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Hypoxic Stress and Inflammatory Pain Disrupt Blood-Brain Barrier Tight Junctions: Implications for Drug Delivery to the Central Nervous System

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Abstract

A functional blood-brain barrier (BBB) is necessary to maintain central nervous system (CNS) homeostasis. Many diseases affecting the CNS, however, alter the functional integrity of the BBB. It has been shown that various diseases and physiological stressors can impact the BBB's ability to selectively restrict passage of substances from the blood to the brain. Modifications of the BBB's permeability properties can potentially contribute to the pathophysiology of CNS diseases and result in altered brain delivery of therapeutic agents. Hypoxia and/or inflammation are central components of a number of diseases affecting the CNS. A number of studies indicate hypoxia or inflammatory pain increase BBB paracellular permeability, induce changes in the expression and/or localization of tight junction proteins, and affect CNS drug uptake. In this review, we look at what is currently known with regard to BBB disruption following a hypoxic or inflammatory insult *in vivo*. Potential mechanisms involved in altering tight junction components at the BBB are also discussed. A more detailed understanding of the mediators involved in changing BBB functional integrity in response to hypoxia or inflammatory pain could potentially lead to new treatments for CNS diseases with hypoxic or inflammatory components. Additionally, greater insight into the mechanisms involved in TJ rearrangement at the BBB may lead to novel strategies to pharmacologically increase delivery of drugs to the CNS.

Keywords

blood-brain barrier; drug delivery; hypoxia; pain; tight junctions

INTRODUCTION

The CNS barriers such as the blood-brain barrier (BBB), blood-cerebrospinal fluid barrier (BCSFB), and blood-spinal cord barrier (BSCB) are physical, biochemical, and metabolic barriers formed to protect the CNS from xenobiotics and potential neurotoxins present in the blood [1, 2]. In addition, CNS barriers assist in the strict regulation of chemical and ionic gradients needed to conduct neural transmission. Although the CNS barriers play an essential role in maintaining tightly regulated CNS homeostasis, they exclude a number of potentially therapeutic compounds and are major obstacles in developing treatments for

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diseases of the CNS [1–4]. The majority of research concerning drug delivery across CNS barriers has focused on the BBB, which will be the primary focus of this review.

The BBB exists at the level of the cerebral microvasculature. Compared to most microvessels outside of the CNS, microvessels in the brain exhibit very low permeability. It has been estimated that close to 98% of all small molecule drugs and nearly all macromolecules are excluded from entering the CNS from the blood [5]. Tight junction (TJ) protein complexes form a seal between brain endothelial cells and contribute to a high transendothelial electrical resistance of 1500–2000 Ω cm^2, which greatly restricts the paracellular permeability of the BBB [6–8]. In addition to the presence of tight junctions, the brain microvasculature lacks fenestrations and exhibits low rates of pinocytosis which further contribute to its low permeability [8]. These properties result in a barrier that is nearly impermeable to polar or high molecular weight molecules. Typically, only small (<500 Da), lipophilic compounds are able to appreciably cross the BBB by transcellular diffusion [9]. Exceptions to this include substrates of uptake transporters expressed at the BBB that promote passage from the blood into the brain. Examples of carrier-mediated transport into the CNS include transporters for glucose, amino acids, nucleosides, and certain neurotransmitters [10]. Even substances predicted to cross the BBB through the transcellular route based on their physicochemical properties often achieve only limited levels in the brain due to the high expression of ATP-binding cassette (ABC) transporters which promote efflux from brain endothelial cells into the blood [11–13]. Due to the ability of the BBB to limit passage of therapeutics through both the paracellular and transcellular routes, drug delivery is one of the most important limitations with regard to treating diseases of the CNS.

Most studies examining brain entry of a therapeutic are performed in healthy animals. It has been known for several decades, however, that various stressors can affect BBB permeability and CNS drug delivery. Studies in rats showed that both immobilization stress and heat stress increase brain entry of intravenously administered Evans blue dye, which indicates that stress can increase permeability of the BBB [14, 15]. Interestingly, the CNS-associated side effects of the acetylcholinesterase (AChE) inhibitor pyridostigmine increased in soldiers taking the drug as a prophylactic against organophosphate poisoning during the Persian Gulf War [16]. Due to pyridostigmine's poor ability to cross the BBB, along with no change in serum AChE activity in these soldiers, the most likely interpretation for the reported increase in CNS side effects is that wartime stress and/or anxiety promoted penetration of pyridostigmine through the BBB [16, 17]. Further studies in mice stressed by forced swim sessions show that 1% of the usual dose of pyridostigmine is required to inhibit 50% of brain AChE activity compared to non-stressed mice [18]. Stressed mice administered pyridostigmine also showed increased BBB permeability to Evans blue and plasmid DNA as well as increased neuronal excitability and oncogene activation [18]. These early studies suggest that stressful stimuli can profoundly impact brain entry of peripherally administered drugs, and studying drug penetration across the BBB in disease models is important to determine correct dosing and achieve desired effects.

