Glial Support of Blood–Brain Barrier Integrity: Molecular Targets for Novel Therapeutic Strategies in Stroke

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Abbreviations

ABC	ATP-binding cassette
ALK	Activin receptor-like kinase
APC	Activated protein C
AQP	Aquaporin
BBB	Blood–brain barrier
BCRP	Breast cancer resistance protein
CNS	Central nervous system
COX	Cyclooxygenase
EAAT	Excitatory amino-acid transporter
EPCR	Endothelial protein C receptor
GSH	Glutathione
GSSG	Glutathione disulfide
H/R	Hypoxia/reperfusion
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
JAM	Junctional adhesion molecule
Keap1	Kelch-like ECH-associated protein 1
MAGUK	Membrane-associated guanylate kinase
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion
MCP	Monocyte chemoattractant protein

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MDR	Multidrug resistance
MMP	Matrix metalloproteinase
MRP	Multidrug resistance protein
NO	Nitric oxide
NOS	Nitric oxide synthase
Nrf2	Nuclear factor E2-related factor-2
NVU	Neurovascular unit
OATP	Organic anion transporting polypeptide
PAR	Proteinase-activated receptor
P-gp	P-glycoprotein
RANTES	Regulated upon activation normal T-cell expressed and secreted
ROS	Reactive oxygen species
r-tPA	Recombinant tissue plasminogen activator
SGLT	Sodium–glucose cotransporter
SLC	Solute carrier
SOD	Superoxide dismutase
TEMPOL	4-Hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl
TGF	Transforming growth factor
TLR	Toll-like receptor
VEGF	Vascular endothelial growth factor
ZO	Zonula occluden

1 Introduction

The blood–brain barrier (BBB) is an essential barrier system that separates the central nervous system (CNS) from the circulation. It is formed by a monolayer of endothelial cells that limit brain uptake of xenobiotics in an effort to maintain cerebral homeostasis. The BBB is also a significant obstacle to CNS drug delivery. In fact, several currently marketed drugs have limited or no efficacy in the brain due to an inability to cross the BBB and attain therapeutic CNS concentrations [1]. Brain microvascular endothelial cells are not intrinsically capable of forming a "barrier." Formation of the BBB requires coordinated cell–cell interactions and signaling from adjacent glial cells (i.e., astrocytes, microglia), pericytes, neurons, and extracellular matrix [2]. Such an intricate relationship implies existence of a "neurovascular unit (NVU)." During ischemic stroke, various NVU cell types are triggered by pathological stimuli. Understanding endothelial and glial cell responses that are involved in modifying the NVU/BBB in the context of stroke provides an opportunity not only to protect BBB integrity but also to target these mechanisms for effective CNS drug delivery. In this chapter, we summarize BBB physiology including interactions with associated cell types/structures (i.e., glial cells, pericytes, neurons, extracellular matrix) of the NVU. Additionally, we highlight endothelial and glial cell mechanisms that contribute to BBB/NVU dysfunction. Finally, we provide insights on how such mechanisms can be targeted in an effort to protect BBB integrity and/or optimize CNS drug delivery for stroke pharmacotherapy.

2 The Neurovascular Unit

The NVU consists of multiple CNS cell types/structures including brain microvascular endothelial cells, astrocytes, microglia, pericytes, neurons, and extracellular matrix [3]. The concept of the NVU emphasizes that the brain response to ischemic stroke occurs via coordinated interactions between these cell types [4, 5]. Decreased BBB functional integrity occurs prior to neuronal injury, which suggests that NVU dysfunction is directly linked to the BBB disruption observed in the setting of stroke [6, 7].

2.1 Components of the Neurovascular Unit

2.1.1 Endothelial Cells and the Blood–Brain Barrier

CNS function requires precise regulation of the brain extracellular space. Additionally, metabolic demands of brain tissue are considerable and account for approximately 20 % of total oxygen consumption [8]. The interface between CNS and systemic circulation must possess mechanisms that can facilitate nutrient transport, exactly regulate ion balance, and provide a barrier to potential toxins. This emphasizes a critical need for both physical and biochemical mechanisms that contribute to BBB barrier properties.

The current understanding of BBB structure is based upon studies by Reese, Karnovsky, and Brightman in the late 1960s [9, 10]. Anatomically, BBB endothelial cells are demarcated by a lack of fenestrations, minimal pinocytotic activity, and presence of tight junctions [11]. Cerebral endothelial cells have elevated mitochondrial content that is required for transport of solutes into and out of the brain thereby contributing to maintenance of CNS homeostasis [12]. Several receptors, ion channels, and influx/efflux transport proteins are expressed in brain microvascular endothelial cells. Transporters are a critical BBB biochemical mechanism that are prominently involved in permitting brain entry of some substances while excluding accumulation of other substances in brain parenchyma [13].

2.2 Molecular Characteristics of the BBB

2.2.1 Tight Junction Protein Complexes

BBB endothelial cells are interconnected by tight junctions (Fig. 1), which are large multi-protein complexes maintained by direct contact with astrocytes [14]. Physiologically, tight junctions form a continuous, almost impermeable barrier that limits paracellular diffusion of blood-borne substances with the exception of small, lipid soluble molecules [11]. The high BBB transendothelial resistance (1500–2000 $\Omega\ cm^2$) further restricts free flow of water and solutes [15]. BBB tight

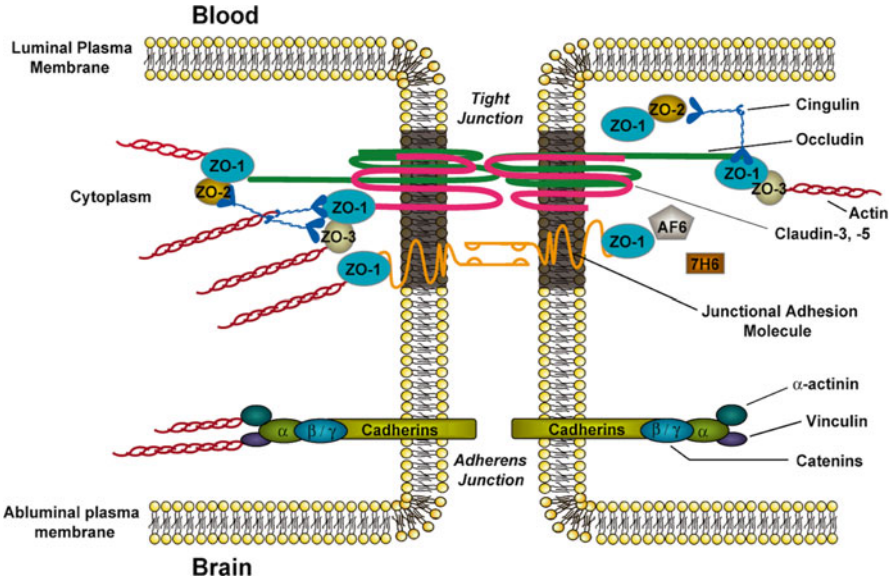

Fig. 1 Basic molecular organization of tight junction protein complexes at the blood–brain barrier. Adapted from Ronaldson & Davis. *Curr Pharm Des.* 18(25): 3624–3644 (2012)

junction formation primarily involves specific transmembrane proteins (i.e., junction adhesion molecules (JAMs), occludin, and claudins (i.e., claudin-1, -3, and -5)) that are linked to cytoskeletal filaments by interactions with accessory proteins (i.e., zonula occluden (ZO)-1, -2, and -3) [16].

