frontiers in Cellular Neuroscience

REVIEW
published: 13 September 2021
doi: 10.3389/fncel.2021.661838

Blood-Brain Barrier Dysfunction Amplifies the Development of Neuroinflammation: Understanding of Cellular Events in Brain Microvascular Endothelial Cells for Prevention and Treatment of BBB Dysfunction

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Specialty section:
This article was submitted to Non-Neuronal Cells, a section of the journal Frontiers in Cellular Neuroscience

Received: *31 January 2021*
Accepted: *09 August 2021*
Published: *13 September 2021*

Citation:
Takata F, Nakagawa S, Matsumoto J and Dohgu S (2021) Blood-Brain Barrier Dysfunction Amplifies the Development of Neuroinflammation: Understanding of Cellular Events in Brain Microvascular Endothelial Cells for Prevention and Treatment of BBB Dysfunction. Front. Cell. Neurosci. 15:661838. doi: 10.3389/fncel.2021.661838

Neuroinflammation is involved in the onset or progression of various neurodegenerative diseases. Initiation of neuroinflammation is triggered by endogenous substances (damage-associated molecular patterns) and/or exogenous pathogens. Activation of glial cells (microglia and astrocytes) is widely recognized as a hallmark of neuroinflammation and triggers the release of proinflammatory cytokines, leading to neurotoxicity and neuronal dysfunction. Another feature associated with neuroinflammatory diseases is impairment of the blood-brain barrier (BBB). The BBB, which is composed of brain endothelial cells connected by tight junctions, maintains brain homeostasis and protects neurons. Impairment of this barrier allows trafficking of immune cells or plasma proteins into the brain parenchyma and subsequent inflammatory processes in the brain. Besides neurons, activated glial cells also affect BBB integrity. Therefore, BBB dysfunction can amplify neuroinflammation and act as a key process in the development of neuroinflammation. BBB integrity is determined by the integration of multiple signaling pathways within brain endothelial cells through intercellular communication between brain endothelial cells and brain perivascular cells (pericytes, astrocytes, microglia, and oligodendrocytes). For prevention of BBB disruption, both cellular components, such as signaling molecules in brain endothelial cells, and non-cellular components, such as inflammatory mediators released by perivascular cells, should be considered. Thus, understanding of intracellular signaling pathways that disrupt the BBB can provide novel treatments for neurological diseases associated with neuroinflammation. In this review, we discuss current knowledge regarding the underlying mechanisms involved in BBB impairment by inflammatory mediators released by perivascular cells.

Keywords: blood-brain barrier, pericyte, astrocyte, microglia, oligodendrocyte, tight junction, neuroinflammation, cytokine

INTRODUCTION

Neuroinflammation is widely observed in neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS). Although inflammatory responses in the central nervous system (CNS) are essentially beneficial for repair of damaged brain tissue, inappropriate inflammatory responses can lead to neuronal dysfunction. CNS homeostasis depends on the balance of innate immunity. Innate immunity is the first line of defense against exogenous pathogens. Pathogen recognition receptors (PRRs) expressed on glial cells and neurons become activated by binding to their ligands, pathogen-associated molecular patterns (PAMPs). PRRs also recognize endogenous substances like damage-associated molecular patterns (DAMPs), leading to sterile inflammation of the brain. Binding of DAMPs to PRRs triggers the activation of glial cells (astrocytes and microglia), which is recognized as a hallmark of neuroinflammation and induces the release of inflammatory mediators and cytotoxic factors that contribute to neurodegenerative pathology (Chitnis and Weiner, 2017; Liddelow et al., 2017). Activated glial cells act as a major source of inflammatory mediators and affect the brain microvascular cells, that compose the blood-brain barrier (BBB), as well as neurons. Glial cell-derived inflammatory mediators including cytokines, chemokines, reactive oxygen species (ROS), and lipid mediators influence BBB integrity.

Neuroinflammation and BBB Dysfunction in Various Diseases

Accumulating evidence has indicated that disruption of the BBB is a common feature of neuroinflammation-mediated neurodegeneration. BBB disruption is observed in patients with neurodegenerative diseases including AD (Bowman et al., 2007), PD (Gray and Woulfe, 2015), ALS (Zlokovic, 2008), and MS (Tofts and Kermode, 1991; Waubant, 2006). The BBB is a highly sophisticated system that restricts the transport of certain plasma proteins and immune cells from the blood to the brain parenchyma. BBB disruption is considered to allow lymphocytes, macrophages, and plasma proteins to enter the brain parenchyma. Infiltration of peripheral immune cells was shown to induce microglial activation (Laurent et al., 2017) and neurodegeneration (Mayne et al., 2020). Various neuroimaging molecules or methods for detecting BBB dysfunction and neuroinflammation in neurodegenerative diseases have been well reviewed elsewhere (Sweeney et al., 2018; Werry et al., 2019).

Neuroinflammation and BBB Dysfunction in Traumatic Brain Injury

Traumatic brain injury (TBI) is classified as mild, moderate and severe TBI. BBB dysfunction is widely observed from mild to severe TBI (O'Keeffe et al., 2020). BBB disruption in mild TBI precedes neuroinflammation responses identified by microglia and astrocyte activation (Wu et al., 2021). BBB disruption occurs within hours after brain injury and can be sustained for years (Hay et al., 2015). Extravasation of plasma proteins including fibrinogen, IgG, and albumin has been observed in the brain of patients with TBI in the acute and chronic phase. TBI-induced neuroinflammation responses can be identified by elevated levels of inflammatory mediators, within hours post-injury (Corrigan et al., 2016; Lier et al., 2020). Microglia and astrocytes are activated by the extravasation of albumin to release cytokines/chemokines and matrix metalloproteinases (MMPs) which disrupt the BBB (Ralay Ranaivo et al., 2012).

Neuroinflammation and BBB Dysfunction in Stroke

In acute ischemic stroke, BBB breakdown occurs within several hours of ischemic onset. This allows plasma proteins to enter the brain parenchyma resulting in vasogenic edema. Early BBB breakdown is mainly due to oxidative stress including ROS. ROS modulate tight junction proteins and the cytoskeleton of brain endothelial cells. Subsequently, neuroinflammation processes induce further BBB breakdown over the following 72 h (da Fonseca et al., 2014). Ischemia-induced cell death, DAMPs, miRNA and oxidative stress activate microglia and astrocytes, leading to the production of inflammatory cytokines. Glial cell-derived matrix metalloproteinases (MMPs) contribute to BBB dysfunction through digesting tight junction proteins at this phase. Neuroinflammatory responses in stroke occur over days to weeks (Xing et al., 2012). BBB dysfunction is sustained during the chronic stages of stroke (Strbian et al., 2008).

Following these acute CNS injuries described above, cerebral edema as a result of BBB dysfunction develops through several phases, including ionic edema, vasogenic edema and hemorrhagic conversion (Stokum et al., 2016). Brain endothelial dysfunction contributes to these processes. In ionic edema which occurs with an intact BBB, ions (Na^+ and Cl^-) and water flux are increased though brain endothelial ion channels and transporters such as Na^+/H^+ exchanger, Na^+/K^+-ATPase, Na^+/K^+Cl^- co-transporter, Sur1-Trpm4, GLUT1 and SGLT1. Perivascular astrocytic aquaporin 4 also contributes to ionic edema formation (Haj-Yasein et al., 2011). Vasogenic edema is characterized by BBB breakdown and extravasation of plasma proteins. This subtype of cerebral edema is mediated by MMP-9 and several inflammatory mediators (Stokum et al., 2016). Intracerebral hemorrhage occurs due to a loss of structural integrity of brain microvessels and allows extravasation of all component of blood. Infiltrating erythrocytes and plasma proteins triggers glial activation and neuronal damage.

Neuroinflammation and BBB Dysfunction in Neurodegenerative Diseases

Multiple sclerosis is an autoimmune and neuroinflammatory disease leading to demyelination and neurodegeneration. BBB dysfunction is an early feature of MS pathogenesis (Alvarez et al., 2011) and triggers immune cell infiltration and plasma protein extravasation. This is supported by fibrinogen deposition in developing lesions (Vos et al., 2005). Infiltrated immune cells release various inflammatory mediators and activate astrocytes and microglia, which contribute to progressive MS (Correale et al., 2017). These events would exacerbate BBB dysfunction.

Amyotrophic lateral sclerosis is characterized by progressive motor neurodegeneration in the brain and spinal cord. Various mutant genes are involved in the inflammatory responses which contribute to the pathogenesis of ALS (Beers and Appel, 2019).

Post mortem tissue analysis revealed that BBB dysfunction occurs in gray and white matter of patient with ALS (Garbuzova-Davis et al., 2012). ALS model mice showed that damage in neurovascular unit occurred prior to neurodegeneration (Miyazaki et al., 2011). Activation of microglia and astrocytes in living patients with ALS was detected by use of ^{11}C-PK11195 PET imaging (Turner et al., 2004) and ^{11}C-Deuterium-L-Deprenyl (DED) PET imaging (Johansson et al., 2007), respectively.

The main pathological hallmarks of PD are dopaminergic neuronal loss and deposition of Lewy bodies, which are mainly composed of aggregated α-synuclein. α-Synuclein induces neuroinflammation associated with PD development (Su et al., 2008). Extracellular α-synuclein activates microglia and astrocytes through Toll-like receptors to release inflammatory mediators (Lee et al., 2010; Fellner et al., 2013). Monomeric α-synuclein induces the release of inflammatory mediators from brain pericytes leading to rat brain endothelial barrier dysfunction (Dohgu et al., 2019). One previous study reported that fibrillar α-synuclein-induced dysfunction of the human brain endothelial barrier when co-cultured with neurons (Kuan et al., 2016). In addition, BBB leakage of fibrin and hemosiderin was observed in the striatum of PD patients (Gray and Woulfe, 2015). Subtle BBB disruption in PD patients is observed in the substantia nigra, white matter and posterior cortical regions (Al-Bachari et al., 2020).

Alzheimer's disease is characterized by the increased production and deposition of misfolded protein amyloid β (Aβ) and tau, and extracellular deposits of Aβ induce neuroinflammation. Activated microglia and astrocytes, as well as the release of various inflammatory cytokines around Aβ plaques, have been observed in AD brains (da Fonseca et al., 2014). In addition, Aβ is reported to impair the integrity of BBB (Chan et al., 2018; Cuevas et al., 2019). According to the two-hit vascular hypothesis in AD (Nelson et al., 2016), BBB dysfunction leads to the infiltration of neurotoxic substances in brain parenchyma and triggers neuroinflammation. Deposition of harmful plasma proteins like fibrin has been observed in a wide range of neurodegenerative diseases (Petersen et al., 2018). Fibrinogen entering the CNS was found to promote microglial activation, leading to the release of inflammatory mediators that caused neuronal damage in AD mice (Merlini et al., 2019). Fibrinogen is not expressed in the brain, and therefore BBB dysfunction is necessary for plasma fibrinogen to cross the BBB and enter the brain parenchyma. Measurement of BBB integrity with dynamic contrast-enhanced magnetic resonance imaging revealed increased BBB permeability to a gadolinium-based contrast agent in the hippocampus in patients with mild cognitive impairment (Montagne et al., 2015). These findings suggest that a lesser extent of BBB dysfunction that allows penetration of small molecules, but not large molecules like fibrinogen, precedes the development of AD.

