Tissue Barriers 4:1, e1138017; January/February/March 2016; © Taylor & Francis Group, LLC

Tight junction modulation of the blood brain barrier: CNS delivery of small molecules

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Abbreviations: BBB, blood brain barrier; CNS, central nervous system; TJ, tight junction; NVU, neurovascular unit; AJ, adherens junction; JAM, junctional adhesion molecule; EAE, experimental autoimmune encephalmyelomitis; C10, sodium caprate; siRNA, small interfering RNA; AMD, age-related macular degeneration; OAT3, organic ion transporter 3; BRB, blood-retina barrier; ZOT, zonula occludens toxin; AD, Alzheimer's disease; Aβ, amyloid β; RAGE, receptor for advanced glycation end products; MS, multiple sclerosis; CypA, cyclophilin A; BMVEC, brain microvascular endothelial cells; GC, glucocorticosteroids; CSR, chronic sleep restriction; FUS, focused ultrasound.

The blood brain barrier (BBB) represents a major obstacle for targeted drug delivery to the brain for the treatment of central nervous system (CNS) disorders. Significant advances in barrier research over the past decade has led to the discovery of an increasing number of structural and regulatory proteins in tight junctions (TJ) and adherens junctions (AJ). These discoveries are providing the framework for the development of novel TJ modulators which can act specifically and temporarily to alter BBB function and regulate paracellular uptake of molecules. TJ modulators that have shown therapeutic potential in preclinical models include claudin-5 and occludin siRNAs, peptides derived from zonula occludens toxin as well as synthetic peptides targeting the extracellular loops of TJs. Adding to the array of modulating agents are novel mechanisms of BBB regulation such as focused ultrasound (FUS). This review will give a succinct overview of BBB biology and TJ modulation in general. Novel insights into BBB regulation in health and disease will also be summarized.

Introduction

Physiological barriers provide the framework for a boundary between circulating blood and interstitial fluid, a pre-requisite for mammalian life. Of the numerous biological barriers, the blood-brain barrier (BBB), situated along blood vessels of the central nervous system (CNS), is perhaps the most selective and tightly regulated, reflecting the brain's critical roles in cognitive function, controlling metabolism and strictly coordinating the functions of peripheral organs. Central to this function is the neuron, a terminally differentiated electrically excitable cell, which requires fine control of both electrophysiological and chemical signals to function efficiently.[1] As such, the brain requires a precise and balanced microenvironment. The BBB is therefore important in regulating the exchange of ions and nutrients between the blood and brain but also to protect delicate neural tissue from potentially damaging blood-borne agents such as pathogens, immune cells and anaphylatoxins.[2] Owing to this specialized barrier, CNS endothelial cells are distinct from endothelial cells of the periphery in several ways, specifically they contain: BBB-specific proteins to control the entry and exit of metabolites across cells (transcellular pathway); highly electrical resistant tight junctions (TJ) to limit the flux between adjacent endothelial cells (paracellular pathway); an absence of fenestrations (pores to allow rapid exchange of molecules between blood and tissue in peripheral endothelial cells) to limit the movement of molecules.[2,3] The low rate of vesicular transport (absorptive transcytosis), in the CNS endothelium, by comparison with other endothelia, is also important in preventing transport of large hydrophilic molecules to the CNS.

The BBB is not a static microenvironment, it is highly dynamic in both homeostatic physiology and indeed in pathology. Such is the impact of the BBB on neural integrity that no brain cell is ever further than ~25 μm from a capillary.[4] As a result of this, crossing the BBB is the favored route for drug delivery to the brain as once the BBB is by-passed, diffusion distances to the site of action are relatively short. As well as a short diffusion distance, the combined surface area of microvessels is 150–200 cm^2/g of tissue which equates to ~15–20 m^2 per adult human brain allowing for a huge area of access to brain parenchyma.[5] However, while an ample network of capillaries exists to target therapeutics toward the brain, the BBB functions to impede this as a result of TJ proteins between adjacent endothelial cells. In addition, an array of transporters actively eflux material out of the brain and numerous enzymes along the capillary systems are highly active in degrading un-wanted material. Such is the degree of restriction, many drugs which are currently approved and clinically enabled cannot cross the BBB in sufficient quantities to be therapeutic.[6]

