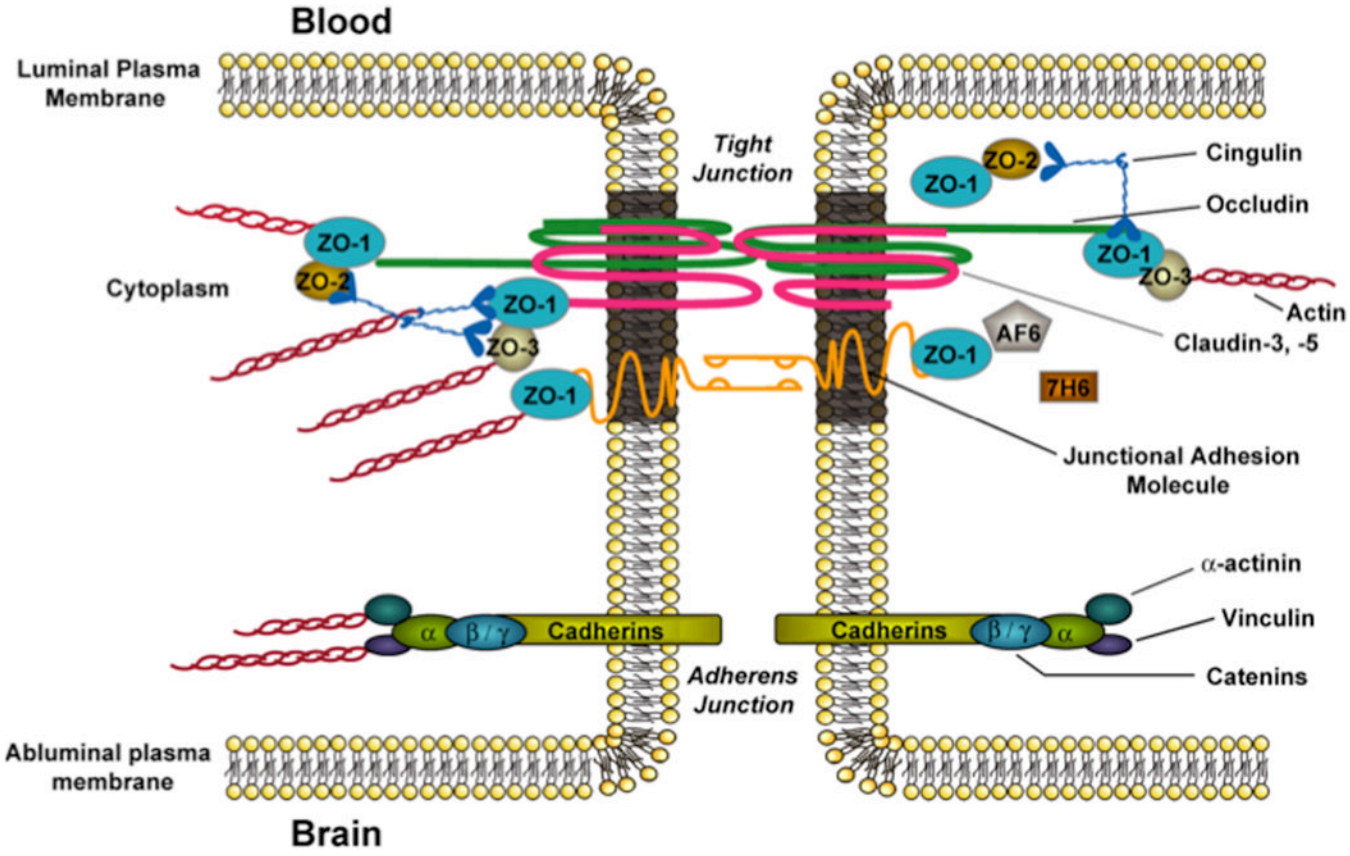

Figure 1.
Basic molecular organization of tight junction protein complexes at the blood-brain barrier. Adapted from Ronaldson & Davis. *Therapeutic Delivery*. **2**: 1016–1041 (2011).