BBB Dysfunction Amplifies Neuroinflammation

It remains unclear whether the lesser extent of BBB dysfunction is a cause or a result of the neuroinflammation and neurodegeneration. Importantly, systemic administration of lipopolysaccharide (LPS), a gram-negative bacterial endotoxin, caused increased BBB permeability to sodium fluorescein in parallel with an increased number of activated microglia (Nishioku et al., 2009). Ju et al. (2018) reported that increased BBB permeability induced by mannitol led to microglial activation. At the least, these findings suggest that a leaky BBB contributes to microglial activation and has the potential for understanding how to reduce the exacerbation of sterile brain inflammation initiated by activation of innate immune responses in glial cells. Once glial cells are activated without any impairment of the brain endothelial barrier function, glial activation triggers BBB dysfunction through the release of inflammatory mediators, and in turn, the leaky BBB allows infiltration of blood-borne inducers of glial activation into the brain, resulting in aggravated brain inflammation and glial activation (**Figure 1**). Thus, BBB dysfunction positively amplifies the development of neuroinflammation, rather than being a pathological result of glial activation and neuroinflammation.

Given that the development of neurodegeneration is associated with neuroinflammation, pharmacological manipulation of BBB dysfunction would be a promising supportive treatment for neurodegenerative diseases. The barrier function of the BBB is mainly determined by tight junctions that seal paracellular gaps between brain microvascular endothelial cells (BMECs). Tight junctions are mainly composed of occludin and claudin-5 transmembrane proteins, and zonula occludens cytoplasmic scaffold proteins, such as ZO-1. The formation and maintenance of tight junctions is regulated by integration of multiple intercellular communications between BMECs and other brain cell types (pericytes, astrocytes, microglia, oligodendrocytes [OLs], and neurons) through various soluble factors (Luissint et al., 2012; Sweeney et al., 2019). However, these cells surrounding BMECs also participate in the process of BBB

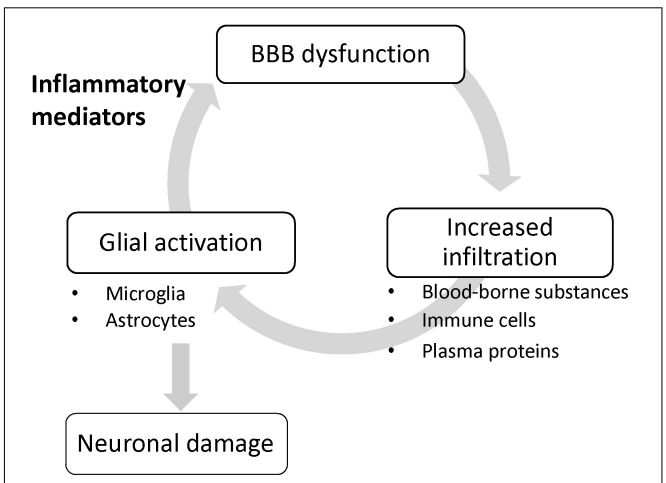

FIGURE 1 | BBB dysfunction amplifies glial activation. Once glial cells are activated without any impairment of the brain endothelial barrier function, glial activation triggers BBB dysfunction through the release of inflammatory mediators, and in turn, the leaky BBB allows infiltration of blood-borne inducers of glial activation into the brain, resulting in aggravated glial activation.

dysfunction through the release of various substances that affect BBB integrity, as described below and presented in **Figure 2**. In this review, we propose two components as therapeutic targets for BBB dysfunction: the non-cellular component, including inflammatory mediators derived from the surrounding cells, and the cellular component, referring to intracellular signaling pathways that lead to disruption of brain endothelial barrier integrity. In particular, we will focus on understanding of the intracellular signaling pathways that lead to BBB dysfunction for the purpose of pharmacologically targeting BMECs. Specifically, we will outline how the brain endothelial barrier is disrupted by all other brain parenchymal cell types, and then discuss inflammatory mediators and related intracellular signaling pathways that lead to BBB dysfunction, mainly with reference to *in vitro* studies, to clarify the cellular events in BMECs.

COMMUNICATIONS BETWEEN BMECs AND OTHER CELL TYPES IN NEUROVASCULAR UNITS LEADING TO BBB DYSFUNCTION

Pericytes

Pericytes surround brain microvessels and communicate with BMECs to stabilize functional microvessels through physical

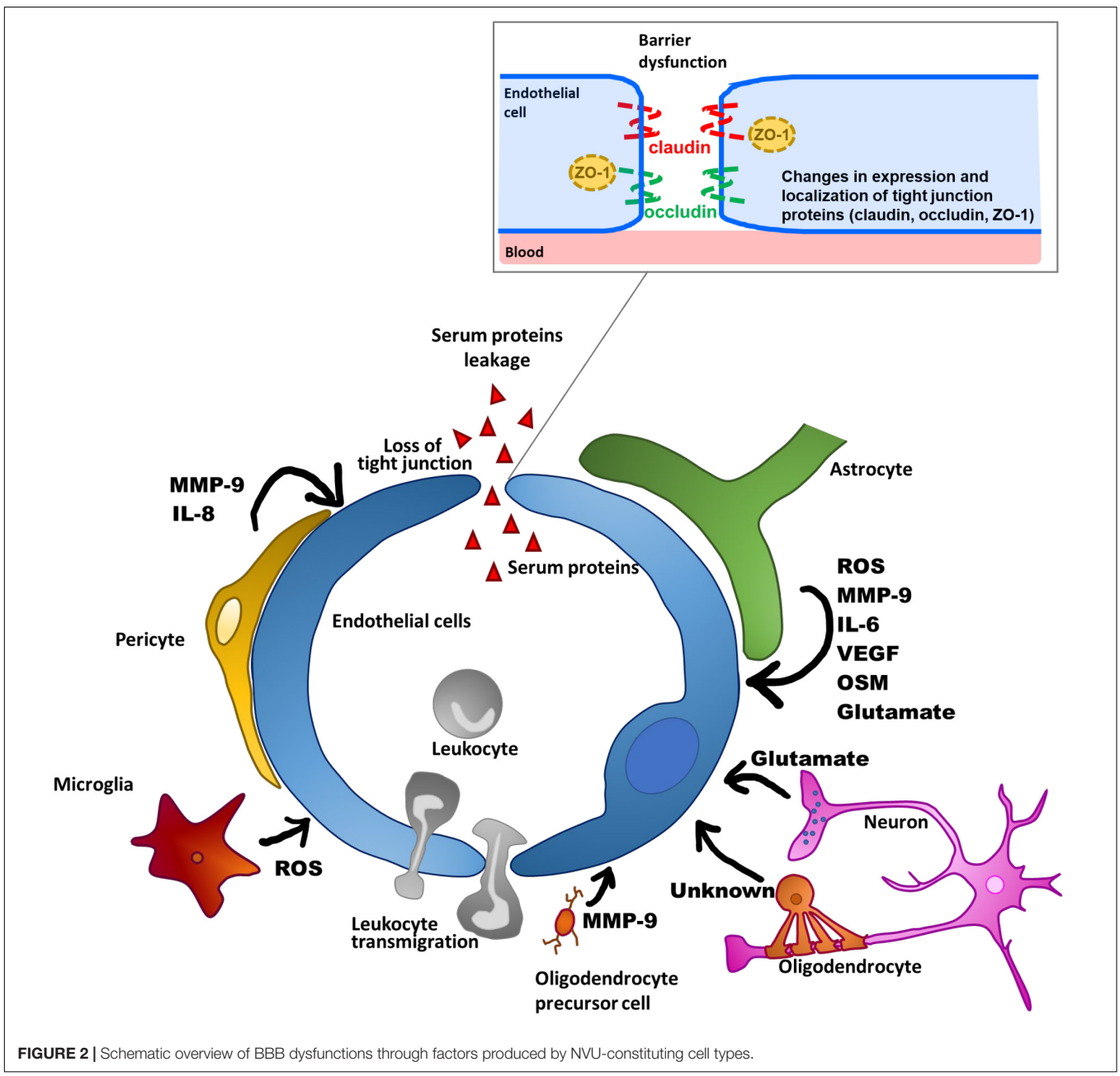

FIGURE 2 | Schematic overview of BBB dysfunctions through factors produced by NVU-constituting cell types.

contacts such as *N*-cadherin-dependent adherens junctions and connexin 43-dependent gap junctions, as well as paracrine signaling *via* soluble factors and their receptors (Caruso et al., 2009; Navarro et al., 2016; Perrot et al., 2020). Previous studies of pericyte-deficient transgenic mice revealed BBB hyperpermeability to neurotoxic serum proteins like fibrin, thrombin, and plasmin, indicating that the presence of brain pericytes in brain microvessels is integral to BBB integrity and normal neuronal function (Bell et al., 2010). Furthermore, in the cortex of pericyte-deficient mice, neuroinflammatory molecules like tumor necrosis factor-α (TNF-α), monocyte-chemoattractant protein (MCP)-1, and intercellular adhesion molecule 1 (ICAM-1) were elevated, and increased Iba1 expression, an index of microglial activation, was observed with age. These findings suggest that the brain pericyte deficit led to impaired communication with BMECs and evoked neuronal inflammation through BBB impairment. Indeed, sepsis model mice showed microglial activation and brain pericyte detachment from the basal lamina following elevation of BBB permeability (Nishioku et al., 2009). Thus, the insufficient pericyte coverage of microvessels observed in CNS disorders like ischemia and TBI (Fernández-Klett et al., 2013; Zehendner et al., 2015) may be implicated in the development of BBB dysfunction and brain inflammation.

Pericytes in the brain microvessels are also likely to contribute to induction of BBB dysfunction under pathological conditions, even though brain pericyte detachment from the basal lamina was not observed. Brain pericytes express several receptors for endogenous cytokines, pathogens, and pathogenic molecules like TNF-α, interleukin (IL)-1β, IL-8, LPS, and α-synuclein, and are implicated in the production of inflammation-related molecules (Matsumoto et al., 2014; Navarro et al., 2016; Dohgu et al., 2019). Interestingly, brain pericytes were much more responsive than other cell types constituting the BBB, such as BMECs and astrocytes, to TNF-α and thrombin stimulation in producing MMP-9 (Takata et al., 2011; Machida et al., 2015). Under pathological conditions, production of MMP-9 in the brain leads to BBB impairment through rearrangement and degradation of tight junction-associated proteins (Bauer et al., 2010; Zhang et al., 2012). Obesity-associated diabetes mice had elevated thrombin levels in their brain and BBB dysfunction, suggesting that BBB dysfunction could be attributed to the release of MMP-9 by thrombin-reactive pericytes (Machida et al., 2017b). Indeed, *in vitro* studies demonstrated that thrombin-induced MMP-9 production by brain pericytes led to increased BBB permeability through morphological disorganization of ZO-1 and decreased expression of ZO-1 and occludin in BMECs (Machida et al., 2017a,b). Based on these findings, the possibility that pericytes localized in microvessels can act as inducers and amplifiers of BBB dysfunction through the release of molecules that lead to increased BBB permeability in BMECs under pathological conditions should be considered.