The dominant idea in early BBB research proposed that endothelial cells lining the lumen of the blood vessel wall was responsible for forming the BBB, however increasing numbers of studies have highlighted the variety of cell types that interact to form an intricate network of crosstalk between cell types to maintain BBB integrity.[5,7] This neurovacular unit (NVU, see **Fig. 1**),

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Submitted: 11/10/2015; Revised: 12/22/2015; Accepted: 12/24/2015
http://dx.doi.org/10.1080/21688370.2015.1138017

primarily comprising endothelial cells, pericytes and astrocytic end-feet can interact with a collection of neurons, microglia and other brain components, helping to explain BBB induction processes and maintenance of BBB integrity in health and disease and how dysfunction of any one of these cell types can influence BBB integrity. Indeed, the NVU has several functions including a) maintenance of homeostasis in ionic composition for optimal synaptic signaling,[8] b) separating the pool of neurotransmitters between the CNS and peripheral nervous system,[5] c) omitting entry of macromolecules such as albumin and plasminogen that are harmful to neurons,[9] d) shielding the CNS from neurotoxins and allowing entry of essential water soluble nutrients and metabolites.[10] Dysfunction of any one of these processes can impact on the health and integrity of the CNS as a whole. Recent studies have identified how TJ deficiencies and disruption of macromolecule transporters (*e.g.* GLUT-1, msfd2a) can influence overall cerebrovascular integrity.[11,12] This work has implications in the study of CNS disorders such as schizophrenia and epilepsy and in the development of novel drug delivery systems based on BBB biology. For example, glial-derived neurotrophic factor can restore dopaminergic neurons in animal models of Parkinson disease, but it cannot cross the BBB.[13]

"Kissing Points" to Seal the Paracellular Space

Over a century has passed since Paul Ehrlich and Edwin Goldmann's seminal experiments identified a clear compartmentalization of the blood and brain. However, while major advances in techniques from electron microscopy to freeze fracture analysis has paved the way for extensive discoveries in BBB biology, it still represents a key impediment to targeted therapeutic delivery to the brain. Indeed, it has been estimated that approximately 100% of large-molecule neurotherapeutics and over 95% of small-molecule drugs cannot circumvent the BBB.[6]

Significant breakthroughs have been made in approaches to deliver drugs across the BBB by employing molecular Trojan horses,[14] encapsulating therapeutics in nanoparticle carriers[15] and by targeting BBB-specific receptors (transferrin, P-glycoprotein).[16] While these methods have had moderate pre-clinical success in circumventing the BBB through the transcellular pathway, manipulating TJs in the paracellular pathway is a promising alternative for rapid, reversible opening of the BBB. Two major junctional complexes are present at the BBB: adherens junctions (AJs) and TJs. AJs are composed primarily of cadherin proteins that span the intercellular cleft and provide stability by linking to the cell cytoplasm by $\alpha/\beta/\gamma$ catenin proteins.[17] The precise role of AJ has yet to be resolved, however it is thought that the molecular components play a key role in maintaining cellular polarity, providing stability, promoting endothelial cell survival and responding to stimuli via interactions with catherin proteins and the actin cytoskeleton. Evidence suggests that AJs are also essential for the formation of TJs.[17] Unlike AJs which are present in all vascular beds, TJs are enriched in the endothelium of the brain microvasculature. TJs appear as continuous, anastomosing, intramembranous networks of strands that interact with TJ proteins on the same cell or on adjacent endothelial cells at so called "kissing points" to eliminate the paracellular space.[18] This fusion of TJs is responsible for impeding the flow of solutes and ions from the blood to brain and vice-versa, in turn creating a dynamic and highly regulatable barrier system.

The predominant TJ proteins are the claudins and occludin. Claudins and occludin also interact with cytoskeletal scaffolding proteins called zonula occludens on the intercellular domain of the plasma membrane to "tether" the TJs to the actin cytoskeleton.[19-21] At three cell contacts, tricellulin and lipolysis-stimulated lipoprotein receptor (LSR) have been identified as potentially regulating paracellular permeability.[22] Other proteins present within the TJ system are the junctional adhesion molecules (JAM) of which several isoforms have been discovered.