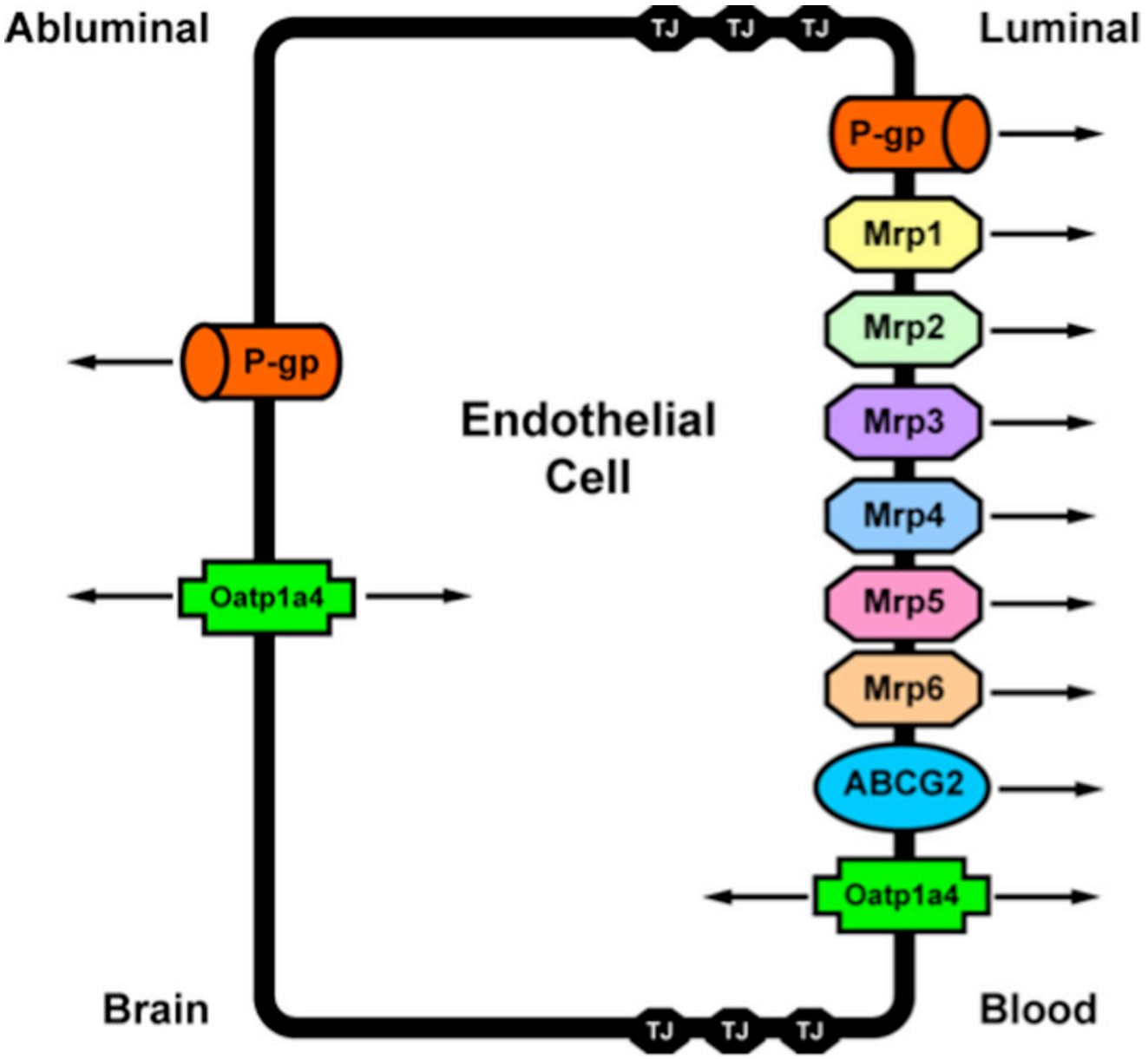

Figure 2.
Endothelial localization of drug transporters known to be involved in transport of opioids at the blood-brain barrier. Adapted from Ronaldson & Davis. *Therapeutic Delivery*. **2**: 1016–1041 (2011).