Several JAM isoforms have been identified at the mammalian BBB including JAM-1, JAM-2, and JAM-3 [3, 16]. JAMs regulate transendothelial migration of neutrophils and monocytes/macrophages [17, 18]. Loss of JAM protein expression is directly correlated with BBB disruption and injury [19]. Additionally, studies in an immortalized human brain endothelial cell line (hCMEC/d3) showed that inflammatory stimulation led to increased JAM movement away from the tight junction and increased paracellular solute diffusion, which further suggests a central role for JAMs in maintaining BBB integrity [20].

Monomeric occludin is a 60- to 65-kDa protein that is highly expressed along endothelial cell margins in brain vasculature [21, 22]. Our group has shown that occludin is a critical regulator of BBB permeability in vivo [21, 22]. Occludin assembles into dimers and higher-order oligomers, a characteristic that is required for restriction of paracellular permeability. Altered occludin expression is associated with BBB dysfunction in several pathologies including hypoxia/aglycemia [23], hypoxia/reoxygenation stress [22], and focal cerebral ischemia [24].

Claudins are 20- to 24-kDa proteins that contribute to the physiological "seal" of the tight junction [25]. In cerebral microvascular endothelial cells, various claudin isoforms have been detected including claudin-1, -3, and -5 [24, 26–28]. In experimental stroke models [19, 29], altered expression of claudin-5 and an associated

enhancement in paracellular permeability has been reported. Claudin-1 expression was shown to decrease at the in vivo BBB following exposure to human amyloid-β ($A\beta_{40}$) peptide [30]. Aβ deposition at the BBB is characteristic of cerebral amyloid angiopathy, a pathological condition that contributes to microvascular injury including hemorrhages and ischemia.

Proper physiological functioning of the BBB, particularly restriction of paracellular solute transport, requires association of JAMs, occludin, and claudins with cytoplasmic accessory proteins. In brain microvascular endothelial cells, membrane-associated guanylate kinase-like (MAGUK) proteins are involved in clustering of tight junction protein complexes to the cell membrane [31]. Three MAGUK proteins have been identified at the tight junction: ZO-1, -2, and -3. ZO-1 links transmembrane tight junction proteins (i.e., JAMs, occludin, claudins) to the actin cytoskeleton [32]. Previous studies have shown that dissociation of ZO-1 from the junction complex is associated with increased permeability, suggesting that the ZO-1-transmembrane protein interaction is critical to tight junction stability and function [33–35]. ZO-1 has been shown to localize to the endothelial cell nucleus under conditions of proliferation or injury [36], following Ca^{2+} depletion [37], and in response to nicotine [27]. Similarly, ZO-2 binds tight junction constituents, signaling molecules and transcription factors [38]. In fact, ZO-2 may act redundantly with ZO-1 as it has been shown to facilitate formation of morphologically intact tight junctions in cultured cells lacking ZO-1 [39]. ZO-3 is expressed at the BBB but its exact role in the formation of tight junctions and/or maintenance of tight junction integrity has not been elucidated [40]. CNS pathologies including hypoxia, cerebral ischemia/reperfusion injury, and focal cerebral ischemia are associated with reduced ZO-1 expression at the BBB [41–43].

2.2.2 Adherens Junctions

Adherens junctions are specialized cell–cell interactions, which are formed by cadherins and associated proteins that are directly linked to actin filaments [44]. Cadherins regulate endothelial function by activation of phosphoinositide 3-kinase signaling, an intracellular pathway that organizes the cytoskeleton and enables complex formation with vascular endothelial growth factor receptor 2. Therefore, cadherin-mediated signaling is essential for endothelial cell layer integrity and for the spatial organization of new microvessels [45]. Barrier-forming endothelium has been shown to express higher levels of cadherin-10 relative to VE-cadherin [46]. In contrast, circumventricular organs and choroid plexus capillaries (i.e., brain microvasculature that is devoid of BBB properties) primarily express VE-cadherin [46]. Optimal cadherin function requires direct association with catenins. At least four catenin isoforms (i.e., β, α, χ, and p120) are expressed at the BBB, with β-catenin linking cadherin to α-catenin, which binds this protein complex to the actin cytoskeleton [47]. In vitro studies by Steiner and colleagues demonstrated that the heparin sulfate proteoglycan agrin also contributes to barrier properties of adherens junction by promoting localization of VE-cadherin and β-catenin to endothelial cell

junctions [48]. Paracellular expression of VE-cadherin and/or β-catenin is associated with BBB repair following focal astrocyte loss in vivo [49]. VE-cadherin expression was also decreased at the BBB in mice subjected to focal cerebral ischemia, suggesting that adherens junction disruption contributes to BBB dysfunction in the context of stroke [50].

2.3 Transporters

For many endogenous and exogenous substances, proteins expressed at the BBB determine their ability to traverse biological membranes. Such transport proteins include ATP-binding cassette (ABC) transporters and solute carrier (SLC) transporters [13]. In order to target transporters for optimization of pharmacotherapy, it is critical to understand localization (i.e., luminal versus abluminal), expression, and activity of endogenous transporters at the BBB endothelium (Fig. 2).

ABC Transporters. ABC transporters require biological energy via ATP hydrolysis to transport drugs and their metabolites against their concentration gradient. ABC drug transporters, specifically P-glycoprotein (P-gp), Multidrug Resistance Proteins (MRPs in humans; Mrps in rodents) and Breast Cancer Resistance Protein (BCRP in