Junctional Complexes of the BBB

Occludin

Occludin was identified as the first integral membrane protein within the TJ of endothelial cells.[23] Occludin is a functional component of TJs and, like the claudin family, has four membrane spanning domains and two extracellular loops.[19] The function of occludin at the TJ was revealed by ectopic expression of chicken occludin in Sf9 insect cells, whereupon it induced the formation of TJ-like structures.[24] Upon introduction of a truncated occludin into MDCK cells, paracellular leakage to small tracers increased, hinting at a role in TJ formation.[25] However, embryonic stem cells lacking occludin could still form intact TJs indicating that occludin is dispensable to barrier formation.[26] Further to this, occludin knockout mice have been reported with a complex phenotype and postnatal growth retardation and brain calcification. However the mice still formed intact TJs. The complex array of abnormalities indicate a potential physiological role of occludin secondary to TJ formation.[27] In fact, a number of studies have now shown that occludin undergoes extensive modifications at the post-transcriptional and post-translational level.[28] Prior to development of disease symptoms in experimental autoimmune encephalomyelitis (EAE), an animal model of brain inflammation, dephosphorylation of occludin occurs suggesting that occludin could be a target for signaling events in EAE.[29] It is also known that occludin plays a key role in redox regulation of TJs. Normoxia conditions promote occludin oligomerization and TJ assembly while oxidative stress associated with inflammation promotes TJ disruption.[30]

Claudins

27 members of the claudin family of proteins have been identified.[31] All claudins have the same structural pattern; they are integral membrane proteins with four membrane spanning domains, two extracellular loops and two cytoplasmic termini: a short chain N terminus and a longer chain C terminus.[32] The C terminus contains a PDZ motif which links claudins to scaffolding proteins ZO-1, ZO-2, ZO-3, MAGI-1, PatJ, PALS1 and MUPP1.[33] They are the major structural component of the TJ and form the backbone of TJs through homotypic and

heterotypic interactions via their extracellular loops.[33,34] At the BBB, claudin-5 is by far the dominant TJ component, but claudin-3, and claudin-12 are also present.[35] Our understanding of the claudins has been drastically improved through genetic knock-in and knock-out models and in *in vitro* models of the BBB. Claudin-5 was identified to form stable TJ networks upon transfection into TJ free MDCK cells concurrent with a selective decrease in permeability to ions.[33,36] The role of claudin-5 in forming the BBB was confirmed in mice genetically altered to lack claudin-5. Nita *et al.* showed that claudin-5 knockout mice have an impaired BBB that was leaky to molecules up to 800 Da in size. However, complete ablation of claudin-5 is lethal, with all mice dying within hours of birth from un-defined causes. Interestingly, the barrier remained intact to molecules greater than 1 kDa indicating other components may be involved in regulating barrier integrity.[37] It is apparent that the claudins have an intrinsic role in regulating BBB permeability (for a review see ref.[38]).

Junctional adhesion molecules

JAMs are integral membrane proteins belonging to the immunoglobulin superfamily. They consist of a single membrane-spanning domain, an extracellular domain with an N terminus and a short cytoplasmic C terminus.[39] The cytoplasmic C terminus contains a PDZ motif that interacts with scaffolding proteins including ZO-1, AF-6, ASIP/Par3 and cingulin.[40,41] JAMs can form homotypic interactions with JAMs on opposing cells and form heterotypic interactions with different JAM family members as well as other adhesion molecules.[42] Through binding to Par3, JAMs promote cell polarity and the localization of ZO-1 and occludin at points of cell contact.[43] Mounting evidence has implicated a role for JAM family members in leukocyte migration across endothelial cell layers.[44,45]

Scaffolding proteins

The ZO (ZO-1, ZO-2, and ZO-3) proteins are members of the membrane-associated guanylate-kinase (MAGUK) protein family. They are the prominent scaffolding proteins linking TJs to the actin cytoskeleton. ZO-1 is essential for endothelial barrier formation, VE-cadherin-mediated cell tension and actomyosin organization through its interaction with F-actin.[46] The ZO proteins contain a PDZ motif on the C terminus to link ZO proteins with transmembrane proteins or with PDZ motifs on other proteins. ZO-1 binds to the claudins, occludin and JAM via PDZ motifs as well as with various components of the cytoskeleton.[40,41,46]