Among the CNS-constituting cell types, including BMECs, astrocytes, and microglia, pericytes are the most sensitive to TNF-α and have a unique cytokine and chemokine release profile, characterized by substantial release of IL-6 and macrophage inflammatory protein-1α (Matsumoto et al., 2014). Furthermore, in a pericyte/microglia co-culture system, TNF-α treatment induced increased expression of inducible nitric oxide synthase (iNOS) and IL-1β in the microglia as an index of microglial activation, whereas the induction was not observed in microglia monoculture in the absence of pericytes. TNF-α-sensitive pericytes evoked microglial activation accompanied by increased iNOS through cooperation between the IκB-nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) and Janus-activated kinase (JAK)-signal transducer and activator of transcription 3 (STAT3) pathways (Matsumoto et al., 2018). In TBI mice, an injury-induced pericyte response characterized by rapidly increased platelet-derived growth factor receptor β (PDGFRβ) expression was observed, followed by activation of microglia (Sakai et al., 2021). Inhibition of PDGFRβ signaling in reactive pericytes in TBI mice suppressed the microglial activation, raising the possibility that the response of pericytes to pathological insults mediates the process of brain inflammation including microglial activation (Sakai et al., 2021). Taken together, accumulating evidence suggests that brain pericytes play a key role as mediators of neuroinflammation.

As well as contributing to propagation of inflammatory responses in the brain *via* the release of inflammatory mediators and activation of microglia, brain pericytes are essential players in the regulation of leukocyte diapedesis in brain tissue (Rudziak et al., 2019). Brain pericytes stimulated by inflammatory mediators like TNF-α, IL-1β, and LPS produce IL-8 and MMP-9 (Pieper et al., 2013). In a pericyte/endothelial cell coculture system, these inflammatory mediators increased neutrophil transmigration, while the addition of neutralizing antibodies against IL-8 attenuated the enhanced neutrophil transmigration. Meanwhile, inhibition of MMP-9 derived from brain pericytes treated with these mediators enhanced adhesion of neutrophils to pericytes, suggesting the possibility that MMP-9 derived from pericytes releases neutrophils attached to pericytes in the brain parenchyma. Collectively, inflammatory reactive pericytes may allow leukocytes to breach the BBB through the released IL-8 and MMP-9, leading to penetration of leukocytes into the brain and the subsequent development of neuroinflammation.

Astrocytes

Astrocytes exist around brain microvessels and are one of the important cellular constituents of the BBB together with brain pericytes. The terminal processes of astrocytes, known as endfeet, express potassium channels and aquaporin-4, which support BBB functions by controlling the ion and water balance (Michinaga and Koyama, 2019). In the healthy adult brain, it is commonly assumed that astrocytes play a role in maintaining BBB integrity, because the integrity in an *in vitro* BBB model involving co-culture of endothelial cells and astrocytes was greater than that in monoculture of endothelial cells (Nakagawa et al., 2009). Studies demonstrated that astrocyte-derived soluble factors, such as glial-derived neurotrophic factor, fibroblast growth factor, and angiopoietin 1, were partly responsible for modulating BBB functions (Igarashi et al., 1999; Lee et al., 2003; Murakami et al., 2008; Lecuyer et al., 2016).

Under pathological conditions, including ischemic injury, reactive astrocytes were shown to be enhanced

(Liu M. et al., 2020) and to secrete inflammatory factors like vascular endothelial growth factor A (VEGF-A), MMPs and MCP-1 (Shan et al., 2019), which can directly or indirectly aggravate BBB disruption. Mice subjected to middle cerebral artery occlusion exhibited increased VEGF-A and MMP-9 expression in astrocytes. Astrocytes cultured under oxygen-glucose deprivation plus reoxygenation conditions produced VEGF-A and MMP-9 *via* the JAK2/STAT3 signaling pathway. In both *in vivo* and *in vitro* brain ischemic models, reductions in VEGF-A and MMP-9 in astrocytes were shown to ameliorate the reduced BBB integrity induced by ischemic stress (Shan et al., 2019). Taken together, these findings directly implicate the astrocyte response to ischemic injury in BBB dysfunction through the production of VEGF-A and MMP-9. Furthermore, cytokines released by reactive astrocytes induce tight junction reorganization in the BBB. IL-6 derived from astrocytes elevated BBB permeability and induced the release of chemokines (Takeshita et al., 2017). Another cytokine in the IL-6 family, oncostatin M (OSM), was expressed in activated astrocytes from MS patients (Ensoli et al., 2002). Recently, OSM was also shown to increase BBB permeability and decrease expression of claudin-5 (Takata et al., 2008, 2018). Therefore, cytokines belonging to the IL-6 family are secreted by astrocytes and mediate BBB impairment during neuroinflammation.

A hallmark of cerebral inflammatory disorders such as MS and encephalopathy associated with virus infection is the appearance of immune cells derived from circulating blood in the brain, together with a loss of BBB integrity (Jaureguiberry-Bravo et al., 2016). Stimulation with TNF-α, IFNγ, and IL-1β enabled astrocytes to release chemoattractants including MCP-1 (Weiss et al., 1998). An *in vitro* study involving co-cultures of endothelial cells and astrocytes examined whether astrocytes can directly influence the transmigration of peripheral blood mononuclear cells (PBMCs) across the endothelial layer. Astrocyte-derived MCP-1 mediated greater monocyte migration across these co-cultures (Weiss et al., 1998). Conversely, in an *in vitro* co-culture model, the presence of astrocytes reduced Aβ-induced PBMC transmigration across endothelial layers as well as Aβ-induced ICAM-1 expression (Spampinato et al., 2019). These findings raise the possibility that astrocyte-derived factors play dual roles in mediating the transmigration of PBMCs across the BBB. Overall, astrocytes participate in both BBB impairment after pathological insults and the development of neuroinflammation.

Microglia

Microglia, as resident immune cells in the brain, play important roles in primary immune responses to protect the CNS against insults such as infection, ischemia, injury, and disease. Resting microglia routinely survey their environments, and are ready to undergo rapid transformation to their activated state in response to inflammatory stimuli (Kettenmann et al., 2011). Activated microglia produce various inflammatory mediators, including cytokines, chemokines, and ROS, and subsequently induce neuroinflammation and neuronal damage (Subhramanyam et al., 2019). Emerging evidence has suggested that microglial activation and neuroinflammation are also associated with BBB dysfunction under pathological conditions (Zlokovic, 2008). Impairment of BBB integrity was shown to occur in parallel with microglial activation in LPS-induced septic encephalopathy model mice (Nishioku et al., 2009). Sumi et al. (2010) established a co-culture model using BMECs and rat primary microglia to investigate the direct interactions between activated microglia and BMECs. They found that LPS-activated microglia induced hyperpermeability to sodium fluorescein, a marker of the paracellular route between adjacent endothelial cells, and decreased the activity of P-glycoprotein in their co-culture system (Sumi et al., 2010; Matsumoto et al., 2012). The alterations by which LPS-activated microglia impaired the endothelial barrier functions were blocked by DPI, an inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Sumi et al., 2010; Matsumoto et al., 2012). They also found that blockade of TNF-α suppressed endothelial dysfunction by LPS-activated microglia (Nishioku et al., 2010). These findings indicate that microglial activation directly leads to brain endothelial barrier dysfunction. A recent study showed that LPS-activated microglia interacted with astrocytes and dramatically increased chemokine release in co-cultures of LPS-activated microglia and astrocytes. Furthermore, addition of LPS-activated microglia to the astrocyte layer in a BBB model (BMEC/pericyte/astrocyte triculture) decreased transendothelial electrical resistance (TEER) and increased sodium fluorescein permeability in the model (Shigemoto-Mogami et al., 2018). Thus, activated microglia directly and/or indirectly disrupt the integrity of the BBB through elevation of pro-inflammatory factors such as cytokines, chemokines and ROS.

Oligodendrocyte Lineage Cells

Oligodendrocyte lineage cells including OLs and oligodendrocyte precursor cells (OPCs) are closely located to BMECs (Pham et al., 2012; Maki et al., 2015). Therefore, it is possible that oligodendrocyte lineage cells exert similar crosstalk with BMECs to pericytes and astrocytes, for the regulation of BBB functions. OPC-specific TGF-β-deficient mice exhibited loss of BBB functions and cerebral hemorrhage (Seo et al., 2014), suggesting that OPCs may be implicated in the upregulation of BBB functions through release of TGF-β. Indeed, in an *in vitro* study, OPC-conditioned medium decreased BBB permeability through BMECs and increased the expression of tight junction-associated proteins such as claudin-5, ZO-1, and occludin (Seo et al., 2014). Furthermore, OPCs exposed to PDGF-BB released from BMECs induced decreased BBB permeability in BMECs, indicating that BMECs can help OPCs to modulate BBB functions (Kimura et al., 2020). Thus, it is considered that the crosstalk between OPCs and BMECs *via* their secreted factors plays important roles in BBB formation and maintenance. In mice with cerebral ischemic injury, OPCs in the damaged white matter expressed increased MMP-9 at an early stage after injury and OPC-derived MMP-9 caused BBB impairment and neutrophil infiltration into the brain, leading to the development of brain dysfunctions, including demyelination (Seo et al., 2013). These findings suggest that stressed OPCs mediate BBB impairment in the injured white matter. Meanwhile, a recent study revealed that transplantation of healthy OPCs protected against BBB disruption in a mouse model of brain ischemia through activation

of the Wnt7a/β-catenin pathway (Wang et al., 2020). These reports suggest that OPCs have a biphasic effect on BBB functions. Therefore, the endothelial BBB functions may be perturbed under pathological conditions in accordance with alterations in OPC functions in the CNS.

Besides OPCs, it was reported that the presence of OLs decreased BBB permeability *via* BMECs through unknown soluble factors (Kimura et al., 2020). Hence, OLs are expected to be implicated in the alterations to BBB functions under pathological conditions. However, there is no direct evidence linking altered interactions between BMECs and OLs with BBB impairment. Further experiments are required to understand the role of OLs in BBB pathology.

Neurons

Brain microvessels has been shown to be positioned within 15 μm of every neuron. This proximity allows neuronal activity to regulate the brain endothelial function (Tsai et al., 2009). BMECs express receptors for neurotransmitters such as glutamate (Lu et al., 2019) and γ-aminobutyric acid (GABA) (Won et al., 2013) and can therefore sense neurotransmitters released from activated neurons. Increased BBB permeability was observed in neurological conditions associated with neuronal hyperexcitability during ischemia, trauma, and epileptic seizure (Tomkins et al., 2008; Friedman, 2011; Schoknecht et al., 2014). These findings raise the possibility that the occurrence of neural activity affects BBB functions. Exposure to glutamate, a major excitatory neurotransmitter, was shown to elicit reduced barrier function in cultured BMECs through activation of *N*-methyl-D-aspartic acid (NMDA) receptors following alterations in occludin expression and phosphorylation (András et al., 2007). Meanwhile, direct cortical imaging in rats revealed that cortical application of glutamate elevated BBB permeability to sodium fluorescein in a dose-dependent manner (Vazana et al., 2016). Taken together, these findings support the view that glutamate derived from activated neurons plays an important role in the mediation of BBB functions. Astrocytes also have the ability to release glutamate and drive this signaling by activating glutamate receptors in endothelial cells (Lu et al., 2019). Thus, further investigations are required to determine whether this neuronal activity is directly causative for the functional phenotype of the BBB under physiological and pathological conditions.