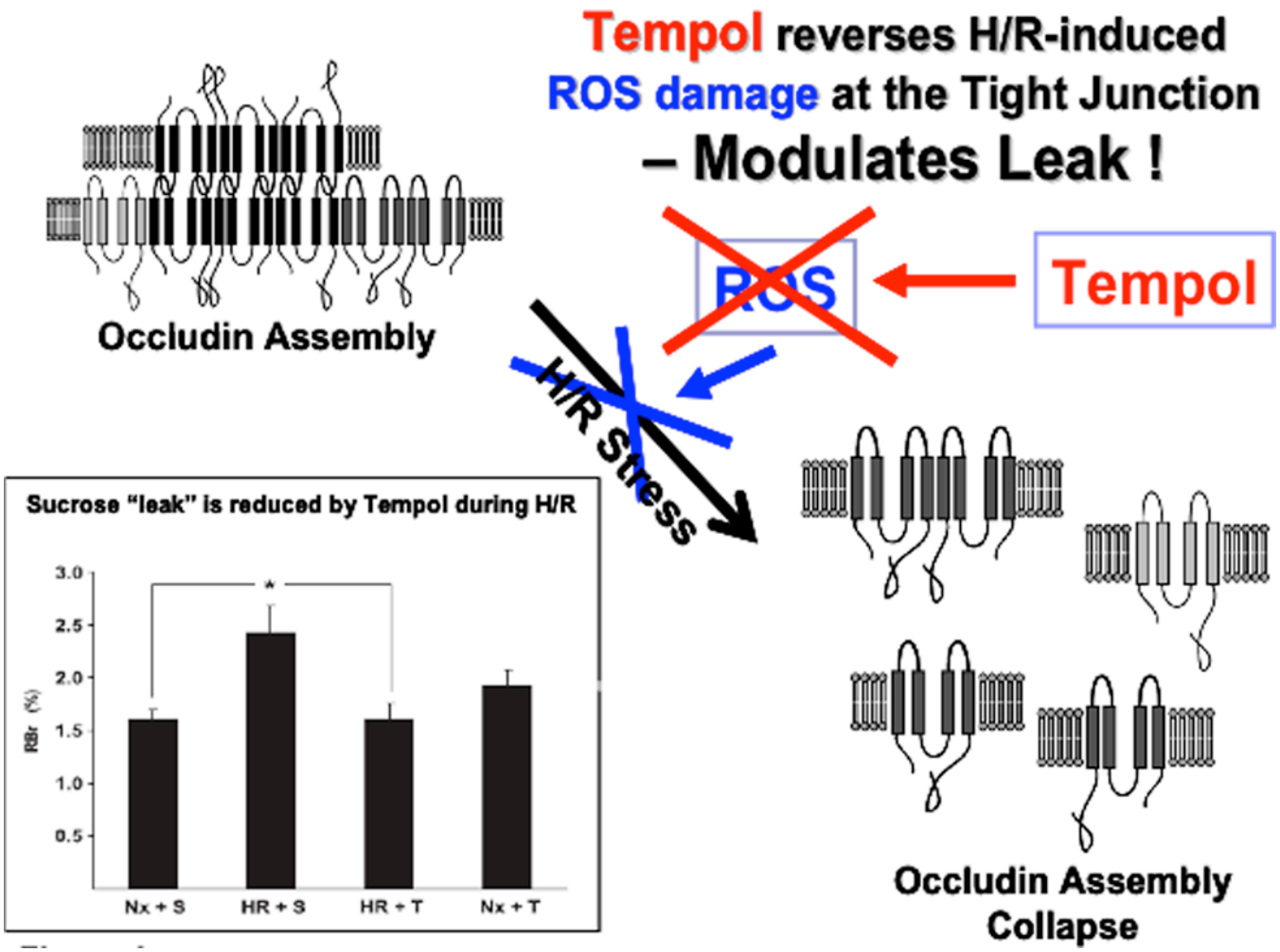

Figure 3.

Effect of TEMPOL on H/R-mediated disruption of the tight junction. ROS and subsequent oxidative stress are known to disrupt assembly of critical TJ proteins such as occludin. Our results show that administration of TEMPOL, by scavenging ROS, prevents disruption of occludin oligomeric assemblies. Furthermore, TEMPOL attenuates the increase in sucrose leak across the BBB observed in animals subjected to H/R stress. Taken together, our studies with TEMPOL demonstrate that the TJ can be targeted pharmacologically in an effort to preserve BBB functional integrity during ischemic stroke.