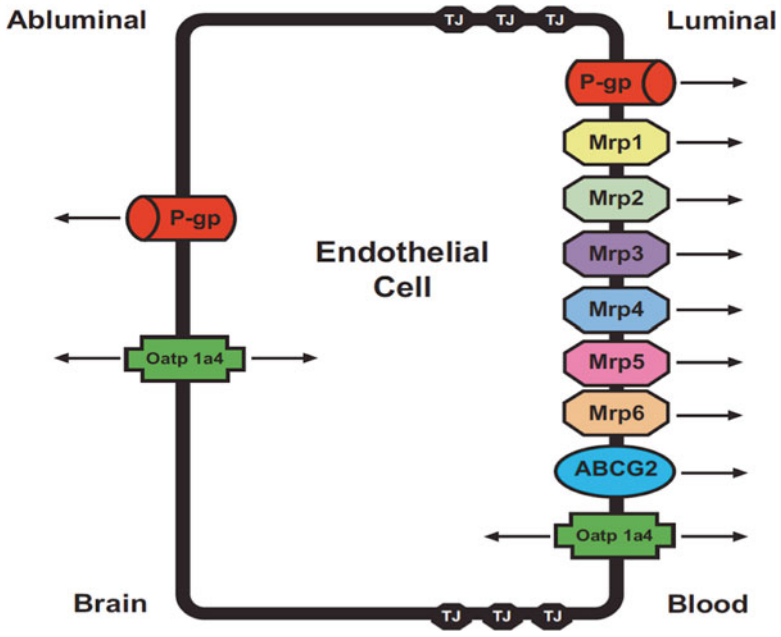

Fig. 2 Endothelial localization of drug transporters known to be involved in transport of therapeutic agents at the blood–brain barrier. Adapted from Ronaldson & Davis. *Curr Pharm Des.* **18**(25): 3624–3644 (2012)

humans; Bcrp in rodents) are involved in cellular extrusion of drugs and greatly contribute to limiting effective therapeutic delivery 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 [13, 51]. 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 [52].

P-gp is a 170-kDa ATP-dependent transporter that primarily functions as a defense mechanism against potentially toxic xenobiotics [53]. P-gp orthologues from different species have greater than 70 % sequence identity [53] 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. In contrast, human MDR1, murine mdr1/mdr3, and rodent mdr1a/mdr1b are involved in drug transport at the BBB endothelium. P-gp has been localized to both the luminal and abluminal membrane of brain microvascular endothelial cells [54]. Abluminal localization of P-gp has also been identified on perivascular astrocyte foot processes [54–56]. Increased expression and/or activity of P-gp has been reported in hippocampal microvessels isolated from stroke-prone spontaneously hypertensive rats [57], which suggests that alterations in P-gp expression and/or activity may be a component of the BBB response to pathological stressors.

The mammalian MRP family belongs to the ABCC group of proteins [58]. Many of the functionally characterized MRP isoforms (i.e., MRP1/Mrp1, MRP2/Mrp2, MRP4/Mrp4, Mrp5, Mrp6) that have the potential to determine CNS drug delivery have been localized to the mammalian BBB [13, 16]. Additionally, the ability of Mrp isoforms to actively efflux the endogenous antioxidant glutathione (GSH) may have significant implications in stroke. GSH is responsible for maintenance of cellular redox balance and antioxidant defense in the brain. It has been demonstrated that functional expression of Mrps is upregulated in response to oxidative stress, a primary component of stroke pathophysiology, which leads to increased cellular efflux of GSH [59]. Enhanced functional expression of Mrp isoforms at the BBB can cause reduced brain and/or endothelial cell concentrations of GSH, an alteration in cellular redox status, and increased potential for cell injury and death.

Several recent studies have demonstrated localization of BCRP at the brain microvasculature, particularly along the luminal side of the BBB [60, 61]. 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 [61, 62] or plays a minimal role in xenobiotic efflux from the brain [63], more detailed analyses have confirmed that BCRP is a critical determinant of drug permeation across the BBB [64, 65]. More recently, oxygen/glucose deprivation was shown to increase the endothelial expression of Bcrp at the mRNA level in an in vitro BBB model [66]. In vivo, Bcrp mRNA expression was observed to be upregulated in the peri-infarct region following reversible middle cerebral artery occlusion (MCAO) [67]. The functional implications of increased Bcrp molecular expression at the BBB/NVU have yet to be determined in the context of stroke.

Solute Carrier (SLC) Transporters: In contrast to ABC transporters, membrane transport of circulating solutes by 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. Perhaps the most viable SLC candidates for transporter targeting are members of the SLC21A/SLCO family, which includes organic anion transporting polypeptides (OATPs in humans; Oatps in rodents). OATPs/Oatps have distinct substrate preferences for amphipathic solutes [68]. OATPs/Oatps are well known to transport 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (i.e., statins), which have been shown to exhibit neuroprotective and antioxidant properties [69, 70]. 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 [71]. Although OATP isoforms are expressed in several tissues, not all exist at the BBB. Immunofluorescence staining of human brain frontal cortex demonstrated OATP1A2 localization at the level of the brain microvascular endothelium [72]. Expression of Oatp1a4, Oatp1c1, and Oatp2a1 has been reported at the rodent BBB [73–78]. Oatp1c1 is selectively expressed at the BBB [74], has relatively narrow substrate specificity, and primarily transports thyroxine and conjugated sterols [75, 76]. Oatp2a1 regulates BBB transport of prostaglandins [73]. It has been proposed that Oatp1a4, a rodent homologue of OATP1A2, is the primary drug transporting Oatp isoform expressed at the rat BBB [79, 80]. Recently, our laboratory demonstrated that Oatp1a4 is a BBB transporter that can be effectively targeted for facilitation of effective CNS drug delivery [77, 78].

2.4 Astrocytes

Astrocytes are the most abundant cell type in the brain and cover over 99 % of cerebral capillaries with their end-feet [3, 81]. Astrocytes are critical in development and/or maintenance of BBB characteristics [14, 82, 83]. Astrocytes may be involved in transient regulation of cerebral microvascular permeability [84], in particular via dynamic Ca^{2+} signaling between astrocytes and the endothelium via gap junctions and purinergic transmission [85, 86]. Additionally, astrocytes play an essential role in regulating water and ion exchange across the brain microvascular endothelium [87]. 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)) that remove excess glutamate from the synapse [88]. Elevated brain levels of glutamate may lead to a pathological condition known as excitotoxicity, which has been implicated in neuronal damage in ischemic stroke [89]. Additionally, astrocytes are known to express volume-regulated anion channels. 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 [90]. Astrocytes are also known to express transport

proteins including P-gp [91], MRP/Mrp isoforms [13, 59], and Bcrp [92]. 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 [51].

2.5 Microglia

Microglia are derived from the monocyte lineage that represents approximately 20 % of the total glial cell population within the CNS [93]. 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 multiple radial cell processes extending from the cell body. Ramified microglia are thought to contribute to CNS homeostasis by participating in extracellular fluid cleansing and neurotransmitter deactivation [51]. 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 [94]. Activated microglia are identified by their larger cell body and short cytoplasmic processes as well as upregulation of cell surface receptors such as CD14 and Toll-like receptors (TLRs) [95, 96]. During an immune response, activated microglia may be further converted into a reactive state, which is characterized by a spheroid or rod-like morphology and the presence of phagocytotic activity. Microglia activation and proliferation has been implicated in the development of neuronal death in ischemic stroke and cerebral hypoxia [97–99]. Furthermore, activation of microglia is associated with dysfunction of the BBB characterized by changes in TJ protein expression and enhanced paracellular permeability [100]. 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 [94]. 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 [101]. 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 [102]. Glutamate receptors [103] and nutrient carrier systems such as GLUT-1 [104] 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 [91], Mrp1 [105], and Mrp4/Mrp5 [106].