TJ's have two main functions. The first is to significantly reduce the permeation of polar solutes and ions from the blood to the brain and vice-versa. This impediment to the flow of ions across the BBB leads to a high electrical resistance *in vivo* of approximately ~1800 $\Omega.cm^2$.[47] Early studies with electron microscopy showed that ionic lanthanum introduced into the cerebral capillary lumen could penetrate the intercellular cleft as far as the TJ where its movement was subsequently impeded.[48] A second function of TJ proteins is to help maintain polarity of cells. This is achieved by restricting the lateral diffusion of membrane lipids and proteins between the apical and basolateral compartments of endothelial cells.[49] While certain substances can cross the barrier via the paracellular route, these are usually extremely small or employ specific mechanisms to move between TJs. For example, T-cell migration is initiated by leukocytes binding to ICAM-1 and -2 expressed on endothelial cell surfaces leading to migration across the transcellular pathway.[50] Indeed, the major route of transport across the BBB is via the transcellular pathway. Neurons require energy to maintain synaptic signaling and this energy need is met by proteins such as GLUT-1 which controls the entry of glucose into the brain. Ion regulation which is critical for optimal synaptic signaling between neurons is maintained by proteins such as Na^+, K^+, and ATPase.[7] Brain endothelial cells also possess specific receptors to control the entry and exit of essential peptides, such as hormones. For example, PST-1 recognizes vasopressin, a pituitary hormone essential for water retention.[51]

TJ Modulating Agents

Chemical

To utilize the paracellular pathway as a means for drug delivery to neuronal regions, it is necessary to regulate the proteins present in the space between endothelial cells. To date, sodium caprate (C10) is one of only a few TJ modulators with clinical relevance. It has been approved in Japan and Sweden as an absorption enhancer for the antibiotic ampicillin through a rectal suppository.[52] It has been shown that a dose of 50 mg per person of C10 significantly increased serum ampicillin concentrations in humans. In the brain endothelium C10 reduces claudin-5 levels, transiently opening the paracellular space.[53] Internal carotid artery infusion of C10 in adult Sprague Dawley rats increases BBB permeability beginning 5 min post injection highlighting its potential use as a reversible BBB modulator.[54] If C10 is to be used as a drug enhancer to the CNS however, its cytotoxic effect on the BBB must be further studied as well as its site-specific mode of action on endothelial cells.

Intra-arterial injection of the hyperosmolar agent mannitol has been used to produce temporary BBB disruption in rats and humans.[55] A lower concentration and intravenously administered mannitol is also used clinically to reduce intracranial pressure following traumatic brain injury (TBI) owing to its osmotic effect and has shown promise in enhancing delivery of chemotherapeutics to brain tumors.[56] Following intra-carotid infusion, mannitol has been purported to exert BBB disruption by shrinking endothelial cells leading to a distinct modulation of the TJ as a whole.[57] Previously, it has been reported that mannitol administration was concurrent with reduced brain S100β levels and significantly elevated serum S100β levels, a hallmark of BBB integrity, indicating a transient opening of the BBB.[58] However, while mannitol shows promise for BBB disruption and enhanced drug delivery,[59] setbacks to its use include the complex surgical procedure required and side-effects that can include focal seizures.

Early research into the design of tight junction modulators focused on improving the absorption of drugs across the BBB. Cereport, a 9 mer synthetic peptide based on bradykinin, showed great promise in the treatment of neurological disorders. In rodent models of glioma and metastatic brain tumors, cereport increased the tumor uptake of chemotherapeutics and prolonged survival.[60] However in a Phase II clinical trial, Cereport proved ineffective in childhood high-grade gliomas and brainstem gliomas.[61] Despite this, it is possible Cereport can still find use in improving the delivery of other neurotherapeutics.

Another approach to the transient modulation of the barrier involves intra-arterial injection of short-chain alkylglycerols, which induce reversible BBB opening concurrent with redistribution of junctional complexes.[62] Brain uptake of methotrexate significantly increased following intra carotid injection of 1-0-pentylglycerol compared to control indicating a distinct modulation of the BBB.[63] However, the use of short chain alkylglycerols is limited due to the invasiveness of the procedure.

Zonula occludens toxin (Zot) is an enterotoxin produced by *Vibrio cholerae*. Zot is capable of reversibly opening the BBB to various molecular weight tracers and chemotherapeutics in bovine brain microvessel endothelial cells. Importantly Zot was shown to be non-toxic and permeability to a range of tracers was increased in a time and dose-dependent manner.[77,79] Further to this, ΔG, a 12 kDa active fragment of Zot can significantly increase the brain distribution of MTX and paclitaxel.[78] These anti-cancer agents are known to have poor brain distribution. Zot is a promising tool for increasing brain penetration of therapeutics through specific modulation of TJs.