The functional integrity of the BBB is compromised in a number of diseases affecting the CNS such as stroke, Alzheimer's disease, multiple sclerosis, and epilepsy among others [19,

20]. While BBB dysfunction is thought to be a contributing factor to the pathogenesis of many CNS disorders, disease-specific alterations in BBB permeability properties may also affect treatment of those diseases. Our laboratory has identified a number of differences in BBB permeability between healthy animals and animals exposed to hypoxia and reoxygenation (H/R) or peripheral inflammatory pain (PIP). H/R, inflammation, and pain are central components of a number of disease states affecting the CNS, and their presence may significantly alter delivery of drugs to the brain. These findings underscore the importance of examining CNS drug delivery in animal models of disease, rather than healthy animals lacking a compromised BBB. In addition, a better understanding of the mechanisms involved in BBB alterations induced by pathological stressors such as H/R and PIP could potentially lead to new methods that rapidly and reversibly modulate BBB TJs to increase paracellular permeability and improve drug delivery to the CNS.

MOLECULAR ORGANIZATION OF TJS AT THE BBB

The TJs at the BBB consist of protein complexes which seal the plasma membrane of brain endothelial cells with the plasma membrane of apposing endothelial cells. These seals greatly restrict the paracellular permeability of substances between the blood and the brain. The major proteins involved in forming the TJ strands at the BBB, as well as most epithelial barriers, are the claudins and occludin, along with the zonula occludens (ZOs), which serve as scaffolding or anchoring proteins. The expression patterns and levels of these proteins are dependent on the tissue type and specific barrier. The TJ complexes are dynamic in nature, and much remains to be discovered about their regulation under both physiological and pathological conditions. Associated with and preceding the formation of TJs are adherens junctions (AJs), which are protein complexes that initiate cell-to-cell contact and promote the maturation, maintenance, plasticity, and tensile properties of epithelial and endothelial cells [21]. The role of AJs in establishing BBB properties is not well understood, but BBB integrity is likely mediated by cross-talk between TJ and AJ complexes [21]. Dysregulation of proteins comprising the AJs is often associated with permeability changes in diseased tissue. For example, the cytokine interleukin 2 changes the composition of the AJ complex and increases the permeability of brain endothelial cells [22]. The role of AJs in regulating the paracellular permeability of the BBB is beyond the scope of this review, but is worthy of future investigation and is the subject of an excellent review by Tietz and Engelhardt [21].

There are 27 different claudin genes with calculated molecular masses of 21–34 kDa [23]. At the BBB, it is likely that the claudin-1, 3, 5, and 12 proteins are expressed while claudin-5 shows the highest mRNA expression level and is thought to be the predominant isoform [24–26]. Claudins are able to reconstitute TJ strands when expressed in fibroblasts and function to tighten the paracellular cleft and form paracellular ion pores [27]. At the BBB, claudin-5 knockout mice show a size-selective permeability to molecules <800 Da, suggesting that manipulation of claudin-5 at the BBB may increase delivery of low MW drugs to the brain [28]. It has been demonstrated that claudins oligomerize or interact in both a *cis* (side to side within the same cell) and *trans* (across the paracellular space) manner and are regulated by a number of post-translational modifications including palmitoylation, phosphorylation, and ubiquitination [25, 29].

Occludin was the first integral membrane TJ protein to be discovered, and its full-length monomers have a molecular weight of ~65 kDa [30]. Occludin possesses a four-transmembrane helix architecture, two extracellular loops, N- and C-terminal cytoplasmic tails, and a MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain [31]. The highly conserved C-terminus interacts with F-actin, ZO-1, 2, and 3, and is involved in the redox sensitive oligomerization of occludin [32–34]. Occludin exists in a number of different isoforms due to splice variants, alternative promoter usage, and oligomerization [31]. At the BBB TJs, occludin forms high molecular weight oligomers that are held together by disulfide bonds in lipid raft microdomains [35]. Interestingly, the TJs in mice lacking occludin are morphologically intact, although a number of histological abnormalities, including brain calcification, are observed [36]. This suggests that occludin plays more of a regulatory role at the TJ. A multitude of phosphorylation and ubiquitination sites on occludin allows for complex regulation of occludin in response to stressors [31]. In addition to occludin, tricellulin and MARVELD3, two occludin-related proteins with MARVEL domains, exist at the TJ [37, 38]. Although the functions of MARVELD3 and tricellulin are not well defined at the BBB, tricellulin has been shown to regulate the permeability of macromolecules at tricellular junctions [39].