INFLAMMATORY MEDIATORS INVOLVED IN BBB DYSFUNCTION AND RELATED INTRACELLULAR SIGNALING PATHWAYS IN BMECs

IL-1β

IL-1β is widely recognized as a proinflammatory mediator associated with leakage of the BBB in CNS pathology in various animal studies. Intrastriatal injection of IL-1β induced a transient increase in BBB permeability (Blamire et al., 2000). IL-1β treatment of BMECs caused an increase in BBB permeability to sodium fluorescein and dextran (Beard et al., 2014; Ni et al., 2017) and a decrease in TEER (de Vries et al., 1996). These findings suggest that IL-1β acts directly on BMECs, resulting in increased BBB permeability. Although IL-1 type I and type II receptors are both expressed on BMECs, IL-1 type II receptors are involved in IL-1β transport across BMECs (Skinner et al., 2009). A 6-h exposure of BMECs to IL-1β did not affect the expression levels of claudin-5 and ZO-1, but did lead to IL-1β-induced brain endothelial barrier dysfunction associated with elevated phosphorylation of ZO-1 through activation of protein kinase C (PKC)θ (Rigor et al., 2012). A longer (24-h) exposure of BMECs to IL-1β decreased claudin-5 expression through transcriptional repression of β-catenin and FoxO1. IL-1β increased both β-catenin and FoxO1 nuclear translocation leading to decreased expression of claudin-5 mRNA. This suppression was dependent on non-muscle myosin light chain kinase (Beard et al., 2014). Furthermore, IL-1β decreased another tight junction-associated protein, occludin. The phosphorylation levels of ERK1/2 and p38 mitogen-activated protein kinase (MAPK) in BMECs were transiently elevated at 15–30 min after IL-1β treatment (Ni et al., 2017), suggesting that MAPK signaling is also involved in the IL-1β-induced BBB dysfunction (see **Figure 3**). Furthermore, IL-1β induced the expression of other inflammatory mediators including TNF-α, CXCL10, IL-8, IL-6, MCP-1, G-CSF, VEGF, and GM-CSF in BMECs (O'Carroll et al., 2015; Krasnow et al., 2017). Although it is not completely understood whether all of these BMEC-derived soluble factors induced by IL-1β disrupt the barrier integrity of BMECs, these factors may participate in the destructive effect of IL-1β on the BBB in an autocrine manner. Importantly, the ability of BMECs to secrete IL-1β in response to oxygen glucose deprivation and TNF-α should also be considered in IL-1β-induced BBB dysfunction (Matsumoto et al., 2014; Chen et al., 2017).

TNF-α

TNF-α is well-known as a proinflammatory cytokine that induces BBB dysfunction. Accumulating evidence has indicated that various systemic and CNS inflammatory diseases induce BBB dysfunction through increased levels of TNF-α. An *in vitro* study using cultured BMECs showed that TNF-α directly interacted with TNF receptor I (p55) or TNF receptor II (p75) (Lucas et al., 1998) to generate increased BBB permeability (Deli et al., 1995b; Lopez-Ramirez et al., 2012; Ni et al., 2017) and decreased TEER (de Vries et al., 1996; Wong et al., 2004). Both TNF receptors act cooperatively to transport TNF-α across the BBB (Pan and Kastin, 2002). TNF-α decreased the expression of the tight junction-associated proteins ZO-1 (Rochfort and Cummins, 2015), claudin-5 (Aslam et al., 2012; Camire et al., 2015), and occludin (Ni et al., 2017). The underlying mechanisms for the TNF-α-induced reductions in tight junction-associated protein expression involve various intracellular signaling pathways. TNF-α reduced claudin-5 promoter activity and mRNA expression through NF-κB signaling (Aslam et al., 2012). Meanwhile, phosphatidylinositol-3 kinase (PI3K) inhibition with LY294002 attenuated the TNF-α-induced loss of claudin-5 expression in BMECs (Camire et al., 2015). These findings suggest that activation of NF-κB and PI3K induces BBB dysfunction mediated by TNF-α. BAY11-7058,

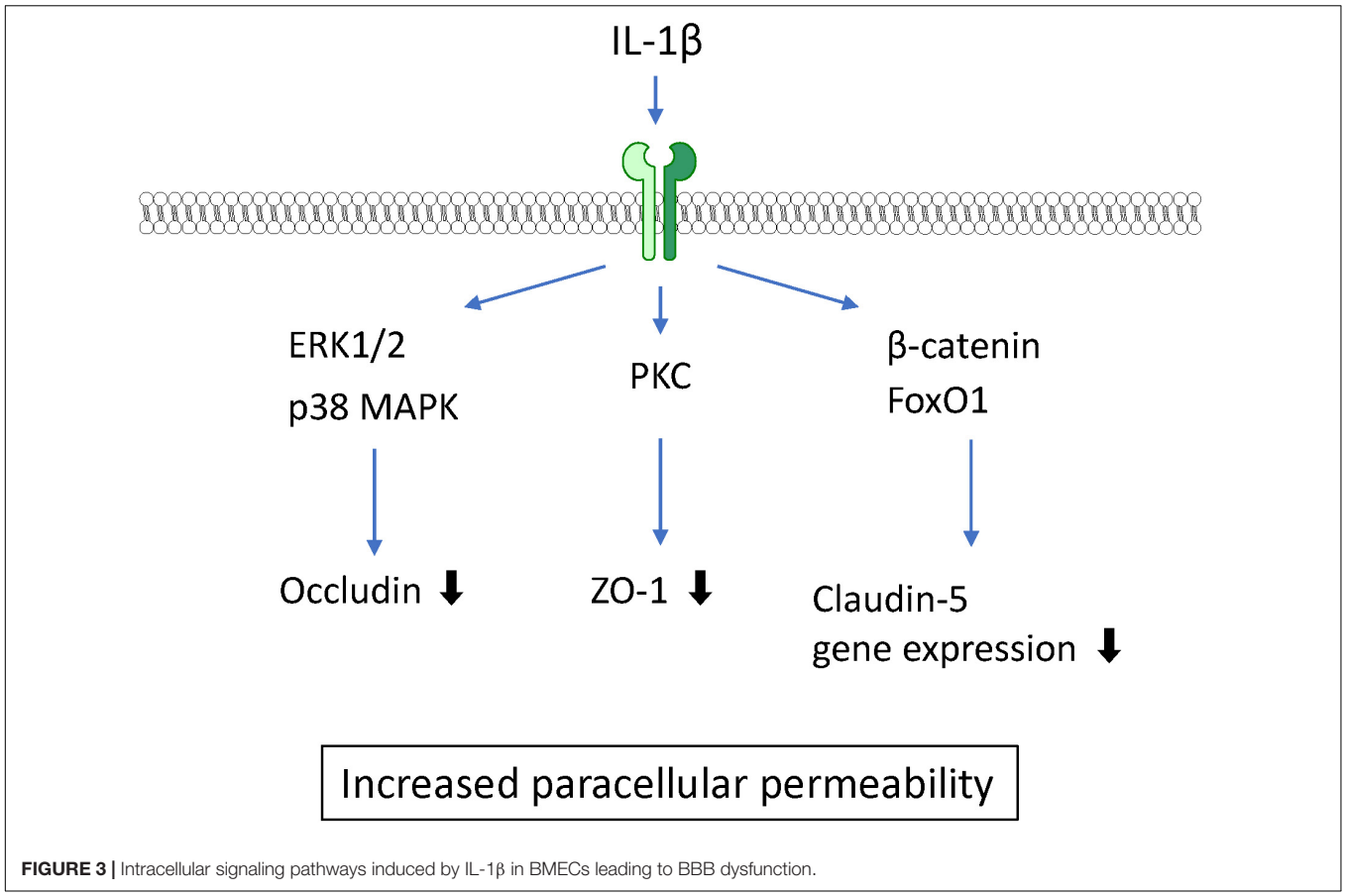

FIGURE 3 | Intracellular signaling pathways induced by IL-1β in BMECs leading to BBB dysfunction.

an NF-κB inhibitor, inhibited the TNF-α-induced increase in BBB permeability (Coelho-Santos et al., 2015). Further studies involving cultures of BMECs are needed to determine whether pharmacological or genetic inhibition of PI3K activation in BMECs is linked to the attenuation of the increased BBB permeability induced by TNF-α. Although both ERK1/2 and p38 MAPK were phosphorylated by TNF-α, only p38 MAPK inhibition with the pharmacological inhibitor SB202190 attenuated the decreased expression of occludin induced by TNF-α without an inhibitory effect on the TNF-α-induced increase in paracellular permeability (Ni et al., 2017). TNF-α-induced activation of ERK1/2 likely mediates an increase in transcytosis (Miller et al., 2005) rather than an increase in paracellular permeability. The c-Jun *N*-terminal kinases (JNK) inhibitor SP600125 partially prevented the TNF-α-induced increase in paracellular permeability (Lopez-Ramirez et al., 2012). TNF-α also activated protein tyrosine kinase and PKC in BMECs (Hudson et al., 1996). Inhibition of PKC attenuated the TNF-α-induced endocytosis (Defazio et al., 2000), but not the paracellular hyperpermeability to fluorescein isothiocyanate (FITC)-dextran (Lopez-Ramirez et al., 2012) in BMECs. However, the PKCα/RhoA pathway mediated the TNF-α-induced F-actin rearrangement leading to decreased TEER (Peng et al., 2011).

In addition to TNF-α-activated intracellular signaling pathways, other factors are involved in the TNF-α-induced BBB dysfunction. Treatment of BMECs with TNF-α induced the production of IL-6, leading to BBB disruption (Rochfort et al., 2016). Involvement of NADPH oxidase and ROS were also reported by the same research group (Rochfort et al., 2014; Rochfort and Cummins, 2015). Alkabie et al. (2016) reported that secreted protein acidic and rich in cysteine (SPARC), a cell-matrix-modulating protein, contributed to the TNF-α-induced BBB dysfunction. They found that TNF-α increased the expression of SPARC in BMECs. SPARC exhibited increased paracellular permeability in parallel with decreased expression of tight junction proteins (Alkabie et al., 2016). The TNF-α-induced decrease in expression of cellular prion protein (PrP^c), a non-pathogenic cellular isoform constitutively expressed in BMECs, mediated the decreased expression of occludin and claudin-5 (Megra et al., 2018). Although the structural links between PrP^c and tight junction-associated proteins are unclear, such links are supported by evidence that PrP^c knockdown in BMECs altered the localization of tight junction proteins. TNF-α induced the production of various inflammatory mediators, including IL-6, IL-17, INF-γ, and MCP-1, in BMECs (Matsumoto et al., 2014). Indeed, combination with other inflammatory mediators (IL-6 and IL-17) potentiated the TNF-α-induced loss of BBB integrity through activation of NF-κB (Voirin et al., 2020). Co-treatment with IFN-γ and TNF-α induced hyperpermeability through activation of caspase-3 and caspase-9 (Lopez-Ramirez et al., 2012) (see **Figure 4** and **Table 1**).