RNA interference

An alternative method for the transient and reversible modulation of the BBB involves the use of RNA interference (RNAi). 15 years have passed since the proof of principle experiments demonstrating synthetic small interfering RNA (siRNA) could produce specific gene knockdown in mammalian cells.[64] Today, siRNA technology is a multi-billion dollar industry. Using siRNAs has shown great promise in the treatment of age-related macular degeneration (AMD) with several therapies having progressed up to and including Phase III clinical trials. SiRNA targeting VEGFR-1 reduced the extent of choroidal neovascularization and decreased retinal neovascularization in oxygen-induced ischemic retinopathy in mice. In a study evaluating the safety and tolerability of siRNA administration, 26 patients responded with limited side-effects following administration of up to 1600 μg of siRNA as well as stabilization or improvement of visual acuity in cases with neovascular AMD.[65] It is clear that RNAi is a promising technology with potential application in treating CNS disorders through targeted disruption of TJ proteins of the BBB.

A number of studies have shown that it is possible to specifically target siRNA to brain capillary endothelial cells for targeted deletion of BBB-specific proteins. siRNA targeting the organic ion transporter 3 (OAT3) *in vivo* in mouse brain capillary endothelial cells could efficiently suppress OAT3 and reduce brain to blood transport of benzyl penicillin.[66] We have shown in numerous studies that siRNA targeting claudin-5 mRNA can reduce the expression levels of the protein and transiently modulate the BBB to molecules up to approximately 1 kDa in size.[67] Furthermore, targeted suppression of claudin-5 through RNAi can reduce water content in the brain following traumatic brain injury and improve cognitive function in mice with focal cerebral edema.[68] RNAi can be utilized to improve the delivery of small molecule therapeutics across the blood-retina barrier (BRB) and BBB. We have shown the therapeutic potential of RNAi directed against TJ proteins at the BRB by enhancing the delivery of neurotherapeutics to the neural retina of rodent models of retinopathy. IMPDH knockout mice are a model of autosomal recessive retinitis pigmentosa. These mice lack an enzyme involved in the *de novo* synthesis of GTP (MW: 523 Da) which is essential for visual transduction. Through targeted suppression of claudin-5 in the neural retina, systemic injection of GTP could bypass the BRB and improve retinal function. Similarly in BalB/c mice with light-induced retinopathy, systemic injection of the calpain inhibitor N-acetyl-Leu-Leu-Met-CHO (ALLM) (MW: 401 Da) could readily diffuse across the BRB and reduce the level of photoreceptor cell death, a hallmark of light-induced retinopathy.[69] Further to this, the use of an adeno-associated virus (AAV) expressing a doxycycline-inducible shRNA targeting claudin-5 mRNA transcripts has been used for the delivery of small molecule therapeutics across the BBB/BRB. With this approach it was possible to attenuate the effects of laser-induced choroidal neovascularization through improved delivery of 17-AAG (MW: 585 Da) and Sunitinib malate (MW: 532 Da), two well characterized VEGF inhibitors, across the BRB.[70] Importantly, this approach specifically downregulated claudin-5 at the BBB/BRB while expression patterns of other TJs remained at normal physiological levels. It is also possible to modulate TJs in specific brain regions, e.g., by direct stereotaxic injection of AAV vectors.

Recently, we have shown that sequential delivery of siRNAs targeting claudin-5 and occludin to co-suppress both proteins can result in soluble human Aβ(1–40) monomers diffusing across the paracellular pathway of the BBB from brain to blood. In Tg2576 mice, a murine model of familial AD, cognitive function improved in tandem with reduced brain levels of Aβ(1–40) and increased serum levels of Aβ(1–40) indicating that it is possible to remove pathogenic agents from the brain to the blood.[71] In summary, targeted suppression of TJs at the BBB/BRB increases paracellular permeability and enhances targeted drug delivery to neuronal regions. Through use of an inducible system, it is also possible to reverse BBB permeability by withdrawal of the inducing agent.