2.6 Pericytes

In addition to glia, pericytes also play a crucial role in the maintenance of BBB homeostasis [107]. Pericytes are flat, undifferentiated, contractile cells that attach at irregular intervals along the capillary walls and communicate with the other cell types of the NVU [108]. 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 [109]. Involvement of pericytes in induction of BBB properties is also exemplified by the observation that proper localization of endothelial proteins (i.e., P-gp, utrophin) requires co-culture with pericytes [110]. 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 [111]. 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 and to CNS drug delivery [112].

2.7 Neurons

Noradrenergic [113], serotonergic [114], cholinergic [115], and GABAergic [116] neurons have been shown to make distinct connections with other cell types of the NVU. 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. Interestingly, disruption of BBB integrity induced by pathophysiological factors (i.e., inflammation, hypertension, ischemia) often accompanies changes in cerebral blood flow and perfusion pressure [117–119] 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.

2.8 Extracellular Matrix

In addition to cellular components of the NVU, 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 [120, 121]. The extracellular matrix serves as an anchor for the endothelium via interaction of laminin and other matrix proteins with endothelial integrin receptors [122]. Matrix proteins can also influence expression of TJ proteins [123, 124]. Proteolysis of extracellular matrix proteins is well known to occur

in response to stroke, an effect that greatly contributes to BBB disruption [125]. Additionally, protein fragments generated from extracellular matrix proteolysis (i.e., perlecan domain V) may have beneficial effects in neuroprotection and post-stroke brain repair [125, 126].

3 Ischemic Stroke

3.1 Overview of Ischemic Stroke

Stroke is a leading cause of death and long-term disability in the United States. Every year, more than 795,000 Americans suffer from either a new or recurrent stroke, which averages one incidence of stroke every 40 s [127]. Of all strokes, 87 % are ischemic [127]. The annual cost of stroke rehabilitation in the United States is an estimated $34 billion [127]. 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 [127].

Stroke is characterized by a heterogeneous spectrum of conditions caused by interruption of blood flow supplying the brain [128]. Such a deficit in cerebral blood flow causes an irreversibly damaged ischemic core and salvageable surrounding neural tissue known as the penumbra [129]. CNS energy requirements are met by brain uptake of oxygen and glucose, which are metabolized to enable phosphorylation of ADP to ATP. When blood flow to the brain is interrupted during stroke, the ischemic core is quickly 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), an event that causes neuronal cell death and development of an infarction [130]. 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 [4]. 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 [131]. 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 [131].

Cell death processes in the ischemic core occur extremely rapidly (i.e., within minutes) thereby rendering this region difficult to protect using pharmacological approaches [4]. In contrast, cells within the ischemic penumbra die more slowly by active cell death mechanisms [4]. The primary goal of drug therapy for acute ischemic stroke is to salvage the penumbra as much as possible and as early as possible [129]. Currently, there is only one therapeutic agent approved by the FDA for acute ischemic stroke treatment, recombinant tissue plasminogen activator (r-tPA) [132]. The primary goal of r-tPA therapy is to restore the 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) [132]. Such hypoxia/reoxygenation (H/R) stress/injury is associated with neuronal apoptosis characterized by cytochrome *c* release, caspase-3 activation, and internucleosomal DNA fragmentation [80]. Pathophysiological mechanisms that can cause neuronal apoptosis during H/R include increased production of reactive oxygen species (ROS) [16, 80]. 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, upregulation of the cellular stress marker heat shock protein-70, and increased nuclear expression of hypoxia-sensitive transcription factors such as hypoxia-inducible factor-1 and nuclear factor-κB [80]. The H/R component of stroke is also associated with decreased brain concentrations of GSH [133], 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 drugs that can be administered alone or in conjunction with r-tPA for "rescue" of neural tissue.

3.2 The Neurovascular Unit in Ischemic Stroke

3.2.1 Disruption of the Blood–Brain Barrier

BBB homeostasis is dependent on discrete interactions between NVU components [134, 135]. Perturbation of extracellular matrix (i.e., type IV collagen, heparan sulfate proteoglycan, laminin, fibronectin, perlecan) disrupts cell-matrix and cell–cell signaling mechanisms critical to NVU function [121, 125]. Proteinases such as matrix metalloproteinases (MMPs) contribute to breakdown of the extracellular matrix and BBB disruption in stroke [136]. This includes MMPs that are activated by HIF-1α-dependent mechanisms (i.e., MMP2) and MMPs whose activation is triggered by pro-inflammatory cytokines (i.e., TNF-α, IL-1β) such as MMP3 and MMP9 [135]. Involvement of MMPs in BBB disruption following ischemic stroke has been reported in experimental stroke models [137, 138]. Furthermore, a recent clinical study demonstrated that MMP9 levels were elevated in stroke patients [139]. MMPs directly compromise the BBB by degrading tight junction constituent proteins such as claudin-5 and occludin [135]. MMP-mediated opening of the BBB in ischemic stroke may be regulated by nitric oxide (NO) signaling [140]. Overall, damage and subsequent opening of the BBB is a key event in development of intracerebral hemorrhage and brain edema following ischemic stroke.

Experimental models of stroke have provided considerable information on solute leak across the BBB. Using the transient MCAO rodent model, Pfefferkorn and Rosenberg demonstrated increased leak of sucrose, a vascular marker that does not typically cross the BBB, in the ischemic hemisphere [141]. 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 [41]. 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 [142]. Jiao and colleagues observed redistribution of various tight junction proteins following 2 h of focal cerebral ischemia, an event that correlated with increased blood-to-brain flux of Evan's blue-albumin [41]. Reorganization of tight junction proteins following focal cerebral ischemia is also mediated by vascular endothelial growth factor (VEGF) [143] and NO [144].

BBB dysfunction and subsequent leak across the microvascular endothelium following focal ischemia enables considerable movement of vascular fluid across the microvascular endothelium and development of vasogenic edema [145]. Recent studies using the MCAO model have shown that water movement across the BBB is exacerbated by enhanced blood-to-brain movement of sodium. Alterations in sodium gradients across the microvascular endothelium dramatically alter oncotic pressure and are facilitated by increased functional expression of Na–K–Cl cotransporter [146], as well as Na–H exchangers NHE1 and/or NHE2 [147]. MCAO studies in spontaneously hypertensive rats demonstrated that the NHE1 transporter is a critical regulator of ischemic-induced infarct volume [148]. Disruption of sodium gradients across the BBB during ischemic stroke can also involve upregulation of sodium-dependent glucose transporters such as sodium–glucose cotransporters (SGLTs). 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 [149].