ZO proteins (ZO-1, ZO-2, and ZO-3) are scaffolding proteins which are localized at TJs, AJs, and/or gap junctions depending on the cell type [40]. ZO proteins are members of the family of membrane-associated guanylate kinase (MAGUK)-like proteins and contain three PDZ domains, one SH3 domain, a GUK domain, and a proline-rich region [40]. At BBB TJs, ZO-1 and ZO-2 link occludin and the claudins to the actin cytoskeleton [34, 41]. ZO-1 is a 220-kDa protein and was the first ZO protein discovered [42]. ZO-2 is a 160-kDa protein which binds to and has a high sequence homology to ZO-1 [43]. The function of ZO-2 at the BBB is not as well characterized as that of ZO-1, but changes in the expression and localization of both proteins are associated with diseases exhibiting BBB disruption [44, 45]. ZO-3 is a 130-kDa MAGUK protein which also localizes at TJs, but not in endothelial cells [46]. In addition to anchoring proteins of TJs, AJs, or gap junctions to the actin cytoskeleton, ZO proteins contain highly conserved functional nuclear localization and export motifs and are likely involved in signal transduction pathways which regulate gene expression and cell behavior [40].

H/R'S EFFECTS ON BBB PARACELLULAR PERMEABILITY

Hypoxia and reoxygenation are important components of many disorders which affect the CNS including stroke [47], dementia [48], obstructive sleep apnea [49], neuroinflammatory disease [50], traumatic brain injury [47], high altitude cerebral edema, and acute mountain sickness [51]. Both hypoxia, which occurs during ischemia, and reoxygenation, which occurs during the subsequent reperfusion phase, contribute to tissue damage resulting from ischemia/reperfusion injury. Reoxygenation and/or reperfusion produce highly toxic reactive oxygen species (ROS) in the mitochondria which overwhelm antioxidant defenses and contribute to cellular damage [52]. A number of studies have shown that TJs at the BBB are altered in ischemic stroke models [53]. Many *in vitro* studies using different culture systems and hypoxic exposures have demonstrated that hypoxia disrupts BBB paracellular permeability to a variety of tracer molecules [54]. We have previously shown that exposure

to 6% O_2 for 1 h followed by 10 min reoxygenation in room air significantly increases BBB permeability to [^{14}C]sucrose, but not Evans blue bound to bovine serum albumin (EB-BSA; ~80 kDa), as measured using *in situ* brain perfusion in rats [55]. The *in situ* brain perfusion technique maintains a constant tracer concentration and perfusate flow rate while eliminating potentially confounding variables associated with peripheral blood flow, metabolism, and clearance [56]. [^{14}C]sucrose is a low molecular weight (342 Da) polar molecule, which is not subject to metabolism in rat brain and is a useful marker of BBB disruption due to its low permeability under physiological conditions [57]. Increased size-dependent permeability to [^{14}C]sucrose, but not EB-BSA, following H/R is likely due to an increase in the paracellular permeability of the BBB. However, it has recently been shown in a stroke model that ischemia increases transcytosis across the BBB [58]. Knowland *et al.* found that the number of caveolae and the rate of transcytosis across the BBB increased 6 h after transient middle cerebral artery occlusion. Under physiological conditions, transcytosis across the BBB is suppressed by major facilitator super family domain containing 2a (Mfsd2a) [59]. Mfsd2a knockout mice display significantly higher rates of vesicular transcytosis across the BBB. Future studies may reveal a role for Mfsd2a dysregulation and increased transcellular permeability at the BBB due to pathological stressors such as hypoxia and/or ischemia.

BBB permeability to [^{14}C]sucrose after an acute hypoxic insult is biphasic in nature with the first increase occurring 10 min after reoxygenation and the second increase occurring at 6–18 h reoxygenation [60]. These biphasic changes in BBB paracellular permeability reflect the dynamic nature of BBB disruption during H/R stress and should be considered when administering drugs after a hypoxic episode has occurred. Although an increase in BBB permeability (i.e., leak) may contribute to the pathophysiology of CNS disorders with an H/R component, it also creates an opportunity to deliver low molecular weight polar drugs to the brain which may not be able to appreciably cross the BBB under physiological conditions. Due to the biphasic nature of BBB changes, it may be possible to increase delivery of certain drugs to the CNS at certain time points after H/R, but not at others.

A better mechanistic understanding of the changes in TJ architecture that occur during H/R may lead to methods that manipulate BBB paracellular permeability in a specific manner in order to increase drug delivery to the CNS for a wide range of diseases. We have shown that 1 h of hypoxia followed by 10 min reoxygenation leads to phosphorylation of occludin, but no changes in the expression of claudin-3 or ZO-1 [55]. Acute hypoxia (6% O_2 for 30–60 min) stimulates total protein kinase C (PKC) activity in rat brain microvessels and increases vascular permeability in the hippocampus to dextrans (4 and 10 kDa) and rat immunoglobulin G (IgG; 150 kDa) [61, 62]. The BBB leak in the hippocampus to dextrans and IgG after 30 min of hypoxia is sealed within 15 min of reoxygenation or pre-treatment with the PKC inhibitor chelerythrine chloride, suggesting that BBB paracellular permeability to macromolecules can be rapidly and reversibly modulated. In contrast to hypoxia alone, H/R causes increased BBB permeability to [^{14}C] sucrose, but not dextrans or IgG, suggesting that BBB disruption exhibits size-dependent differences between the hypoxia phase and the reoxygenation phase. These permeability changes are associated with increased expression of the novel PKC isoform PKC-θ and the atypical isoform PKC-ζ. Hypoxia causes these PKC isoforms to translocate from the cytoplasm towards the TJs,