Peptidomimetics

Peptidomimetics is the study of peptides designed to mimic endogenous short-chain proteins. When introduced, the modified peptide acts by adjusting the molecular properties of the endogenous peptide such as stability or biological activity. Peptides have previously been used to beneficial effect in the targeting of cancer cells and directing them toward apoptotic fates.[72] With regard to the BBB, peptidomimetics have the potential to modulate BBB permeability and improve drug delivery. Several

peptides have been synthesized to mimic the extracellular loop domains of a number of TJs. It has been shown that a peptide targeting the second extracellular loop of occludin (OCC2) could reversibly deplete occludin protein levels, decrease transepithelial electrical resistance and concomitantly increase paracellular flux of tracer molecules.[73] However, while occludin levels were decreased, expression levels of ZO-1, ZO-2, cingulin and E-cadherin remained unchanged indicating a selective depletion of occludin from the TJ in Xenopus kidney epithelial cells. In a similar study it was found that a peptide targeting the first extracellular loop of occludin could selectively increase paracellular permeability to mannitol.[74] The disparity of these results may be down to the species of origin of occludin. In the former study, peptides corresponding to chicken occludin were designed whereas in the latter, peptides corresponding to human occludin were designed.

Peptides have been designed targeting the claudins as well. A peptide emulating the second half of the first extracellular domain of claudin-1 can reversibly effect TJ structure and function in T84 intestinal epithelial cells.[75] C1C2 is another claudin-1 peptidomimetic emulating the second extracellular loop. This peptide can reversibly modulate various barriers *in vivo* by preferential interaction with claudin-1 and claudin-5 resulting in redistribution of claudins and occludin to the cytosol.[76]

The BBB in health and disease: a requisite for modulation

A functional, intact BBB is essential for maintaining a microenvironment with the right balance of ions and nutrients for efficient neural signaling. A compromised BBB however can lead to an imbalance in the flux of ions and molecules across the BBB when TJs or transport processes are impaired as well as increased extravasation of immune cells. The consequences of BBB breakdown are prevalent in neuropathology.

Alzheimer disease

Alzheimer's disease (AD) is a CNS disorder characterized by the accumulation of amyloid-β (Aβ) peptides and the hyperphosphorylation of the microtubule associated protein tau and cerebrovascular alterations leading to a gradual decline in cognitive function.[80] Previous reports have shown that Aβ can disrupt BBB integrity contributing to AD-related pathogenesis.[81,82] The receptor for advanced glycation end products (RAGE) is the major route for the movement of Aβ from the blood to brain. RAGE is thought to mediate Aβ-induced TJ dysfunction. RAGE levels are elevated in the brain endothelium of many AD patients.[83] In BMEC cultures *in vitro*, changes in TJ expression were concurrent with increased RAGE expression. By specifically antagonizing RAGE expression with neutralizing antibodies, Aβ-induced changes in TJ expression could be reversed.[84] Recently, using sophisticated high-resolution imaging of the living brain, Zlokovic *et al* discovered that the BBB becomes progressively leaky with age, starting from the hippocampus; the brain region responsible for learning and memory.[85] In a follow-up study, it was discovered that aberrant expression of PICALM-1, a high risk genetic factor for AD, was associated with diminished clearance of Aβ.[86]

Neuroinflammation

While this review has surveyed the use of tight junction modulators to selectively decrease the levels of specific TJs, pathologies arise where it may be necessary to strengthen the BBB, particularly in response to inflammatory insults. Breakdown of the BBB has been shown in conditions such as multiple sclerosis (MS) and stroke, the consequences of which can lead to damaging serum proteins invading the brain parenchyma and affecting neural integrity. As such, in certain neuropathologies, restoring barrier integrity may prove to be a promising approach. Indeed, in claudin-1 knock-in mice, it has been shown that claudin-1 positive microvessels showed significantly reduced BBB leakiness to blood borne tracers and plasma proteins. Increased barrier tightness was associated with a reduced disease burden in the chronic phase of experimental autoimmune encephalomyelitis (EAE), an animal model of MS.[87] Similarly, selective loss of claudin-3 correlates with immune cell infiltration into the CNS and BBB leakiness, exacerbating conditions in EAE.[88]

ApoE, a gene strongly linked to AD pathogenesis, has been reported to negatively affect BBB function.[89] ApoE knockout mice and mice expressing a human apoE4 gene had compromised BBB integrity owing to dysregulated cyclophilin A (CypA). CypA upregulation activates the NF-κB signaling pathway and secretion of matrix metalloproteinase-9 from pericytes. This in turn precedes neurodegeneration.[90] Furthermore, a range of neuroinflammatory cytokines have been linked to alterations in TJs. For example, CCL2 (previously monocyte chemoattractant protein-1 (MCP1) downregulates occludin, ZO-1 and caveolin-1 expression. Treating brain microvascular endothelial cell (BMVEC) cultures with cavtratin, a synthetic peptide encoding the caveolin-1 scaffolding domain, reversed CCL2-mediated TJ disruption.[91]