Functional BBB integrity is disrupted by production of ROS and subsequent oxidative stress (Fig. 3). Production of superoxide anion, a potent ROS generated when molecular oxygen is reduced by only one electron, is a known mediator of cellular damage following stroke [150, 151]. Superoxide dismutase (SOD) enzymes tightly control biological activity of superoxide anion, a by-product of normal physiological processes. Under oxidative stress conditions, superoxide is produced at high levels that overwhelm the metabolic capacity of SOD. This phenomenon is supported by the observation that infarct size and cerebral edema were markedly reduced in mice engineered to overexpress SOD as compared to wild-type controls [150]. Increased levels of superoxide also contribute to BBB endothelial dysfunction [152, 153]. BBB damage can be intensified by conjugation of superoxide and NO to form peroxynitrite, a cytotoxic and pro-inflammatory molecule. Peroxynitrite causes injury to cerebral microvessels through lipid peroxidation, consumption of endogenous antioxidants (i.e., reduced GSH), and induction of mitochondrial failure [80, 154]. Peroxynitrite is known to induce endothelial damage by its ability to nitrosylate tyrosine, leading to functional modifications of critical proteins [155]. Peroxynitrite formation at the BBB becomes more likely with activation of endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS) because NO rapidly diffuses through membranes and reacts with superoxide anion [154].

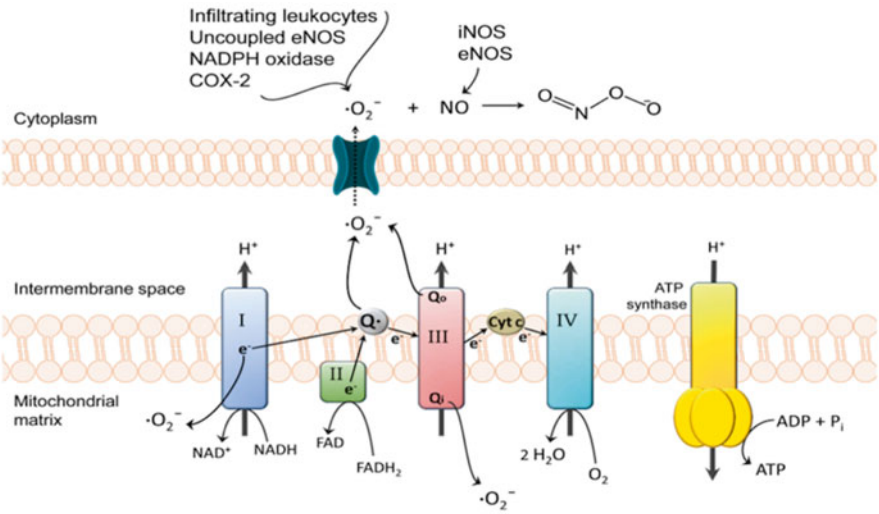

Fig. 3 Generation of reactive oxygen species (ROS) in brain microvascular endothelial cells. During disease, mitochondrial superoxide levels increase via NO inhibition of cytochrome complexes and oxidation of reducing equivalents in the electron transport chain. Complex I as well as both sides of complex III (i.e., Qi and Qo sites) are the most common sources of mitochondrial superoxide. Superoxide generated within the intermembrane space of mitochondria can reach the cytosol through voltage-dependent mitochondrial anion channels. Superoxide levels further increase via cyclooxygenase-2, NADPH oxidase, uncoupled eNOS, and infiltrating leukocytes. The resulting high levels of superoxide coupled with the activation of NO-producing eNOS and iNOS increases the probability of peroxynitrite formation. Peroxynitrite-induced cellular damage includes protein oxidation, tyrosine nitration, DNA damage, poly(ADP-ribose) polymerase activation, lipid peroxidation, and mitochondrial dysfunction. Adapted from Thompson and Ronaldson (2014). *Adv Pharmacol.* **71**: 165–209

Inflammatory stimuli are critical mediators of BBB dysfunction in the setting of stroke. Previous research has demonstrated that inflammatory mechanisms in focal cerebral ischemia are mediated through pro-inflammatory cytokines TNF-α and IL-1β, which appear within 2–6 h following ischemic insult [156]. Pro-inflammatory signaling induces adhesion molecules and subsequent transmigration of activated neutrophils, lymphocytes, or monocytes into brain parenchyma [157]. Physiologically, expression of vascular adhesion molecules such as ICAM-1 and VCAM-1 are minimally detectable at the BBB; however, their expression is dramatically increased in response to diseases such as stroke [157, 158]. Pro-inflammatory mediators also alter functional expression of endogenous BBB transporters and tight junction proteins. For example, TNF-α increased expression of P-gp but decreased BCRP expression in hCMEC/d3 cells [159]. Production and secretion of TNF-α and IL-1β has been observed to alter the expression of occludin and ZO-1 [160], suggesting involvement of inflammation in exacerbating paracellular leak during stroke.

3.3 Ischemic Stroke and Glial Support of the BBB

It is well established 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. In the inferior colliculus, focal astrocyte loss demarcated by reduced GFAP immunoreactivity directly corresponded with decreased paracellular localization of critical tight junction proteins claudin-5, occludin, and ZO-1 [83]. At the same time points that tight junction proteins were downregulated, an increase in leak of dextran (10 kDa) and fibrinogen was observed [83], which suggests a significant disruption of the BBB due to astrocyte cell death. The results of this study and others [161, 162] illustrate the requirement of intercellular 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 [163]. Astrocytes secrete VEGF and fibroblast growth factor-2 (FGF-2), which promote angiogenesis and regulate biological transport at the BBB [164].

Astrocytes are essential contributors to the brain immunological response during ischemic stroke. Astrocytes maintain focal contacts with neighboring microglia and maintain these cells in a dormant, ramified state [94]. Regulation of microglia by astrocytes is prevented by inflammatory signaling, thus enabling microglia to elicit an immune response [94]. Astrocytes can 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 [165]. This enhancement is also facilitated by astrocytic secretion of chemokines such as macrophage inflammatory protein-1α/β (MIP-1 α/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 [166, 167] and to increased brain parenchymal concentrations of tPA [168]. 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 [166]. 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 [169] as well as transforming growth factor-β (TGF-β) [170]. Cytokines such as TNF-α, IL-1α, IL-1β, and interferon-γ can trigger the endothelium and activate processes involved in BBB disruption. Although secretion of TGF-β1 may play a neuroprotective role in brain parenchyma [170], 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 capillaries that lack pericytes, contain fewer endothelial cells and are shorter in length [84]. Indeed, pharmacological

targeting of TGF-β signaling at the level of the brain microvascular endothelium may be an efficacious approach for the protection of BBB integrity and/or preservation of angiogenic responses to 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 pro-inflammatory cytokines can induce deleterious processes in astrocytes themselves via upregulation of iNOS [171]. 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 [172, 173]. Reactive astrocytosis is associated with disruption of tight junctions between adjacent brain microvessel endothelial cells and increased BBB permeability [174].