concurrent with a redistribution of ZO-1, claudin-5, and occludin away from the TJs [62]. This suggests a mechanism by which hypoxia activates PKC isoforms, leading to phosphorylation of TJ-associated proteins and their translocation away from the TJ. Although H/R-induced disruption of the BBB is associated with edema and neurotoxicity during ischemic stroke [53], these observations also implicate PKC as a potential target to rapidly modulate BBB paracellular permeability and increase drug delivery to the CNS. A number of post-translational modifications of occludin are associated with H/R, suggesting that occludin may also be a target to modify BBB paracellular permeability. H/R has been shown to induce phosphorylation of occludin, promote its trafficking to higher density lipid rafts, and reduce disulfide bonded occludin oligomeric assemblies [55, 63, 64]. The reduction of oligomeric disulfide bonds is likely redox-dependent, as administration of the ROS scavenger 4-hydroxy TEMPO (tempol) before exposure to H/R prevents disulfide bond reduction, increased punctate localization of occludin, and increased permeability to [^{14}C]-sucrose at the BBB [63].

Longer durations of hypoxia also affect BBB paracellular permeability. Mice exposed to 6% O_2 for 24 h followed by 1 h reoxygenation show an increased permeability to Evans blue which is mediated by von Willebrand factor [65]. Exposure to 8% O_2 for 48 h disrupts continuity of ZO-1 and occludin at the TJ and increases BBB permeability to sodium fluorescein in mice. These effects are mediated, in part, by matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor [66]. It is clear that hypoxic stress can mediate TJ structural alterations and paracellular permeability at the BBB, which likely contributes to the pathogenesis of neurological diseases with an H/R component. Further elucidation of the molecular mechanisms leading to TJ disruption during H/R may yield targets that can be pharmacologically manipulated to rapidly and reversibly increase delivery of therapeutics to the CNS while minimizing potentially dangerous consequences associated with a large BBB disruption for an extended period of time.

PIP EFFECTS ON BBB PARACELLULAR PERMEABILITY

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. Commonly used pain medications include non-steroidal anti-inflammatory drugs (NSAIDs) and opioids. Most pre-clinical analgesia studies are performed in healthy animals, but it is becoming increasingly clear that nociceptive stimuli can significantly alter the delivery of analgesics to the brain by modulating BBB permeability. It is important that these changes are taken into account so drugs can be properly dosed and unwanted side effects can be avoided.

Several different models of PIP, including hind paw injection of λ-carrageenan, complete Freund's adjuvant (CFA), or formalin, have all shown increases in BBB permeability to [^{14}C]sucrose, but not EB-BSA, as measured by *in situ* brain perfusion [67]. As is seen in the H/R model, the hind paw λ-carrageenan PIP model exhibits biphasic BBB permeability to [^{14}C]sucrose with the first phase occurring at 1–6 h and the second phase at 48 h [68]. Changes in BBB permeability following λ-carrageenan injection are associated with a decrease in occludin and an increase in ZO-1 protein expression [67, 68]. In addition, there are significant reductions in co-immunoprecipitation of occludin, ZO-2, and actin with ZO-1

[68]. λ-Carrageenan also induces trafficking and localization changes in occludin and ZO-1, which is evident using subcellular fractionation techniques [69]. Similar to what occurs following H/R, λ-carrageenan disrupts disulfide-bonded occludin oligomeric assemblies, which may impair occludin's ability to restrict BBB paracellular permeability [69]. At 72 h post-CFA injection, increased brain entry of [^{14}C]sucrose is associated with a decrease in occludin and an increase in claudin-3 and claudin-5 in brain microvessels, although the claudin-5 appears to be distributed throughout the cell rather than localized primarily at the TJ [70, 71].