It is known that several cytokines can cross the BBB[92] and that numerous cytokines are released in neuroinflammatory situations. LPS and TNF-a have been shown to induce BBB breakdown with TNF-α, a proinflammatory cytokine, acting through the reorganization of actin filaments to stress fibers leading to increased paracellular clearance of sucrose and inulin.[93,94] TNF-α has been shown to mediate HIV-1 infection in the brain via opening of the paracellular route.[95] Similarly, Haemophilius influenza type b LPS induced BBB permeability.[96] A key mediator of BBB permeability is the proinflammatory cytokine IL-1 β which induces phenotypic plasticity and permeability in brain microvessels resulting in BBB permeability through induction of hypoxia inducible factor-1 and vascular endothelial growth factor-A (VEGF-A).[97] VEGF-A acts directly on BMVEC to downregulate claudin-5 and occludin protein and mRNA *in vitro* and in mouse models EAE.[98] Further to this, transforming growth factor-β (TGF-B) has been implicated in regulating BBB permeability. Following inflammatory pain, reduced TGF-B levels were associated with increased BBB permeability to sucrose in a rodent model.[99]

These studies show that targeting cytokine mediators of BBB breakdown is a possible therapeutic intervention to promote BBB stability in response to neuroinflammation to prevent potentially damaging blood-borne agents entering the brain

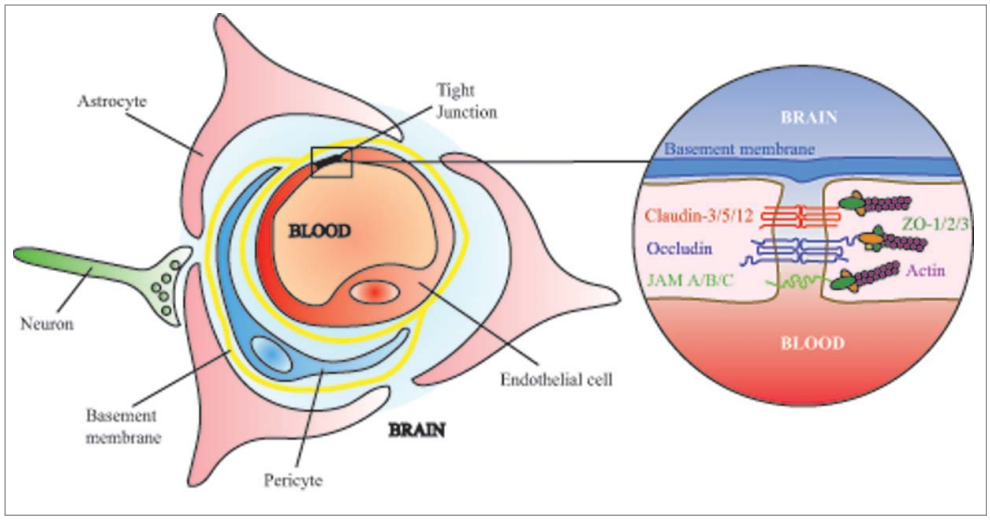

Figure 1. The neurovascular unit is an intricately developed system of endothelial cells, astrocytes and pericytes that can interact with neurons, microglia and other brain components to impart specific properties on the blood-brain barrier. Within endothelial cells of the central nervous system, tight junctions limit the paracellular diffusion of all but the smallest solutes, ions and lipid soluble molecules.

parenchyma. Indeed, a recent study showed that knockdown of MMP-2 and MMP-9 by RNAi attenuated MMP-mediated TJ downregulation and BBB permeability in cultured BMVEC.[100,101] Also, treating MS patients with glucocorticosteroids (GC) improved BBB integrity.[102] *In vitro* studies showed that sera from MS patients could induce BBB breakdown by downregulating claudin-5, occludin and upregulating Mmp-9. These effects could be reversed somewhat by GCs.[103]

Novel concepts of BBB regulation

While pathological changes in BBB function are now well understood to occur in a wide range of CNS disorders, less is known about BBB structure and function in common biological processes. For instance, novel studies have begun to decipher the role of the BBB during sleep behavior as well as implicating endogenous miRNAs in maintenance of BBB function.