Although astrocyte injury is a critical determinant of BBB dysfunction in the setting of ischemic stroke, endothelial damage can also be induced via immune stimulated microglia [175]. 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 [175]. Activated microglia produce pro-inflammatory cytokines in response to cerebral ischemia [176], all of which can trigger BBB disruption. In the setting of ischemic stroke, cytokine production in microglia is mediated by NF-kB signaling [177]. 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 [135]. In neuroinflammation, COX2 activates sphingomyelinases leading to release of ceramides, an event that leads to activation of p38 mitogen-activated protein kinase (MAPK) and subsequent secretion of pro-inflammatory cytokines [178–180]. Taken together, these observations point to a critical role for microglia in the inflammatory response to ischemic stroke.

3.4 Targeting the Neurovascular Unit in Ischemic Stroke

3.4.1 Targeting the Tight Junction

The BBB and associated glial support network of the NVU are clearly 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 [21, 22, 26, 78, 148, 181]. Changes in BBB integrity under H/R conditions were demarcated by enhanced brain accumulation of ^{14}C-sucrose [22, 26, 181], 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 4–10 kDa) in hippocampal and cortical microvessels [182]. These alterations in BBB permeability in animals subjected to H/R stress were directly associated with an increase in the

expression of HIF-1α and NF-kB in nuclear fractions isolated from intact microvessels [183]. 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 [26]. Changes in BBB permeability to ^{14}C-sucrose and dextrans were directly correlated with modified organization and/or expression of constituent TJ proteins including occludin, claudin-5, and ZO-1 [22, 26, 182]. 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 [21]. These changes in tight junction organization 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 [181].

Production of ROS and subsequent oxidative stress has been shown to alter the BBB expression of claudin-5 and occludin leading to increased paracellular solute leak [184]. 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, nitrogen dioxide, and the carbonate radical. TEMPOL readily crosses the BBB and has been previously shown to provide neuroprotection as a free radical scavenger in several models of brain injury and ischemia [185, 186]. 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 [22]. This reduction in ^{14}C-sucrose leak was associated with a preservation of occludin localization and occludin oligomerization at the TJ [22]. 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 (Fig. 4). 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 [22]. Taken together, these observations provide evidence that the tight junction 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 (i.e., vascular protection).

3.5 Targeting Endogenous BBB Transporters

The ability of a drug to elicit a pharmacological effect at the level of the BBB requires achievement of efficacious concentrations within CNS. This therapeutic objective is dependent upon multiple mechanisms of transport that may include uptake into the brain by an influx transporter and/or extrusion by an efflux

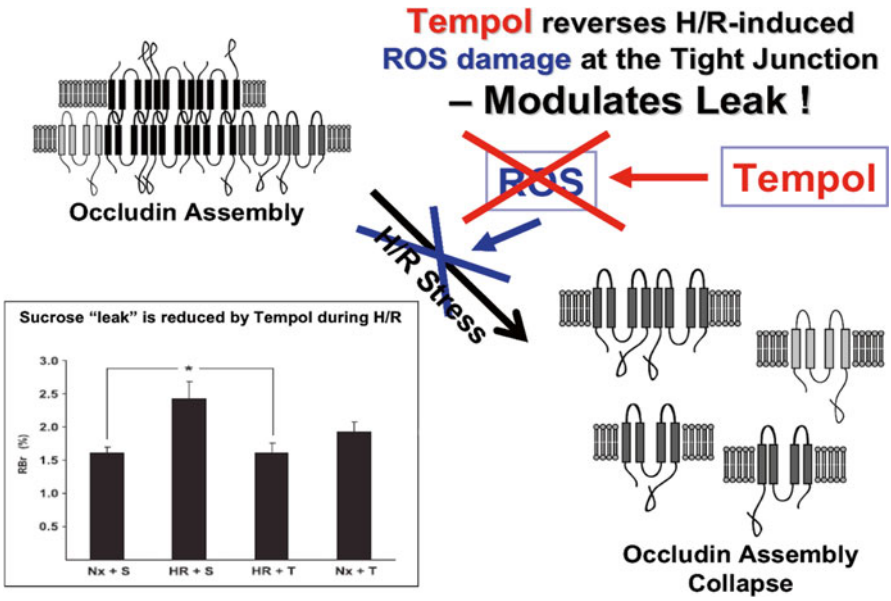

Fig. 4 Effect of TEMPOL on H/R-mediated disruption of the tight junction. ROS and subsequent oxidative stress are known to disrupt assembly of critical TJ proteins such as occludin. Our results show that administration of TEMPOL, by scavenging ROS, prevents disruption of occludin oligomeric assemblies. 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. Adapted from Ronaldson & Davis. *Curr Pharm Des.* **18(25)**: 3624–3644 (2012)

transporter. For many drugs, it is this balance between influx and efflux that determines if a drug will elicit a therapeutic effect in the brain or at the BBB. The complexity of drug transporter biology is further underscored by the observation that functional expression of transporters can be dramatically altered by oxidative stress [59, 187, 188]. A thorough understanding of regulation and functional expression of endogenous BBB transporters in both health and disease is essential for effective pharmacotherapy. Furthermore, such information will enable effective targeting of transporters and/or transporter regulatory mechanisms, thus allowing endogenous BBB transport systems to be exploited for purposes of improving CNS drug delivery and/or conferring BBB protection.

Considerable research has focused on studying mechanisms that limit endothelial membrane transport by describing the role of P-gp in restricting drug uptake from the systemic circulation [1, 189–191]; however, clinical trials targeting P-gp with small molecule inhibitors have been unsuccessful in improving pharmacotherapy due to inhibitor toxicity and/or enhanced tissue penetration of drugs [192, 193]. An alternative approach for optimizing delivery of drugs is to focus on BBB transporters that are involved in blood-to-brain transport. One intriguing candidate is Oatp1a4 (Fig. 5), which is known to transport HMG-CoA reductase inhibitors

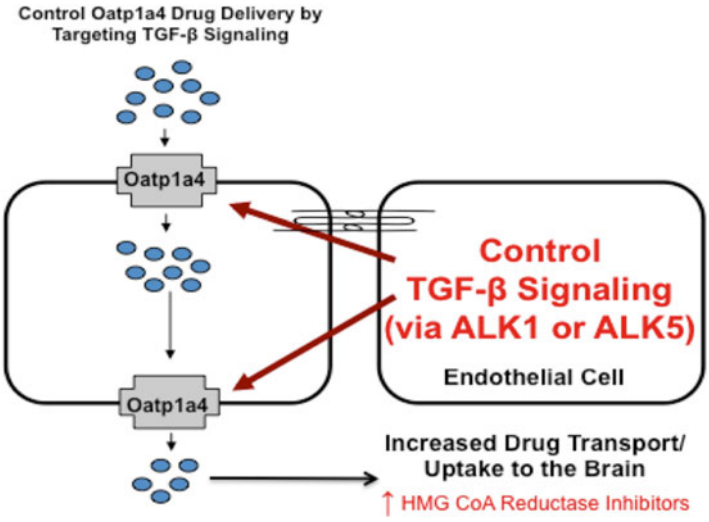

Fig. 5 Targeting Oatp transporters at the BBB for optimization of CNS drug delivery. Results from our studies demonstrate that targeting Oatp transporters during pathophysiological stress can modify CNS drug delivery. Oatp1a4 facilitates brain delivery of drugs that may exhibit efficacy in treatment of peripheral inflammatory pain or cerebral hypoxia such as statins and opioid peptide analgesics. The TGF-β signaling pathway enables control of Oatp isoforms by targeting TGF-β receptors (i.e., ALK1, ALK5) with small molecule therapeutics