PIP is a complex physiological response to tissue injury or infection and, not surprisingly, involves a number of both peripheral and central cell types. Many different cellular signaling pathways and mechanisms likely mediate BBB functional alterations during PIP. The NSAID diclofenac reduces brain uptake of [^{14}C]sucrose during PIP, suggesting that cyclooxygenase (COX) signaling is able to mediate BBB disruption [72]. Peripheral nerve block induced by injection of the voltage-gated sodium channel blocker bupivacaine into the hind leg prevents increased BBB permeability to [^{14}C]sucrose as well as changes in the expression of ZO-1, claudin-5, and occludin 1 h after λ-carrageenan hind paw injection [73]. These observations suggest that nociceptive input is an important factor contributing to BBB alterations during PIP. We have also shown that transforming growth factor-beta (TGF-β1) signaling is involved in BBB changes caused by PIP. Following λ-carrageenan injection, serum levels of TGF-β1 and expression of the TGF-β receptor activin receptor-like kinase 5 (ALK5) at the BBB significantly decrease [74]. Intraperitoneal injection of TGF-β1 before induction of PIP prevents increased BBB permeability to [^{14}C]sucrose. Administration of the selective ALK5 inhibitor SB431542 to control rats increases BBB permeability to [^{14}C]sucrose, and in rats injected with λ-carrageenan, the BBB permeability to [^{14}C]sucrose is significantly increased compared to rats injected with λ-carrageenan and administered vehicle. These observations suggest that TGF-β1 helps maintain BBB integrity through ALK5 signaling. The same study demonstrates that administration of SB431542 significantly increases claudin-3, claudin-5, and phosphorylation of occludin in brain microvessels. SB431542 induces alterations in TJ protein expression in conjunction with an increase in BBB permeability to [^{14}C]sucrose, suggesting that ALK5 signaling is a potential target for manipulating CNS drug delivery through the paracellular pathway. In addition to COX, TGF-β signaling, and nociceptive input, we have shown that ROS are involved in the BBB paracellular permeability changes mediated by λ-carrageenan. When tempol is administered prior to λ-carrageenan injection, an increase in BBB permeability to [^{14}C]sucrose is attenuated and there is reduced breakage of disulfide bonded occludin oligomeric assemblies [75]. Pre-treatment with tempol reduces BBB permeability to [^{14}C]sucrose and disulfide bond breakage of occludin oligomers in both H/R and PIP models, suggesting that ROS production is an important mediator of acute changes to BBB paracellular permeability in disease states with an inflammatory or hypoxic component. Early changes in BBB permeability following induction of PIP may also be due to activation of the sympathetic nervous system. Prevention of BBB disruption with bupivacaine after λ-carrageenan injection indicates that neural transmission, and possibly sympathetic nervous system activation, is involved in BBB functional alterations during PIP [73]. Indeed, studies have shown epinephrine or norepinephrine increase BBB permeability to molecules as large

as IgG in several *in vitro* and *in vivo* models [59, 76–80]. The effect of sympathetic nervous system activation on BBB functional integrity during PIP is an area in need of further investigation.

Our data suggest that PIP would increase blood to brain transport of low molecular weight drugs due to alterations in TJs and paracellular permeability at the BBB. Indeed, this is the case for the opioid analgesic codeine. A significant increase in BBB permeability to [^{3}H]codeine (300 Da) is found 3 and 48 h after λ-carrageenan injection [81]. The degree of codeine-induced anti-nociception, as measured by the tail flick test, in rats given a hind paw injection of λ-carrageenan is also greater than in control rats, which further suggests that PIP increases delivery of codeine from the blood to the CNS. Similar to the situation with [^{14}C]sucrose, BBB permeability to [^{3}H]codeine during PIP was also attenuated by tempol [75]. Interestingly, while BBB permeability to [^{14}C]sucrose and [^{3}H]codeine is increased during PIP, there is a decrease in BBB permeability to [^{3}H]morphine (285 Da) [82]. Although morphine and codeine are structurally similar (codeine is morphine 3-methyl ether), morphine is a substrate for the ABC efflux transporter P-glycoprotein (P-gp) at the BBB [83]. During PIP, P-gp traffics from the nucleus to the luminal plasma membrane of brain endothelial cells where it is well positioned to efflux morphine back into the bloodstream [84]. Therefore, while PIP may increase BBB permeability of certain drugs (e.g., codeine) through the paracellular route, other drugs, which are substrates for efflux transporters (e.g., morphine), may exhibit lower BBB trans-cellular permeability if the functional expression of their efflux transporter(s) is also increased. Alternatively, an increase in the functional expression of endogenous drug uptake transporters at the BBB, such as organic anion transporting peptide 1a4 (Oatp1a4), has been shown to increase CNS delivery of Oatp1a4 substrates such as the opioid peptide [D-penicillamine(2,5)]-enkephalin (647 Da) during PIP and atorvastatin (559 Da) during H/R [85, 86]. These observations indicate that targeting drug uptake transporters at the BBB may aid in the delivery of certain drugs to the CNS through transcellular routes. Targeting drug uptake transporters at the BBB to selectively increase delivery of their substrates to the CNS is an interesting strategy that is beyond the scope of this review, but is reviewed elsewhere in this issue of *AAPS Journal*.

Further complicating pain management is the observation that ancillary pain medications may alter the efficacy of opioids, possibly by modifying BBB permeability. When administered to patients before tonsillectomy, gabapentin and diclofenac reduce post-operative opioid consumption, which may be due to the ability of these drugs to modulate P-gp transport activity at the BBB [87, 88]. We have shown that diclofenac can alter P-gp activity and morphine's effects on nociception in rats [89]. These findings emphasize that the disease state, specific drug(s), time course, and potential drug-drug interactions in question all need to be carefully considered when developing regimens to optimize CNS drug delivery. The dynamic nature of the BBB under pathophysiological conditions precludes generalized statements about BBB permeability to therapeutics. A better understanding of BBB changes induced by PIP may lead to improved treatments for pain as well as novel methods to carefully increase BBB paracellular permeability to therapeutic agents. Although induction of pain with the purpose of improving drug delivery to the CNS is likely not a clinically useful strategy, a precise understanding of the signaling pathways involved in BBB alterations during PIP may lead to the ability to pharmacologically

manipulate BBB permeability and increase brain delivery of small molecule drugs in a highly specific and transient manner.