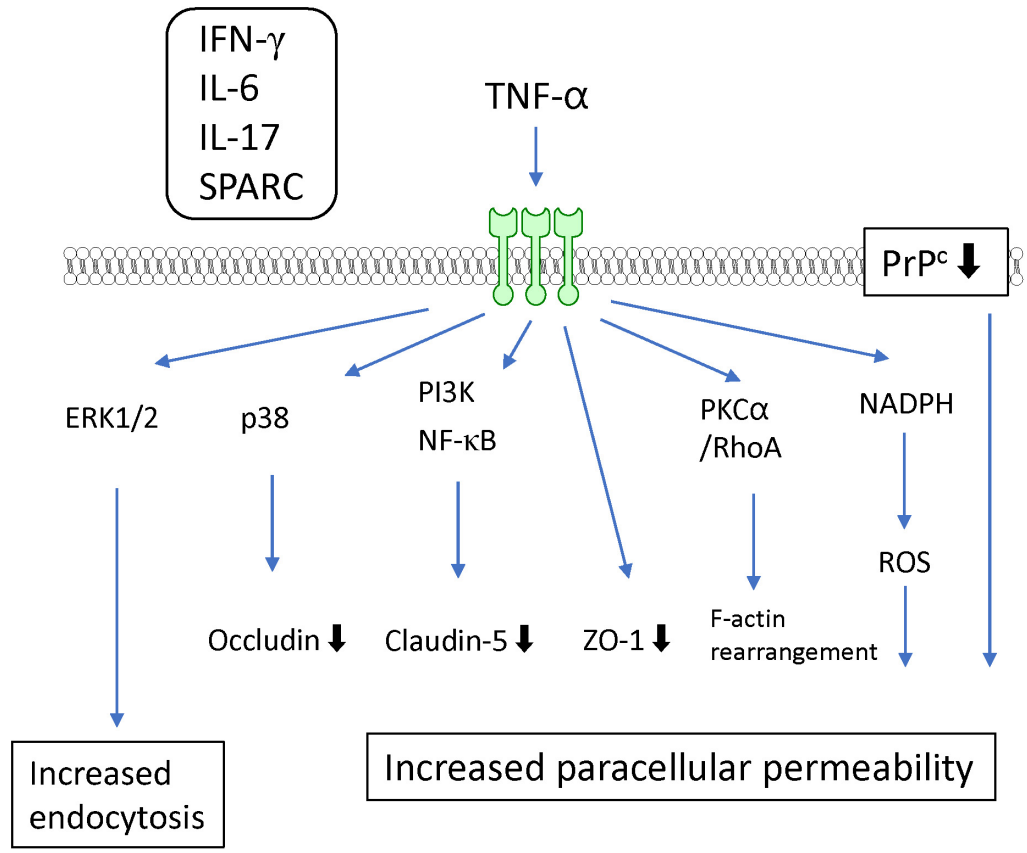

FIGURE 4 | Intracellular signaling pathways induced by TNF-α in BMECs leading to BBB dysfunction. TNF-α induced release of IFN-γ, IL-6, IL-17 and SPARC by BMECs. They contribute to TNF-α-induced BBB dysfunction.

IL-6

IL-6 induced an increase in BMEC permeability and a decrease in expression of the tight junction proteins claudin-5, occludin, and ZO-1 (Voirin et al., 2020). The decreased expression of tight junction proteins induced by IL-6 was mediated by ROS generation in BMECs (Rochfort et al., 2014). In addition, endogenous IL-6 secreted by BMECs contributed to the dysfunction of barrier integrity in an autocrine manner through its receptor glycoprotein 130 (gp130) (Dohgu et al., 2011; Rochfort et al., 2016). However, Takata et al. (2018) reported that BMECs were less sensitive to IL-6 at the same concentration as OSM, another member of the IL-6 family. This lower sensitivity of BMECs to IL-6 could be explained by the lower activation level of STAT3 induced by IL-6. Although IL-6 itself possesses the ability to induce BBB dysfunction, IL-6 may act as a potentiator of other inflammatory mediators such as TNF-α and IL-17 and/or an inducer of microglial activation (Matsumoto et al., 2014, 2018) (see **Table 1**).

Oncostatin M

Oncostatin M is a member of the IL-6 cytokine family. OSM receptors are expressed on BMECs and form heterodimers with gp130 to transduce OSM signaling (Bauer et al., 2007; Takata et al., 2018). When BMECs were treated with OSM, increased BBB permeability, reduced TEER, and decreased expression of claudin-5, a tight junction-associated protein, were observed (Takata et al., 2008, 2018). In MS and TBI associated with neuroinflammation, OSM was elevated in the brain and BBB functions were impaired (Ruprecht et al., 2001; Minagar and Alexander, 2003; Oliva et al., 2012; Logsdon et al., 2018). Taken together, these findings implicate elevated OSM in the brain in the impairment of BBB functions under pathological conditions with neuroinflammation. Our previous study demonstrated that OSM was an intensive molecule for induction of BBB dysfunction *via* prolonged activation of STAT3 following JAK activation (Takata et al., 2018). Although other cytokines belonging to the IL-6 family, IL-6 and LIF, also induced transient activation of STAT3 in BMECs, their activity for downregulation of BBB functions was markedly lower than that of OSM. These findings suggest that prolonged OSM-induced STAT3 activation is largely conducive to impairment of BBB functions. Thus, under pathological conditions accompanied by increased OSM and activation of STAT3 in the brain, impaired BBB functions could allow harmful molecules to penetrate into the brain.

Besides the downregulation of tight junction-associated proteins, the expression of ICAM-1, which controls immune cell trafficking across the BBB, was increased after exposure of

TABLE 1 | Inflammatory mediators involved in BBB dysfunction and related cellular events in BMECs.

Mediators	Species	Cellular events in BMECs	References
IL-1β	Human	Permeability (FITC-Dextran) ↑ TEER ↓ Occludin expression ↓ Phosphorylation levels of ERK1/2 and p38 MAPK ↑	Ni et al., 2017
	Human	TEER ↓ Claudin-5 and ZO-1 expression → Phosphorylation of ZO-1 through activation of PKCθ ↑	Rigor et al., 2012
	Porcine	TEER ↓ Transport of IL-1β through IL-1 type II receptor	Skinner et al., 2009
	Rat	TEER ↓	de Vries et al., 1996
	Mouse	Permeability (sodium fluorescein, TRITC-dextran) ↑ Permeability (albumin) → TEER ↓ Claudin-5 expression ↓ β-catenin and FoxO1 nuclear translocation ↑	Beard et al., 2014
TNF-α	Human	Permeability (FITC-dextran) ↑ TEER ↓ JNK signaling ↑ PKC signaling ↑ (not associated with TNF-α induced hyperpermeability)	Lopez-Ramirez et al., 2012
	Human	Permeability (FITC-dextran) ↑ TEER ↓ Occludin expression ↓ p38MAPK signaling ↑	Ni et al., 2017
	Human	TEER ↓	Wong et al., 2004
	Human	Permeability (FITC-dextran) ↑ Occludin expression ↓ Claudin-5 expression ↓ ROS ↑ NADPH oxydase ↑ ZO-1 expression ↓ IL-6 expression ↑	Rochfort et al., 2014 Rochfort and Cummins, 2015 Rochfort et al., 2016
	Human	Expression of secreted protein acidic and rich in cysteine (SPARC) ↑ Permeability (FITC-dextran) ↑ TEER ↓ ZO-1 expression ↓ Occludin expression ↓	Alkabie et al., 2016
	Human	Expression of cellular prion protein (PrPc) ↓ Permeability (FITC-dextran) ↑	Megra et al., 2018
	Bovine	Permeability (sucrose) ↑ Permeability (inulin) ↑	Deli et al., 1995b
	Bovine	Transcytosis (FITC-holotransferrin) ↑ ERK1/2 signaling ↑	Miller et al., 2005
	Rat	TEER ↓	de Vries et al., 1996
	Rat	Permeability (sodium fluorescein) ↑ TEER ↓ NF-κB signaling ↑	Coelho-Santos et al., 2015
	Rat	Endocytosis (horseradish peroxidase) ↑ PKC signaling ↑	Defazio et al., 2000
	Mouse	Claudin-5 expression ↓ NF-κB signaling ↑	Aslam et al., 2012
	Mouse	Claudin-5 expression ↓ PI3K signaling ↑	Camire et al., 2015
	Mouse	TEER ↓ F-actin rearrangement PKCα/RhoA signaling pathway↑	Peng et al., 2011
Combination of TNF-α and INF-γ	Human	Permeability (FITC-dextran) ↑ Activation of caspase-3 and caspase-9 ↑	Lopez-Ramirez et al., 2012
Combination of TNF-α, IL-6 and IL17	Mouse	Permeability (sodium fluorescein) ↑ TEER ↓ NF-κB transcription factor DNA binding activity ↑	Voirin et al., 2020
IL-6	Human	Permeability (FITC-dextran) ↑ Occludin expression ↓ Claudin-5 expression ↓ ROS ↑	Rochfort et al., 2014
	Mouse	Permeability (sodium fluorescein) ↑ TEER ↓ ZO-1 expression ↓ Claudin-5 expression ↓	Voirin et al., 2020
OSM	Human	Expression of intercellular adhesion molecule-1 ↑ Expression of vascular cell adhesion molecule-1 →	Ruprecht et al., 2001
	Rat	Permeability (sodium fluorescein) ↑ TEER ↓ Claudin-5 expression ↓ JAK/STAT3 signaling pathway ↑	Takata et al., 2008, 2018
Lipid mediators			
PGE2	Human	Permeability (FITC-Dextran) ↑ through activation of EP3 and EP4	Dalvi et al., 2015
	Bovine	Permeability (FITC-Dextran) ↑	Mark et al., 2001
TXA2	Human	Occludin expression ↓ Claudin-5 expression ↓ Activate the ROCK-PTEN-Akt-eNOS pathway	Zhao et al., 2017
LPA	Human	Permeability (Texas red-Dextran) ↑ Occludin expression ↓ Claudin-5 expression ↑	Kim et al., 2018
	Rat	TEER ↓ Activate the LPA–LPA6–G12/13–Rho pathway	Masago et al., 2018
	Mouse	TEER ↓ Phosphorylation of occludin and claudin-5 through activation of Rho Kinase ↑	Yamamoto et al., 2008
S1P	Human	TEER ↑ through activation of S1PR5 receptor	van Doorn et al., 2012
	Human	TEER ↑	Alves et al., 2019
	Human	TEER ↓	Wiltshire et al., 2016
	Rat	TEER ↓ Permeability (Sodium fluorescein) ↑	Nakagawa and Aruga, 2020
Serum amyliod A (SAA)	Rat	Permeability (Sodium fluorescein) ↑ TEER ↓ Claudin-5 expression ↓	Matsumoto et al., 2020
Autoantibody			
Antibody from patients of NMO (target antigen: glucose-regulated protein 78)	Human	Permeability (FITC-Dextran) ↑ Permeability (IgG) ↑ NF-κB nuclear translocation ↑ Claudin-5 expression ↓	Shimizu et al., 2017

(Continued)

TABLE 1 | (Continued)

Mediators	Species	Cellular events in BMECs	References
Antibody from patients of PCD with LEMS (target antigen: glucose-regulated protein 78)	Human	Permeability (FITC-Dextran) ↑ TEER ↓ Claudin-5 expression ↓ NF-κB nuclear translocation ↑	Shimizu et al., 2019
Antibody from patients of SPMS (target antigen: galectin-3)	Human	TEER ↓ Claudin-5 expression ↓ ICAM-1 expression ↑	Shimizu et al., 2014; Nishihara et al., 2017

human BMECs to OSM (Ruprecht et al., 2001). In peripheral organs, it was reported that neutrophil-derived OSM augmented P-selectin-dependent neutrophil rolling on postcapillary venules through gp130-dependent signaling in endothelial cells (Setiadi et al., 2019). Thus, OSM-activated adhesion molecules may evoke neutrophil penetration across the BBB, contributing to the neurotoxicity process in neuroinflammatory diseases such as MS. Under pathological conditions, elevated OSM in the brain should be considered as a possible causal factor for the development and progression of neuroinflammation arising through penetration of harmful molecules and immune cells in the circulating blood across the BBB (see **Table 1**).