(i.e., statins). Recent evidence suggests that statins can act as ROS scavengers independent of their well-documented effects on cholesterol biosynthesis [70]. Specifically, studies in dogs demonstrated that atorvastatin reduced the expression of oxidative and nitrosative stress markers (i.e., protein carbonyls, 4-hydroxy-2-noneal, 3-nitrotyrosine) and increased brain GSH levels [70, 194]. Interestingly, Cui and colleagues showed, in vivo, that atorvastatin administration during the acute phase of cerebral ischemia prevented increases in BBB permeability [195]. More recently, simvastatin was demonstrated to preserve barrier function following experimental intracerebral hemorrhage in an in vivo study involving MRI measurements of T_{1sat}, a marker of BBB integrity [196]. Taken together, these studies suggest that targeted delivery of statins may be an effective strategy for neuroprotection and/or BBB protection in the setting of stroke. We have shown, in vivo, that Oatp1a4 is a BBB transporter target that can be exploited to optimize CNS delivery of drugs, including statins [77, 78].

Although pathophysiological stressors can modulate BBB transporters, such changes must be controlled to provide optimal delivery of drugs. For example, we have demonstrated increased functional expression of Oatp1a4 only after 1 h hypoxia followed by up to 1 h reoxygenation [78]. If Oatp1a4 is to facilitate effective delivery of drugs (i.e., statins), its functional expression must be reliably controlled over a more desirable time course than is possible by relying solely on disease mechanisms. This objective can be accomplished by pharmacological targeting of Oatp regulatory pathways such as the TGF-β system [77, 78, 80]. TGF-βs

are cytokines that signal by binding to a heterotetrameric complex of type I and type II receptors [197]. The type I receptors, also known as activin receptor-like kinases (ALKs) propagate intracellular signals through phosphorylation of receptor-specific Smad proteins (i.e., (R)-Smads). At the BBB, only two ALK receptors (ALK1, ALK5) have been identified [28]. We have shown that pharmacological inhibition of TGF-β/ALK5 signaling can increase Oatp1a4 functional expression [77, 78]. This observation suggests that targeting of the TGF-β/ALK5 pathway may enable control of BBB Oatp1a4 expression and/or activity, thereby providing novel strategies for improved CNS drug delivery and/or BBB protection in stroke.

Optimization of drug delivery is not the only benefit that can be achieved from targeting transporters. BBB transporters mediate the flux of endogenous substrates, many of which are essential to the cellular response to pathological insult. One such substance is the endogenous antioxidant GSH. During oxidative stress, GSH is rapidly oxidized to glutathione disulfide (GSSG). Therefore, the redox state of a cell is represented by the ratio of GSH to GSSG [198]. In vitro studies using human and rodent brain microvascular endothelial cells have demonstrated that hypoxia reduces intracellular GSH levels and decreases the GSH:GSSG ratio, suggesting significant oxidative stress at the level of the BBB [199–201]. Using an in vivo model, oxidative stress was shown to cause BBB disruption characterized by altered expression/assembly of tight junction proteins occludin, claudin-5, and ZO-1 [21–23, 26, 35, 182]. These tight junction modifications correlated with increased BBB permeability to sucrose, an established vascular marker [22, 181], and dextrans [182]. Such increases in BBB permeability can result in leak of neurotoxic substances from blood into brain and/or contribute to vasogenic edema. BBB protection and/or repair in stroke are paramount to protecting the brain from neurological damage. One approach that can accomplish this therapeutic objective is to prevent cellular loss of GSH from endothelial cells by targeting endogenous BBB transporters (Fig. 6). BBB transporters that can transport GSH and GSSG include MRPs/Mrps. Both GSH and GSSG are substrates for MRP1/Mrp1 [59, 202, 203], MRP2/Mrp2 [204], and MRP4/Mrp4 [205]. It is well known that increased cellular concentrations of GSH are cytoprotective while processes that promote GSH loss from cells are damaging [206]. Therefore, it stands to reason that pharmacological targeting of Mrps during oxidative stress may have profound therapeutic benefits including vascular protection at the level of the BBB. Using the known Mrp transport inhibitor MK571, Tadepalle and colleagues showed that inhibition of Mrp1-mediated GSH transport resulted prevented GSH depletion in primary cultures of rat astrocytes [203]. Indeed, the effect of Mrp transport inhibition at the BBB and its effect on endothelial redox status and barrier integrity require further study.

Previous studies have shown that Mrp expression and/or activity can change in response to oxidative stress [59, 207]. Altered BBB expression of Mrps may prevent endothelial cells from retaining effective GSH concentrations. A thorough understanding of signaling pathways involved in Mrp regulation during oxidative stress will enable development of pharmacological approaches to target Mrp-mediated efflux (i.e., GSH transport) for the purpose of preventing BBB dysfunction in diseases with an oxidative stress component. One intriguing pathway is signaling mediated by nuclear factor E2-related factor-2 (Nrf2), a sensor of oxidative stress [188, 208]. In the presence of ROS, the cytosolic Nrf2 repressor Kelch-like ECH-associated

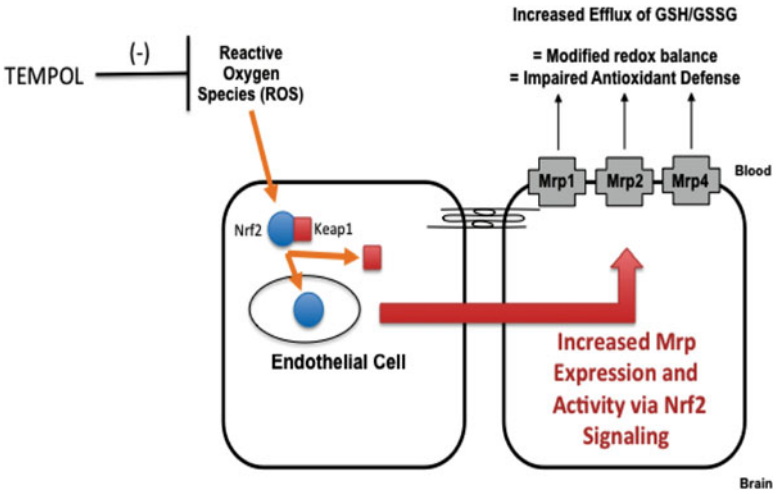

Fig. 6 Prevention of BBB Dysfunction by Targeting Mrp Isoforms in Cerebral Endothelial Cells. Results from our laboratory demonstrate increased expression of Mrp1, Mrp2, and Mrp4 at the BBB following an H/R insult. Furthermore, H/R stress is known to suppress GSH levels and increase GSSG concentrations in the brain. We propose that changes in GSH/GSSG transport occur during H/R as a result of altered functional expression of at least one Mrp isoform. Since Nrf2, a ROS sensitive transcription factor, is known to regulate Mrps, we hypothesize that this pathway is a critical regulatory mechanism for Mrps at the BBB. TEMPOL, a ROS scavenging antioxidant, is a pharmacological tool that can be utilized in order to understand how targeting activation of the Nrf2 pathway can control Mrp expression/activity