In addition to PIP, migraine is also associated with changes in BBB permeability [90]. Opening of the BBB, as measured with gadolinium-enhanced MRI using gadolinium diethylenetriamine penta-acetic acid (938 Da), preceded vasogenic cortical edema in a patient with familial hemiplegic migraine [91]. In contrast, no BBB penetration of [^{11}C]dihydroergotamine (584 Da) was detected in six patients suffering from migraine without aura, suggesting that different types of migraine may show differences in BBB permeability [92]. An increase in BBB permeability to Evans blue is also seen in animal models of migraine where cortical spreading depression (CSD) is induced by topical application of KCl [93, 94]. Evans blue leakage is associated with an increase in MMP-9 expression and activity and a decrease in ZO-1 expression in brain microvessels, and is prevented by the MMP inhibitor GM6001 [93]. These observations suggest BBB impairment may be important in the pathophysiology of migraine. The role of BBB changes in migraine and models of chronic or neuropathic pain warrants further investigation. Treatment of all types of pain could potentially be aided by a better understanding of how the BBB dynamically responds to the various mediators involved in the establishment and maintenance of pain. For a brief summary of BBB alterations induced by hypoxia or pain, please refer to Table I and/or Fig. 1.

FUTURE DIRECTIONS

A number of unanswered questions remain regarding BBB disruption induced by hypoxic stress or inflammatory pain. An increase in BBB paracellular permeability is typically thought of as detrimental to CNS function, but it may play a physiological role to aid in the response to or recovery from stressful stimuli such as hypoxia or peripheral inflammation. TJ rearrangement at the BBB may allow certain nutrients in the blood greater access to the CNS in response to H/R or PIP. Alternatively, greater BBB paracellular permeability may allow the brain to more effectively clear potential CNS toxins which form as byproducts to the brain's response to H/R or PIP. Further investigations into the physiological role of BBB paracellular opening may lead to improved treatments for pain and/or disease states with a hypoxic component.

It is also unknown to what extent the BBB is disrupted due to H/R or PIP. It is clear from *in situ* brain perfusion after H/R or PIP that the BBB is more permeable to [^{14}C]sucrose, but EB-BSA added to the perfusate does not penetrate the brain. There is a large size difference between [^{14}C]sucrose (342 Da) and EB-BSA (~80 kDa). A more accurate estimation of the size of the paracellular leak induced by H/R or PIP could inform and enhance the design of drugs capable of crossing a compromised BBB.

A better understanding of the mechanisms that lead to TJ alterations at the BBB is also needed. For example, pre-treatment with tempol protects BBB integrity against H/R or PIP challenges, but it is unknown what pathways downstream from ROS production are responsible for this protection. Changes in BBB permeability to [^{14}C]sucrose can be seen in as little as 1 h in both models of H/R and PIP, suggesting that rapid modulation of BBB

paracellular permeability is possible. Occludin distribution within brain endothelial cells is different after 1 h hypoxia than it is after 1 h hypoxia followed by 10 min reoxygenation which demonstrates TJ protein modification and redistribution can occur within minutes [62]. The biphasic nature of BBB changes induced by H/R and PIP also indicate that increases in paracellular permeability are reversible. Elucidation of the pathways which control post-translational modifications of TJ proteins during H/R or PIP may yield targets which can be rapidly and reversibly modulated to increase blood to CNS delivery of low molecular weight drugs exhibiting poor BBB penetration under physiological conditions.

In addition to the specific pathways involved in H/Rand/or PIP-induced alterations of BBB TJ proteins, it would be useful to know which cell types are involved in initiating these changes. Brain endothelial cells are in constant communication with other cells such as pericytes, astrocytes, microglia, and neurons which together comprise the neurovascular unit. Nerve block preceding λ-carrageenan hind paw injection prevents changes in BBB permeability to [^{14}C]sucrose and TJ protein expression, suggesting that neural transmission is a necessary component of BBB modulation during PIP. The neuronal signaling pathways involved in this modulation, however, are unknown and represent potential targets to alter BBB paracellular permeability. Additionally, it has been shown that microglia can enhance morphine's effectiveness and are thought to have a causal role in the pathogenesis of neuropathic pain [95, 96]. Microglia are activated in the frontal cortex, parietal cortex, and thalamus within 3 h after PIP induction, but their potential role in modifying BBB permeability has not been thoroughly investigated [97]. Astrocytes interact with brain endothelial cells [4, 98] and are involved in the development and maintenance of inflammatory and chronic pain [99, 100], but their influence on BBB permeability in pain has also not been investigated. Recent evidence indicates pericytes play an important role in regulating BBB-specific gene expression and transcellular permeability [101]. *In vitro* studies suggest that both pericytes and astrocytes are involved in BBB maintenance after prolonged hypoxia, but the mechanisms await further clarification [102]. Leukocytes are activated in response to H/R and/or peripheral inflammation and release inflammatory mediators which can affect BBB function [103, 104]. A better understanding of how circulating leukocytes and cells of the neurovascular unit impact the BBB in response to H/R or PIP may lead to better treatments of these diseases or improvements in drug delivery.