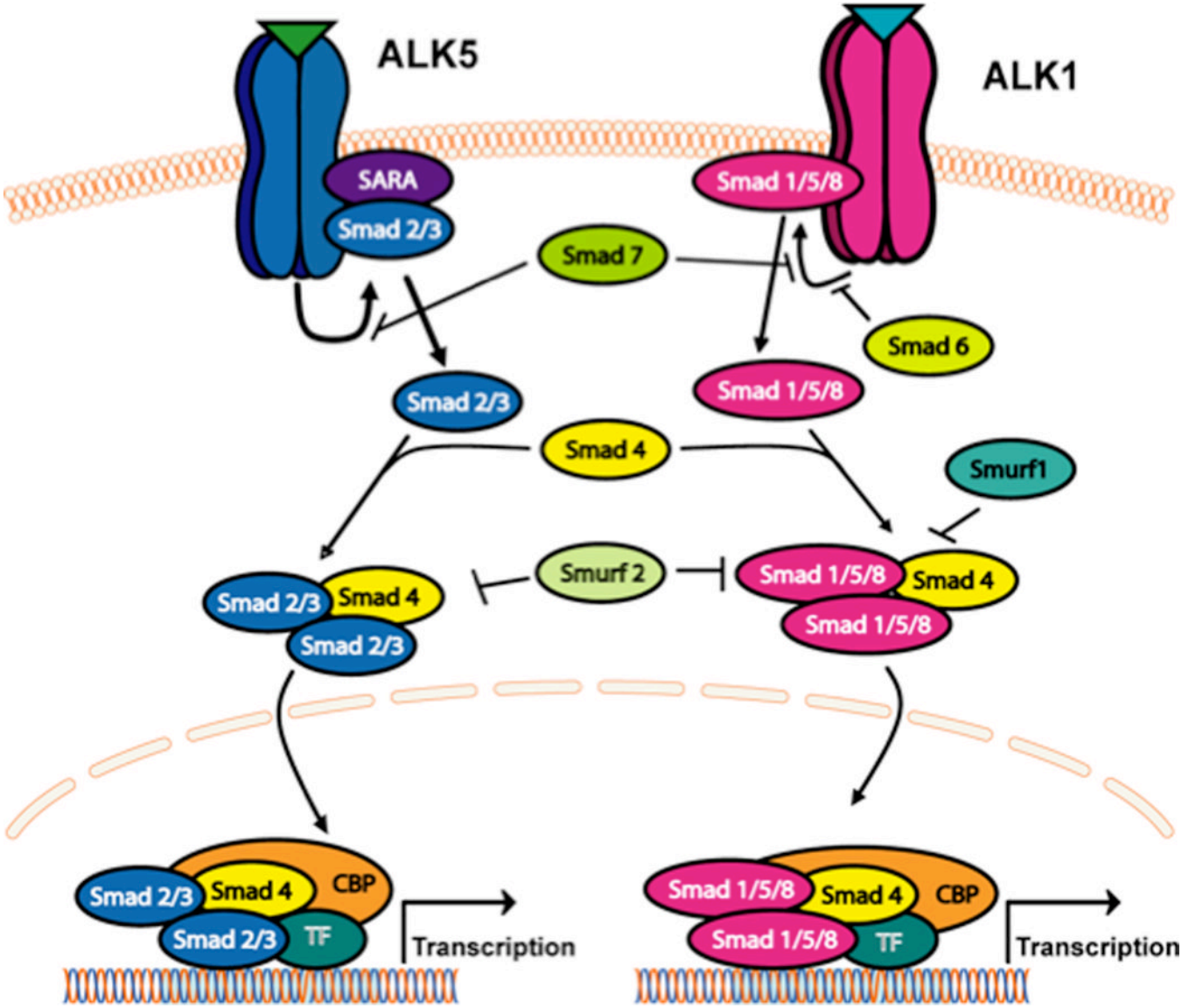

Figure 4.

The transforming growth factor-β (TGF-β) signalling pathway. Intracellular signalling molecules associated with TGF-β signalling at the blood-brain barrier. Signals elicited by the TGF-β pathway involve two cell surface receptors at the brain microvascular endothelium, which are designated activin receptor-like kinase (ALK)-1 and ALK-5. ALK1 transduces signals via phosphorylation of Smad proteins -1, -5, and -8 while ALK5 signals by phosphorylation of Smad2 and Smad3. Once phosphorylated, these Smad proteins bind to the common Smad (i.e., Smad4), thereby forming a protein complex that can translocate to the nucleus and affect transcription.