Sleep-wake cycle

The sleep-wake cycle is a complex process regulated by the body's circadian rhythm. It is thought that sleep functions in energy and nervous system recuperation.[104] Sleep is an essential homeostatic process for humans and sleep loss is associated with increased risk of developing cardiovascular disease, obesity, type 2 diabetes and various neuropsychological disorders.[105-108] Recent studies have found that chronic sleep restriction (CSR), a form of sleep deprivation, can increase BBB permeability. CSR was associated with reduced GLUT-1 and TJ protein expression at the BBB, reduced brain uptake of glucose and increased brain uptake of sodium fluorescein and biotin tracer molecules. Increased BBB permeability and decreased TJ expression were reversed following 24 h of sleep recovery indicating a return of BBB integrity.[109] Parallel work by Gomez-Gonzalez *et al* found that REM sleep restriction increased BBB permeability to Evans blue dye and that brief periods of sleep recovery rapidly restored BBB function. BBB breakdown was associated with invaginations of the plasma membrane called caveolae at brain endothelial cells.[110] Similar work by Xie *et al.* highlighted the role of sleep in regulating waste removal from the interstitial compartments in the brain and spinal cord (the so-called "*glymphatic*" system).[111] These studies indicate the fundamental importance of the sleep-wake cycle in regulating brain homeostasis and maintaining an intact BBB.

miRNA

BBB dysfunction is a major hallmark of many neurological disorders. miRNA's are critically involved in nearly all developmental and physiological processes playing key roles in the post transcriptional regulation of gene expression. Recent studies have identified altered miRNA expression levels in several CNS disorders including brain tumors, neurodegeneration and MS,[112-114] however there has been little insight on the role of miRNA on BBB integrity. Lopez-Ramirez *et al.* identified miR-155 as negatively regulating BBB integrity. In EAE, miR-155 suppression abrogated CNS extravasation of systemic tracers.[115] Reijerkerk *et al* showed that there is a miRNA expression signature in human brain endothelial cells. Specifically, miR-125a-5p regulates TJ expression and improves barrier tightness. Interestingly, the expression levels of these BBB associated miRNAs were all diminished in MS patient capillaries.[116] The therapeutic application of miRNAs has the potential to establish normal brain functioning, particularly in neurological disorders with an endothelial cell-based pathology.

Focused ultrasound

Numerous studies have documented the use of focused ultrasound (FUS) coupled with circulating microbubbles to reversibly open the BBB to enhance delivery of therapeutic agents to the brain. FUS employs low-frequency ultrasound waves to precise neuronal regions to specifically increase BBB permeability by widening TJs.[117] The potential of FUS has previously been reported where dopamine D(4) receptor-targeting antibodies could cross the BBB following FUS. Also, intravenous injection of anti-amyloid β antibodies have been delivered across the BBB following FUS and significantly reduced Aβ plaques 4 days post treatment in a transgenic mouse model of Alzheimer's disease.[118] In fact, a number of therapeutic agents known to be impeded by the BBB have been successfully delivered to the brain using FUS, including chemotherapeutics, siRNA, Herceptin and stem cells.[119-122] Further studies on the precise mechanism of BBB

modulation are required as well as a detailed study of the side-effects of FUS, particularly if to be used in AD pathologies covering large brain areas, and the potential damage to brain tissue before FUS achieves any clinically relevant results. However if these studies are fruitful, FUS could provide a much-needed and non-invasive clinical means for bypassing the BBB and improving drug delivery into the CNS.

Outlook

Significant advances have been made in the past decade relating to BBB modulation to enhance therapeutic drug delivery to the CNS. However, while RNAi remains a promising approach to TJ modulation, extensive studies are required to ensure transient modulation of the BBB doesn't facilitate movement of potentially damaging blood-borne agents to the brain and to ensure toxic side-effects are minimal. Similarly, for novel techniques like FUS, a thorough understanding of the mechanisms behind transient BBB opening are required before the technique can approach the clinic. Despite these requirements, modulation of TJs remains a key area of research from a clinical and neuropathological point of view and promises to make important findings in the treatment of severe CNS disorders like AD, MS and brain tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

Funding was provided by the Science Foundation Ireland (grant no. 12/YI/B2514).

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