Other factors which have been linked to regulation of BBB permeability may also be important with respect to the BBB's response to H/R or PIP. It has recently been shown that the gut microbiota influence the establishment of the BBB [105]. Germ-free mice show increased BBB permeability compared to pathogen-free mice with a normal gut flora. These BBB permeability changes start with intrauterine life and are maintained after birth and into adulthood. The TJ proteins occludin and claudin-5 are also significantly decreased in germ-free mice [105]. It has also been demonstrated that aging induces breakdown of the BBB in the hippocampus of humans [106]. Both the gut microbiome and aging may also affect responses to pain and inflammation. Investigation of the effects of the gut microbiota or aging on BBB disruption during H/R or PIP may yield further insight into mechanisms which compromise BBB functional integrity and influence CNS drug delivery.

Although the focus of this review has been on the BBB, other CNS barriers may also be disrupted by hypoxia or PIP and are also potential targets for drug delivery. The BCSFB has not been the focus of as much research as the BBB, but evidence suggests that substances in the CSF may achieve widespread distribution within the brain along cerebral perivascular spaces [107]. The BCSFB may be disrupted by hypoxia or inflammation, and drugs entering the CSF through a compromised BCSFB could potentially reach targets throughout the brain along cerebral perivascular spaces [108]. The spinal cord transmits nociceptive input from the periphery to the brain and is involved in chronic neck and back pain. It has been shown that the BSCB is disrupted in several different models of pain. Following partial sciatic nerve ligation, a model of neuropathic pain, the lumbar spinal cord is more permeable to intravenously injected Evans blue for up to 4 weeks [109]. The BSCB also shows increased permeability to sodium fluorescein as well as the plasma proteins fibronectin and IgG. These changes are associated with decreased expression of ZO-1 and occludin and are prevented by intrathecal administration of TGF-β1. These results demonstrate that in addition to the BBB, TGF-β1 signaling is involved in TJ protein expression at the BSCB. Spinal cord injury causes increased BSCB permeability to Evans blue and induces edema in the spinal cord [110]. These changes are attenuated by pre-treatment with *p* -chlorophenylalanine, indomethacin, ibuprofen, and nimodipine, indicating a role for serotonin, COX signaling, and calcium channels in BSCB changes induced by spinal cord injury. Spinal cord ischemia-reperfusion injury has been shown to increase the extravasation of Evans blue into the spinal parenchyma and is associated with upregulation of MMP-9 and a reduction in claudin-5, occludin, and ZO-1 [110]. Understanding mechanisms which disrupt the BCSFB or BSCB following hypoxia, pain, or inflammation may lead to novel treatments targeting cells throughout the brain or spinal cord.

CONCLUSIONS

Disruption of the BBB is associated with many diseases affecting the CNS. While impaired BBB integrity is typically thought of as a contributing factor in CNS pathologies, alterations in the permeability of the BBB to therapeutics must also be considered. In models of hypoxic stress or inflammatory pain, BBB permeability is significantly altered when compared to healthy animals. Increases in paracellular permeability due to alterations in BBB TJ complexes may allow low molecular weight drugs greater access to the CNS from the blood. H/R- or PIP-induced changes in the functional expression of drug efflux pumps or drug uptake transporters may also alter CNS penetration of drugs which are substrates of the various transporters expressed at the BBB. The mechanisms involved in TJ alterations and BBB permeability changes during H/R or PIP are only beginning to be unraveled. A detailed understanding of how to rapidly, transiently, and safely manipulate the molecular composition of TJs at the BBB may lead to methods which both optimize CNS drug delivery and limit detrimental neurological consequences associated with a compromised BBB.