Lipid Mediators

Lipid mediators are bioactive lipids with important roles in many physiological and pathological conditions including inflammation, atherosclerosis, ischemia, and cancer. In response to various stimuli, lipid mediators are rapidly synthesized by specific enzymes. The produced intracellular lipid mediators are secreted into the extracellular space where they act as extracellular signaling molecules like local hormones or autacoids through G protein-coupled receptors (GPCRs). The majority of lipid mediators are produced by multistep enzymatic pathways from lipids that are cellular membrane constituents. These lipid mediators can be classified according to their structures, such as arachidonic acid-derived eicosanoids (e.g., prostaglandins, leukotrienes), lysophospholipids (e.g., lysophosphatidic acid, sphingosine 1-phosphate), and others (Shimizu, 2009; Murakami, 2011). These mediators stimulate the cells that constitute the BBB and modulate BBB functions (see **Figures 5**, **6** and **Table 1**).

Eicosanoid

Prostanoids and leukotrienes are a subclass of eicosanoids produced from arachidonic acid through continuous enzyme reactions including the cyclooxygenase and lipoxygenase pathways. Among the prostanoids, prostaglandin E2 (PGE_2), prostaglandin D2 (PGD_2), prostaglandin F2α ($PGF_{2\alpha}$), prostacyclin (PGI_2), and thromboxane A2 (TXA_2) are known to be major bioactive prostanoids. These prostanoids exert their effects by activating their cognate GPCRs (Murakami, 2011). Several studies have indicated that PGE_2 has the ability to regulate BBB permeability depending on its receptor subtypes (EP1–EP4). PGE_2 treatment of cultured BMECs increased the permeability to fluorescein-conjugated dextran (Mark et al., 2001). Meanwhile, increased production of PGE_2 induced by exposure of BMECs to arachidonic acid increased the permeability of BMEC monolayers through activation of EP3 and EP4 (Dalvi et al., 2015). A series of studies involving pharmacological or genetic inhibition of PGE_2 receptors indicated that EP1 and EP3 contributed to BBB breakdown in ischemic stroke models (Fukumoto et al., 2010; Ikeda-Matsuo et al., 2011; Frankowski et al., 2015). Although EP2 activation in the brain was shown to be a detrimental factor in stroke, neurodegenerative diseases, and status epilepticus (Woodling and Andreasson, 2016; Li et al., 2020; Nagib et al., 2020), the direct effect of EP2 on regulation of BBB functions has not been fully elucidated. Meanwhile, EP4 was reported to play a beneficial role in the brain, including attenuation of the BBB dysfunction induced by stroke (Liang et al., 2011; DeMars et al., 2018). A recent study showed that PGE2-EP1/EP4 interactions induced downregulation of *N*-cadherin, connexin-43, and R-Ras leading to reduced pericyte-BMEC interactions. The impaired interactions between BMECs and pericytes evoked BMEC destabilization and hyperpermeability (Perrot et al., 2020). Prostanoid TXA_2 also modulated BBB functions. Stimulation of the TXA2 receptor (TP receptor) on BMECs by hyperglycemia or a TP receptor agonist impaired the integrity of the BBB *via* the ROCK-PTEN-Akt-eNOS pathway (Zhao et al., 2017). Meanwhile, a TP receptor antagonist reduced the brain damage induced by ischemic stroke through preserved expression of tight junction proteins and prevention of microglial activation (Yan et al., 2016). Other prostanoids such as PGI_2, PGD_2, and $PGF_{2\alpha}$ contributed to modifications of brain function including blood-flow regulation and neuroprotection (Ahmad, 2014; Muramatsu et al., 2015; Mohan et al., 2018; Rojas et al., 2019). However, the direct effects of these prostanoids on BBB functions and their putative cell signaling pathways remain unclear. Therefore, systematic examinations of the effects of prostanoids on the regulation of BBB functions are warranted.

In addition to prostanoids, cysteinyl leukotrienes (CysLTs) act as potent proinflammatory mediators and are considered to modulate BBB functions. CysLTs including LTC_4, LTD_4, and LTE_4 are metabolites of arachidonic acid *via* the lipoxygenase pathway that stimulate cognate receptors named cysteinyl leukotriene receptors (CysLTR1–3) (Shimizu, 2009; Murakami, 2011). Injection of LTC_4, LTE_4, or LTB_4 into the brain parenchyma induced BBB hyperpermeability (Black and Hoff, 1985). Furthermore, studies on various animal models including ischemic stroke, AD, MS, and epilepsy indicated that pharmacological inhibition of CysLTRs had beneficial effects such as improvement of BBB dysfunction, inhibition of microglia and/or astrocyte activation, and inhibition of neuroinflammation (Gelosa et al., 2017; Chen et al., 2020) (see **Table 1**).

Lysophospholipids

As another type of bioactive signaling lipids, lysophospholipids are synthesized from phospholipids and sphingolipids. Lysophospholipids including lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) induce a variety of

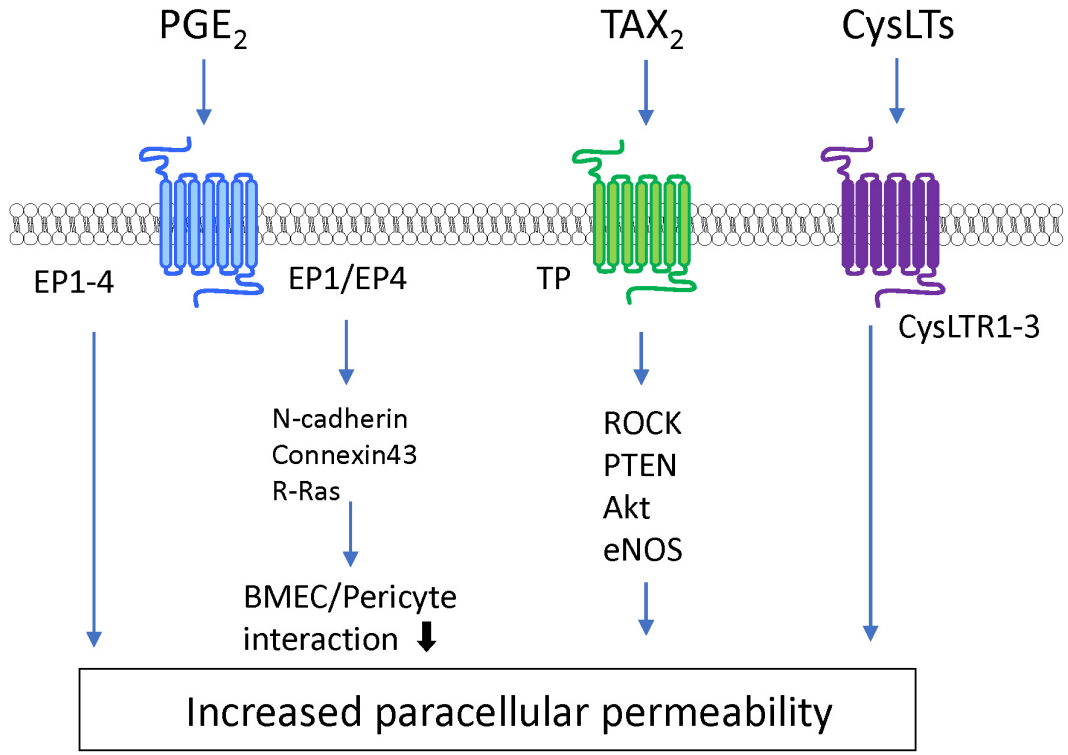

FIGURE 5 | Intracellular signaling pathways induced by eicosanoids in BMECs leading to BBB dysfunction.

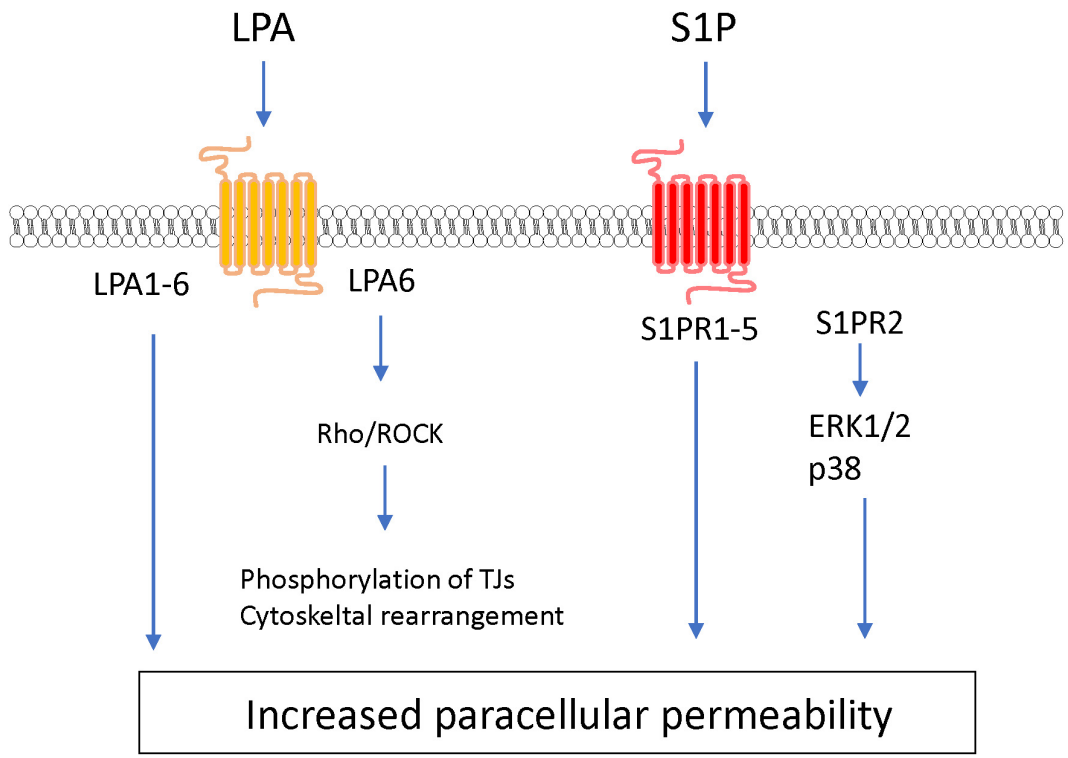

FIGURE 6 | Intracellular signaling pathways induced by lysophosholipids in BMECs leading to BBB dysfunction.

cellular physiological responses *via* cognate GPCR-mediated signaling pathways.