protein 1 (Keap1) undergoes structural alterations that cause dissociation from the Nrf2-Keap1 complex. This enables Nrf2 to translocate to the nucleus and induce transcription of genes that possess an antioxidant response element at their promoter [209, 210]. It has been demonstrated that activation of Nrf2 signaling induces expression of Mrp1, Mrp2, and Mrp4 [188, 207, 209, 211]. An emerging concept is that Nrf2 acts as a double-edged sword [210]: on one hand, Nrf2 is required for protecting tissues from oxidative stress; on the other, its activation can lead to deleterious effects. Therefore, an alteration in the balance of Mrp isoforms via activation of Nrf2 signaling may adversely affect redox balance and antioxidant defense at the brain microvascular endothelium. Indeed, this points towards a need for rigorous study of pharmacological approaches (i.e., use of antioxidant drugs such as TEMPOL) that can modulate Nrf2 signaling and control expression of Mrp isoforms and/or GSH transport at the BBB.

3.6 Targeting Glial Support of the BBB

In addition to the BBB endothelium, glial cells (i.e., astrocytes, microglia) are potential therapeutic targets in treatment of stroke. As noted above, glia play a crucial role in regulating BBB functional integrity in health and disease through release

of trophic factors that maintain tight junction 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.

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 [212]. Both PAR-1 and PAR-2 are expressed on the cell surface of astrocytes [213] and microglia [214, 215] as well as on the endothelial cell surface [216]. PAR-1 has been implicated in cytoprotective mechanisms [217, 218] while PAR-2 is involved in regulation of inflammatory responses [219]. Recent research has focused on pharmaceutical development of agonists targeted to the PAR-1 receptor such as activated protein C (APC) [216]. In a mouse model of transient cerebral ischemia, APC was shown to reduce ischemic brain damage and promote neovascularization and neurogenesis, suggesting that pharmacological targeting of the PAR-1 receptor may be an efficacious approach for the treatment of ischemic stroke [220]. 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 [221]. Although native APC exhibits cytoprotection in stroke models, its use is limited by bleeding complications [222]; however, a mutant form of APC termed 3K3A-APC has been discovered that exhibits considerable cytoprotective efficacy without complications of bleeding [218]. 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 [218]. Furthermore, 3K3A-APC improved the functional outcome and reduced the infarction size at a level that was significantly better than APC in the in vivo murine distal MCAO model [218], which implies that 3K3A-APC offers a safer and more efficacious alternative to APC in pharmacological targeting of the PAR-1 receptor. In the presence of r-tPA, 3K3A-APC reduced significant reduced infarct volume following focal cerebral ischemia in mice and embolic stroke in rats by up to 65 % [223]. More recently, a phase I clinical trial of 3K3A-APC demonstrated that this therapeutic was well tolerated in healthy adult volunteers [224], which provides an impetus to study the effects of 3K3A-APC in stroke patients. 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 [225].

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 [226]. 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 hem-

orrhage in a murine experimental stroke model [175]. In vitro, inhibition of microglial activation with minocycline limited ischemic damage in cultured endothelial cells and reduced superoxide release following oxygen–glucose deprivation [175]. More recently, combination therapy of minocycline and candesartan, a pro-angiogenic drug, was shown to improve long-term recovery in Wistar rats subjected to MCAO [227]. In vivo, minocycline was observed to reduce the frequency of hemorrhage in a murine model of cerebral amyloid angiopathy [228]. Currently, minocycline has been incorporated into clinical trials involving stroke patients. Results of these studies demonstrated that minocycline administration, both alone and in combination with r-tPA, improved functional neurological outcome following ischemic stroke [226].

TLRs are highly expressed in human CNS tissue, particularly by astrocytes and microglia [229]. Targeting these receptors has emerged as a promising goal for therapeutic control of ischemic stroke, primarily because TLRs are involved in BBB dysfunction and NVU ischemic injury [230]. While mRNA for TLRs 1–10 have been detected in murine microglia [231], all except TLR10 have been reported in human microglia [232]. Astrocytes possess a much more limited complement of TLRs since mRNA for TLRs 2, 4, 5, and 9 have been detected in murine astrocytes [233] and only TLR3 mRNA in human astrocytes [234]. 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 before TLR-based stroke therapeutics can reach development [230]. This field has shown promise as demonstrated by recent data in a murine model of cerebral ischemia where CNS delivery of TAT-hsp70 was shown to confer BBB protection via reduction of microglial activation, an effect that may be due to targeting of hsp70 to TLR2/4 [235].

4 Conclusion

The field of BBB biology, particularly the study of tight junction protein complexes and endogenous transport systems, has rapidly advanced over the past two decades. It is now well established that tight junction protein complexes are dynamic in nature and can organize and reorganize in response to ischemic stroke. These changes in tight junctions 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. Now, it is beginning to be appreciated that endogenous BBB transporters can facilitate uptake of therapeutics from blood to the brain, thereby rendering these proteins, potential molecular targets for pharmacotherapy. Additionally, MRPs may represent viable molecular targets for BBB vascular protection in the setting of ischemic stroke. Molecular machinery involved in regulating these endogenous BBB transport systems (i.e., TGF-β/ALK5 signaling, Nrf2

pathway) are just now being fully characterized. These crucial discoveries have identified multiple targets that can be exploited for optimization of CNS delivery of therapeutic agents or for protection against BBB dysfunction. Perhaps targeting of currently marketed or novel drugs to influx transporters such as Oatp1a4 or to efflux transporters such as Mrp1, Mrp2, or Mrp4 will lead to significant advancements in ischemic stroke treatment. Identification and characterization of intracellular signaling pathways that can regulate the functional expression of uptake or efflux transporters provides yet another approach for pharmacological control of transporter systems in an effort to precisely 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 tight junction protein complexes, transporters, and intracellular signaling pathways at the BBB/NVU and how these systems can be effectively targeted for improved stroke therapy.

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