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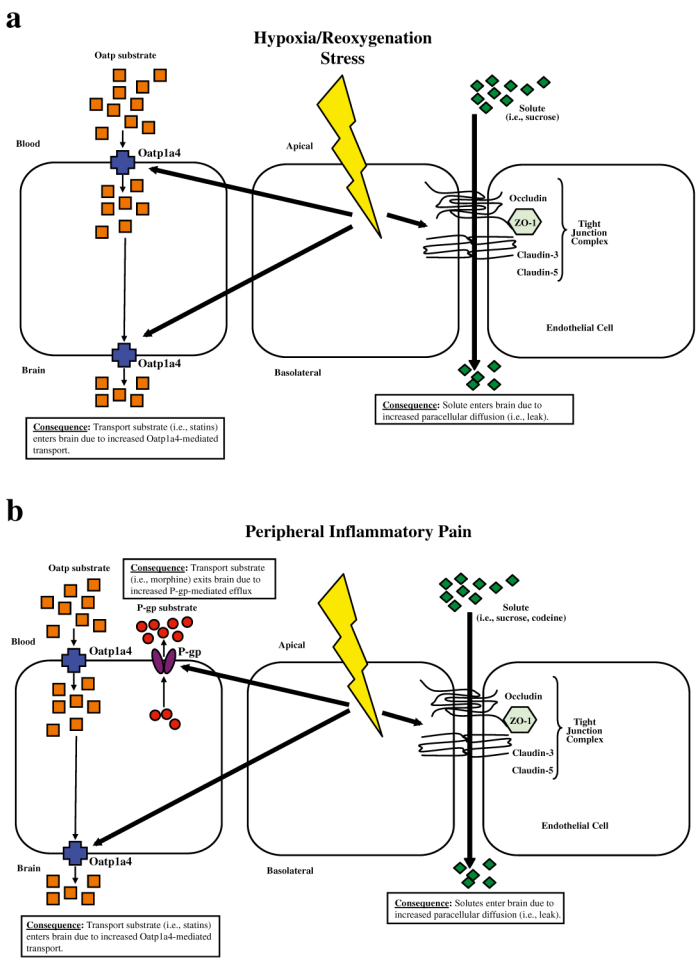

Fig. 1.

Effects of hypoxia/reoxygenation (H/R) stress (**a**) or peripheral inflammatory pain (PIP) (**b**) on tight junction protein complexes and endogenous transporters at the blood-brain barrier (BBB). H/R and PIP lead to dysregulation of the tight junction complex that is demarcated by altered expression of claudin-3, claudin-5, occludin, and ZO-1. Additionally, functional expression of Oatp1a4 is increased, leading to enhanced brain uptake of Oatp transport substrates including therapeutic agents. During PIP, functional expression of Oatp1a4 and P-gp is increased, leading to enhanced uptake of Oatp1a4 substrates and enhanced efflux of P-gp substrates from the brain. These processes result in an increase in BBB paracellular permeability and changes in brain delivery of drugs which are substrates for drug transporters that are functionally altered during H/R or PIP

Table I

Summary of BBB Alterations in Hypoxia and Pain Models *In Vivo*

Model	BBB paracellular permeability changes	Tight junction protein changes	Associated mechanisms
6% O_2 for 30–60 min	Increased to dextrans (4 and 10 kDa) and IgG in hippocampus [58]	Loss of occludin, claudin-5, ZO-1 immunoreactivity; changes in occludin trafficking, increased claudin-5 membrane expression [58, 60]	PKC-θ and PKC-ζ activation [58, 60]
6% O_2 followed by reoxygenation	Increased to [^{14}C]sucrose after 10 min and 6–18 h reoxygenation [53]	Increased occludin phosphorylation; changes in occludin localization and trafficking; disruption of disulfide bonded occludin oligomeric assemblies [53, 59, 60]	PKC activation, ROS generation [53, 59, 60]
6% O_2 for 24 h	Increased to Evans blue [61]	?	Mediated by von Willebrand factor expression [61]
8% O_2 for 48 h	Increased to sodium fluorescein [62]	Decreased occludin expression; changes in occludin and ZO-1 localization [62]	MMP-9 activation and vascular endothelial cell growth factor signaling [62]
Hind paw λ-carrageenan injection (inflammatory pain model)	Increased permeability to [^{14}C]sucrose at 1–6 h and 48 h; increased permeability to [^{3}H]codeine at 3 and 48 h [63, 72]	Decreased occludin expression; disruption of disulfide bonded occludin oligomeric assemblies; changes in occludin and ZO-1 trafficking; increased ZO-1 expression; decreased binding of occludin and ZO-2 to ZO-1 [63–65]	ROS generation; COX signaling; TGF-β1/ALK5 signaling; neural transmission [68–71]
Hind paw CFA injection (inflammatory pain model)	Increased permeability to [^{14}C]sucrose at 72 h [63]	Decrease in occludin expression; increase in claudin-3, claudin-5 and ZO-1 expression; changes in claudin-5 localization [66, 67]	?
Hind paw formalin injection (inflammatory pain model)	Increased permeability to [^{14}C]sucrose at 1 h [63]	Increased ZO-1 expression [63]	?
Topical application of KCl to brain (migraine model)	Increased to Evans blue [84, 85]	Decreased ZO-1 expression [84]	MMP activation [84]

ALK5 activin-like kinase 5, *COX* cyclooxygenase, *IgG* immunoglobulin G, *MMP* matrix metalloproteinase, *PKC* protein kinase C, *ROS* reactive oxygen species, *TGF-β1* transforming growth factor β1, *ZO-1* zonula occludens 1