Lysophosphatidic acid is a bioactive lysophospholipid that induces cellular responses *via* specific GPCRs (LPA1–6). LPA is produced from lysophosphatidylcholine by the action of autotaxin (Hao et al., 2020). Several studies have indicated that LPA treatment of BMECs induces BBB impairment. Masago et al. (2018) reported that LPA stimulation of BMECs induced decreases in TEER in an LPA dose-dependent manner and disrupted the structural integrity of tight junction proteins. The same research group further investigated the putative cell signaling pathway involved in the LPA-induced BBB impairment. The barrier reduction was improved by silencing of LPA6 or treatment with a Rho-associated protein kinase (ROCK) inhibitor (Masago et al., 2018). Other research groups reported that LPA induced phosphorylation of tight junction proteins (claudin-5 and occludin) and cytoskeletal rearrangements *via* the Rho-ROCK pathway (Yamamoto et al., 2008; Kim et al., 2018). Taken together, these findings indicate that LPA stimulates LPA receptors coupled with $G_{12/13}$ to activate the Rho-ROCK pathway, leading to an increase in BBB permeability.

S1P is synthesized by phosphorylation of sphingosine derived from sphingolipids by sphingosine kinases 1 and 2 (Sphk1 and Sphk2). Intracellular S1P is secreted into the extracellular space *via* S1P transporters (e.g., Abca1 and Spns2) where it acts as an extracellular signaling molecule through GPCRs (S1PR1–5) coupled with G_q, G_i, $G_{12/13}$, and Rho proteins (Hao et al., 2020; Pluimer et al., 2020). S1P regulates a number of biological processes including vascular development and function. The direct effect of S1P on cultured BMECs remains controversial. Some research groups have reported barrier enhancement following treatment with S1P (van Doorn et al., 2012; Alves et al., 2019), while other research groups found that S1P decreased barrier properties in BMECs (Wiltshire et al., 2016; Nakagawa and Aruga, 2020). Given that S1P has different effects on endothelial barrier function depending on the S1PRs involved (Camm et al., 2014), the differences between these conflicting findings may be caused by altered expression of S1PRs in different cell types and culture conditions. *In vitro* studies using endothelial cells derived from non-brain tissues indicated that S1PR1 strengthens the barrier function, while S1PR2 reduces the barrier function (Camm et al., 2014; Kerage et al., 2014). *In vivo* studies demonstrated that endothelial-specific S1PR1 knockout mice had facilitated small-molecule-selective BBB opening (Yanagida et al., 2017). Conversely, S1PR2 contributed to induction of BBB impairment. Several research groups reported that S1PR2 played a critical role in induction of BBB dysfunction in ischemic stroke and experimental autoimmune encephalomyelitis models (Cruz-Orengo et al., 2014; Kim et al., 2015). Cao et al. (2019) revealed that activation of p38 MAPK and ERK1/2 signaling stimulated by S1P-S1PR2 interactions was involved in oxidative stress-induced BMEC barrier impairment. Another research group indicated that S1PR2 expressed in pericytes contributed to increased pericyte migration and reduced *N*-cadherin expression *via* the NF-κB p65 signaling pathway (Wan et al., 2018). Meanwhile, S1P/S1PR3 signaling mediated proliferation of pericytes *via* the Ras/pERK pathway, which may be involved in scar formation after spinal cord injury (Tang et al., 2018). Finally, stimulation of S1PR3 on astrocytes enhanced expression of inflammatory genes through RhoA signaling (Dusaban et al., 2017).

Much of the evidence regarding the roles of lipid mediators in regulating endothelial barrier functions has been accumulated using non-brain-derived endothelial cells. It is widely accepted that brain capillary endothelial cells have several characteristic differences compared with peripheral endothelial cells. Furthermore, Daneman et al. (2010) indicated that numerous signaling cascades and metabolic pathways in BMECs differed from those in peripheral endothelial cells. Thus, further studies using brain-derived endothelial cells are needed to elucidate the actual roles of lipid mediators in BBB functions (see **Table 1**).

Serum Amyloid A

Serum amyloid A (SAA) is a major acute-phase protein and its serum level increases by up to 1,000-fold during acute inflammation (Ye and Sun, 2015). A pivotal role for SAA in mediating the pathological processes of brain disorders has also been identified. SAA was elevated in brain disorders such as cognitive impairment, depression, and ischemic stroke (Shang et al., 2018). SAA was also upregulated in subjects with impaired BBB relative to subjects with intact BBB (Bowman et al., 2018). These findings suggest that SAA may alter BBB integrity and impair neurological function in pathological states. Matsumoto et al. (2020) recently reported that recombinant Apo-SAA impaired brain endothelial integrity associated with decreased claudin-5 protein expression in rat brain endothelial cells (RBECs). The pleiotropic functions of SAA are mediated by several receptors including formyl peptide receptor 2 (FPR2), Toll-like receptor 4 (TLR4), TLR2, CD36, P2X receptor 7, and RAGE (Ye and Sun, 2015). Binding of SAA to multiple SAA receptors simultaneously activates several intracellular signaling pathways including the PI3K/Akt, MAPK, and NF-κB signaling pathways (Ye and Sun, 2015; Yu et al., 2017; Sack, 2018). Activation of these signaling pathways was involved in the loss of claudin-5 in endothelial cells (András et al., 2005; Schreibelt et al., 2007; Huang et al., 2016). Apo-SAA induced phosphorylation of NF-κB and p38 MAPK in RBECs (Matsumoto et al., 2020). Furthermore, co-treatment with SAA and high-density lipoprotein (HDL), an apolipoprotein of SAA in circulating blood, recovered the brain endothelial barrier function and decreased claudin-5 expression in Apo-SAA-treated RBECs. In addition, HDL also reduced the phosphorylation of NF-κB and p38 MAPK in the treated cells (Matsumoto et al., 2020). These findings suggest that SAA may downregulate claudin-5 expression *via* multiple signaling pathways, leading to brain endothelial barrier dysfunction (see **Table 1**).

Autoantibody

Blood-brain barrier dysfunction has been reported in several immune-mediated neuroinflammatory diseases, including neuromyelitis optica (NMO) and multiple sclerosis (MS). Accumulating evidence has indicated that autoantibodies in patients with these diseases directly mediate BBB dysfunction (Shimizu et al., 2018; **Table 1**). IgG from secondary progressive

MS (SPMS-IgG) sera induced decreased TEER and claudin-5 expression, as well as increased VCAM-1 expression in human BMECs (Shimizu et al., 2014). One previous study reported that the target molecule of SPMS-IgG for inducing BBB dysfunction was galectin-3, and galectin-3 antibodies in the SPMS sera influenced NF-κB p65 signaling (Nishihara et al., 2017). These findings suggested that galectin-3 autoantibodies were the factors responsible for inducing BBB dysfunction in progressive MS. NMO patient-derived IgG (NMO-IgG) caused the hyperpermeability and decreased levels of claudin-5 expressions through NF-κB nuclear translocation in human BMECs (Shimizu et al., 2017). In addition, the absorption of GRP78-specific antibodies from NMO-IgG resulted in decreased nuclear translocation of NF-κB in human BMECs, suggesting that GRP78 autoantibodies directly induce BBB dysfunction in BMECs. Besides NMO-IgG, IgG derived from patients of paraneoplastic cerebellar degeneration (PCD) with the autoimmune disease Lambert-Eaton myasthenic syndrome (LEMS) was found to impaired the barrier integrity in human BMECs (Shimizu et al., 2019). NF-κB nuclear translocation resulting in BBB dysfunction was inhibited when GRP78-specific IgG was removed from PCD-LEMS IgG. Based on these data, identifying autoantibodies mediating BBB dysfunction might lead to the discovery of target molecules in BMEC to regulate BBB function under immune-mediated neurological diseases (see **Table 1**).

MicroRNAs

MicroRNAs (miRNAs) are small non-coding RNA (21–25 nucleotides) and inhibit translation of target mRNA by binding to 3′ untranslated regions. Several miRNAs have been shown to play a critical role in the development of BBB dysfunction in CNS diseases involving neuroinflammation, such as stroke, TBI, dementia and cerebral infections (Ma et al., 2020). **Supplementary Table 1** shows miRNAs associated with the downregulation of BBB integrity in BMECs (Bukeirat et al., 2016; Fang et al., 2016; Hu et al., 2018; Toyama et al., 2018). Under ischemic conditions, the expression levels of miR-212/132, -21-3p, -30a, -130a, and -182 were increased in BMECs, and these miRNAs induced hyperpermeability, decreased TEER and downregulated levels of tight junction-associated proteins in BMECs (Wang et al., 2018, 2021; Burek et al., 2019; Ge et al., 2019; Zhang et al., 2020). The increased miR-424-5p in BMECs treated with Aβ induced decreased TEER and downregulated levels of ZO-1 and occludin in BMECs (Lin et al., 2019). Stimulation of BMECs with homocysteine, HIV-1 Tat C, cytokine, or