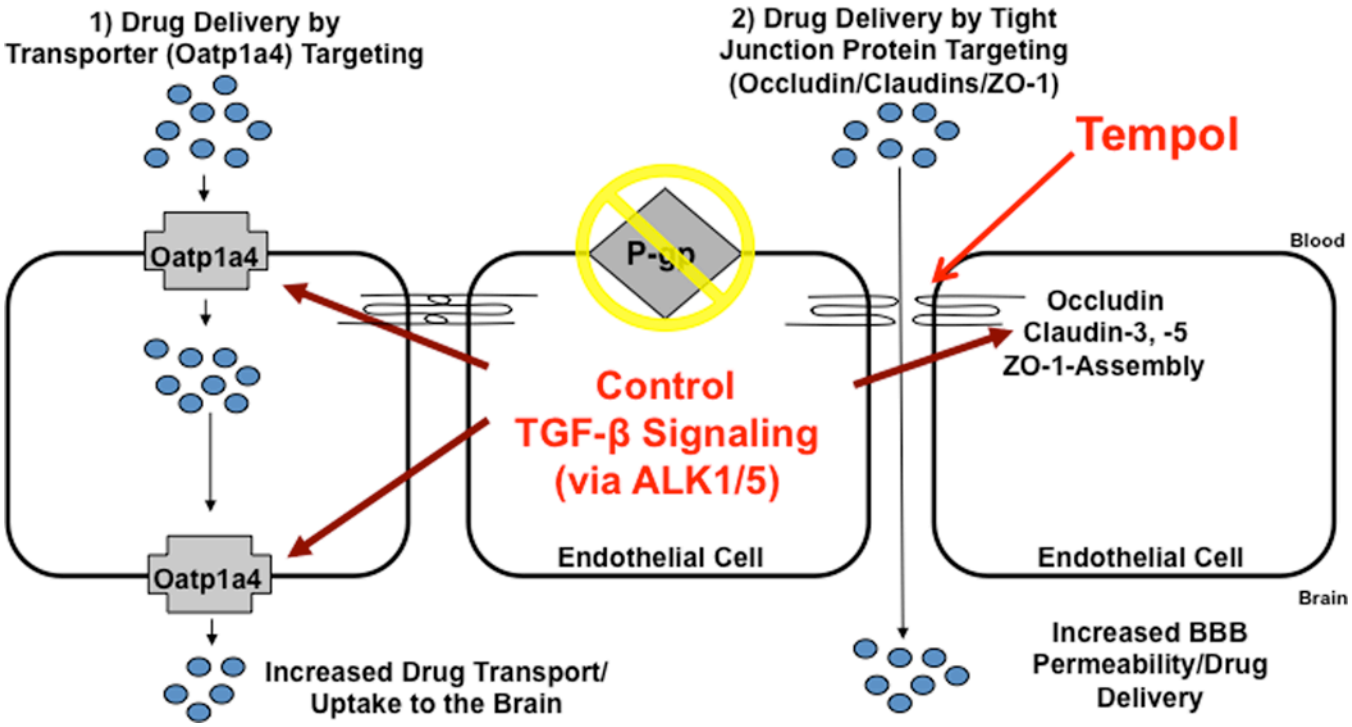

Figure 5. Opportunities for targeting the BBB to optimize CNS drug delivery

Results from our recent studies demonstrate that CNS drug delivery can be modified by targeting specific molecular structures of the BBB during pathophysiological stress such as H/R. TJ functional integrity can be maintained by scavenging ROS with an antioxidant drug such as TEMPOL. Targeting the TJ provides an opportunity to prevent blood-to-brain solute leak during ischemic stroke, thereby enabling control of CNS drug concentrations. In situations where increased uptake of therapeutics into the brain may be desirable, transporters such as Oatp1a4 can be targeted. Oatp1a4 facilitates brain delivery of drugs that may exhibit efficacy in treatment of ischemic stroke such as statins and opioid peptide analgesics. The TGF-β signalling pathway offers an opportunity to control both TJs and transporters by targeting TGF-β receptors (i.e., ALK1, ALK5) with small molecule therapeutics such as SB431542. Although P-gp is also a critical determinant of CNS drug delivery, caution must be exercised when targeting this transporter to enable greater uptake of therapeutic agents into brain parenchyma. This warning arises from evidence obtained from several laboratories including our own that have shown that enhanced brain delivery of drugs can lead to CNS toxicity and unexpected adverse drug reactions.