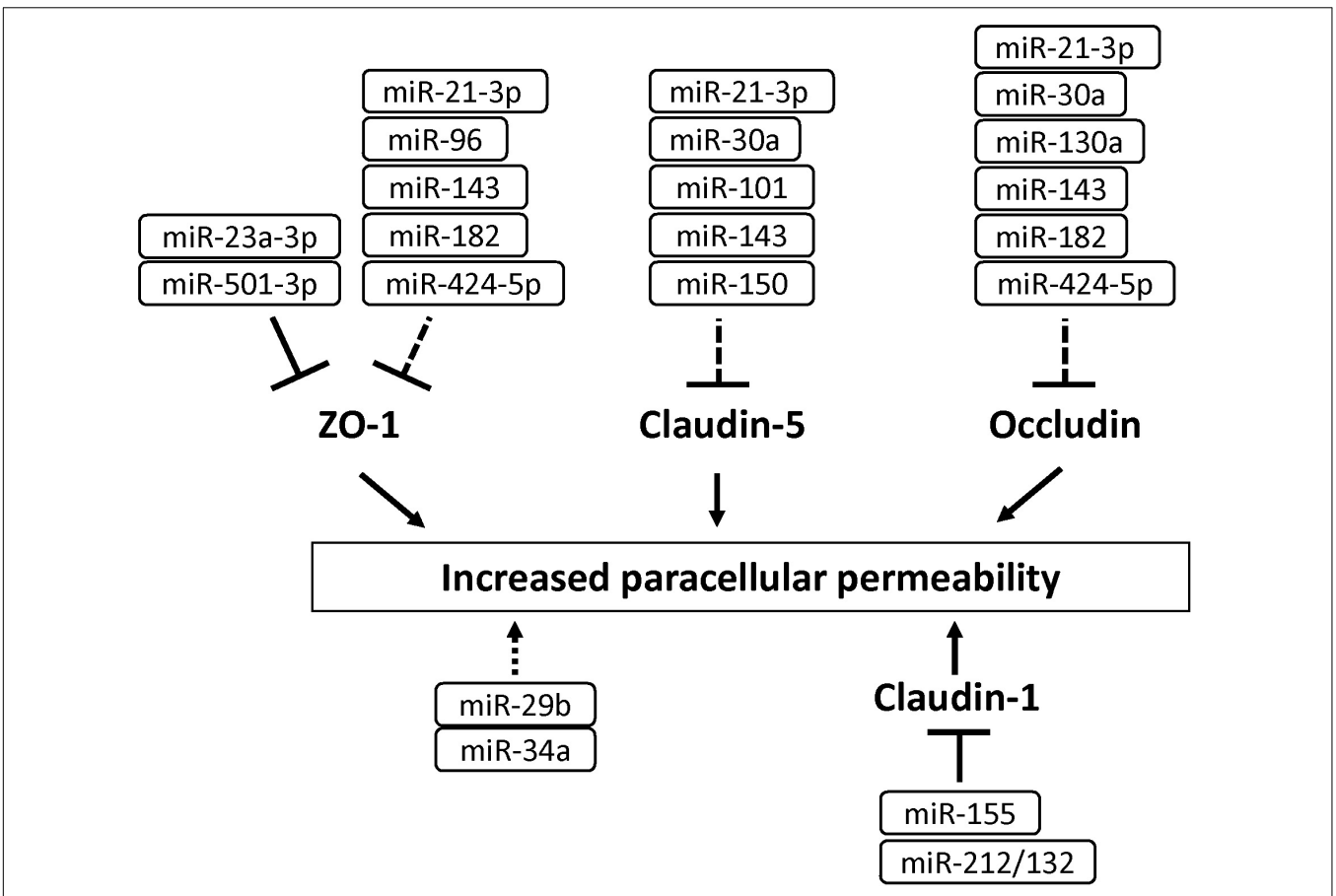

FIGURE 7 | MicroRNAs involved in the down-regulation of tight junction protein levels and BBB function in BMECs. Solid and dashed lines indicate that miRNAs directly and indirectly down-regulate tight junction-associated proteins, respectively.

methamphetamine elicited the downregulation of BBB integrity through the modulation of miR-29b, -96, -101 or -143 levels in BMECs, respectively (Mishra and Singh, 2013; Kalani et al., 2014; Bai et al., 2016; Zhang et al., 2018). Based on these data, the regulation of miRNA as critical therapeutic targets should be considered to maintain BBB function under pathological conditions (see **Figure 7** and **Supplementary Table 1**).

Long Non-coding RNAs

Long non-coding RNAs (lncRNAs) are defined as RNA transcripts with lengths exceeding 200 nucleotides that do not encode proteins (Quinn and Chang, 2016). Lnc RNAs affect various molecular events including gene transcription, translation, splicing and protein interaction. Accumulating studies have demonstrated that lncRNAs induced cerebral endothelial pathology which is associated with stroke and Alzheimer's disease (Yin et al., 2014; Zhang et al., 2016). Increased LINC00094 and LINC00662 in amyloid-β-treated BMECs mediated BBB dysfunction through the down-regulation of tight junction-associated proteins (Zhu et al., 2019; Liu Q. et al., 2020). The increased lncRNA small nucleolar RNA host gene 3 (Snhg3) levels in BMECs led to the BBB hyperpermeability under the condition of intracerebral hemorrhage (Zhang et al., 2019). The increased LOC102640519 expression in BMECs negatively regulated the expressions of ZO-1, claudin-5 and occludin in ischemic conditions (Wu et al., 2018). These findings suggest that lncRNA is a possible therapeutic target for treating the impaired BBB under the pathological conditions (see **Figure 8** and **Supplementary Table 1**).

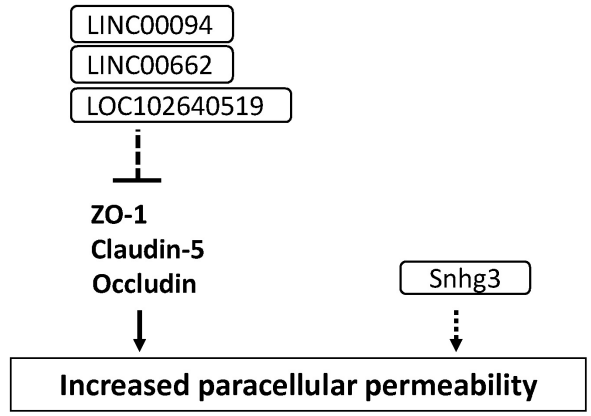

FIGURE 8 | Long non-coding RNAs involved in the down-regulation of tight junction protein levels and BBB function in BMECs. Dashed line indicates indirect down-regulation by lncRNAs. Dashed arrow indicates unknown mechanisms.

TABLE 2 | Signaling pathways in BMECs that strengthen BBB integrity.

Signaling molecules	Species	Cellular events in BMECs	References
AMPK	Rat	AMPK activation ↑ Permeability (sodium fluorescein) ↓ Permeability (Evans blue albumin) ↓ TEER ↑	Takata et al., 2013
cAMP	Bovine	cAMP ↑ TEER ↑	Rubin et al., 1991
	Bovine	cAMP ↑ Permeability (sucrose) ↓ Permeability (inulin) ↓	Deli et al., 1995a
	Porcine	cAMP ↑ TEER ↑ Claudin-5 expression ↑ cAMP/PKA signaling pathway ↑ Phosphorylation of claudin-5 ↑	Ishizaki et al., 2003
Wnt/β-catenin	Human	Wnt/β-catenin signaling pathway ↑ Permeability (Lucifer Yellow salt) ↓ Claudin-5 expression ↑ Claudin-3 expression ↑	Paolinelli et al., 2013
	Human	Wnt signaling ↑ β-catenin expression ↑ Permeability (sodium fluorescein) ↓ Electrical impedance ↑ Claudin-1 expression ↑	Laksitorini et al., 2019

SIGNALING PATHWAYS IN BMECs THAT STRENGTHEN BBB INTEGRITY

AMPK

5′-Adenosine monophosphate-activated protein kinase (AMPK) is a signaling molecule that regulates BBB functions. Activation of AMPK in BMECs induced upregulation of BBB integrity, featuring increased TEER and decreased BBB permeability to tracers such as sodium fluorescein and Evans blue (Takata et al., 2013). Exposure of mice to LPS, a mediator of neuroinflammation, evoked BBB extravasation of IgG and Evans blue and decreased expression of tight junction proteins including claudin-5 through decreased AMPK activation (Wang et al., 2017). Reduced AMPK signaling in BMECs is likely to be involved in BBB impairment under neuroinflammatory conditions. Indeed, activation of AMPK by metformin, AICAR, and melatonin alleviated the impaired BBB functions under pathological conditions in mouse models of ischemic stroke and sepsis (Wang et al., 2017). Considering these findings, regulation of AMPK activity in BMECs is a therapeutic target for restoring impaired BBB functions in neuroinflammatory diseases (see **Table 2**).

cAMP

Intracellular cyclic adenosine monophosphate (cAMP) elevation is a well-known factor that induces strong barrier properties in endothelial cells. Treatment of cultured BMECs with a cell-permeable cAMP analog, adenylate cyclase activator, and a phosphodiesterase inhibitor increased the barrier function, by increasing TEER and decreasing paracellular permeability (Rubin et al., 1991; Deli et al., 1995a, 2005). Ishizaki et al. (2003) found that cAMP acted on tight junctions in the BBB to increase

TABLE 3 | Therapeutic drug candidates for modulating BBB integrity.

Therapeutic drug candidates	Species	Cocultured cells with BMECs	Experimental conditions	Cellular events in BMECs	References
Minocycline	Human	-	Oxygen deprivation	TEER ↑ Claudin-5 expression ↑ Occludin expression ↑ ZO-1 expression ↑	Yang et al., 2015
Alogliptin	Human	-	OGD/reoxygenation	Permeability (FITC-Dextran) ↓ Occludin expression ↑ ZO-1 expression ↑	Hao et al., 2019
Zafirlukast	Human	-	OGD/reoxygenation	Permeability (FITC-Dextran) ↓ Occludin expression ↑ ZO-1 expression ↑	Zeng et al., 2020
Siponimod	Human	Astrocytes	Physiological conditions	Permeability (FITC-Dextran) ↓ Claudin-5 expression ↑ ZO-1 expression ↑	Spampinato et al., 2021
		Astrocytes	TNF-α and INF-γ treatment	PI3K/Akt signaling pathway↑ Permeability (FITC-Dextran) ↓ TEER ↑ Claudin-5 expression ↑ ZO-1 expression ↑	Spampinato et al., 2021
Memantine	Human	Pericytes and astrocytes	Amyloid-β_{1-42} treatment	LINC00094 expression ↓ Permeability (horseradish peroxidase) ↓ TEER ↑ Claudin-5 expression ↑ Occludin expression ↑ ZO-1 expression ↑	Zhu et al., 2019
Metformin	Rat	-	Physiological conditions	AMPK activation ↑ Permeability (sodium fluorescein) ↓ Permeability (Evans blue albumin) ↓ TEER ↑	Takata et al., 2013
Pitavastatin	Rat	-	Physiological conditions	Mevalonate pathway ↓ Permeability (sodium fluorescein) ↓ Permeability (Evans blue albumin) → TEER ↑ Claudin-5 expression ↑	Morofuji et al., 2010
Cilostazol	Rat	-	Physiological conditions	cAMP↑ Permeability (sodium fluorescein) ↓ TEER ↑	Horai et al., 2013
	Rat	Pericytes and astrocytes	OGD/reoxygenation	TEER ↑	Horai et al., 2013
	Rat	Pericytes and astrocytes	TGF-β1 treatment	TEER ↑	Takeshita et al., 2014
	Rat	Pericytes and astrocytes	AGEs treatment under OGD/reoxygenation	TGF-β1 ↓ TEER ↑ Claudin-5 expression ↑	Takeshita et al., 2014
Candesartan	Rat	Astrocytes	OGD/reoxygenation	Permeability (sodium fluorescein) ↓ TEER ↑ Improved localization of claudin-5 and occludin	So et al., 2015
Perampanel	Rat	Astrocytes and neurons	Glutamate treatment	Permeability (sodium fluorescein) ↓ TEER ↑	Chen et al., 2020
	Rat	Astrocytes and neurons	Traumatic neuronal injury model	Permeability (sodium fluorescein) ↓ TEER ↑	Chen et al., 2020
Ruxolitinib	Rat	Pericytes	OSM treatment	Permeability (sodium fluorescein) ↓ TEER ↑ (JAK/STAT3 signaling pathway in pericytes ↓)	Takata et al., 2019
Lithium	Mouse	-	OGD/reoxygenation	Wnt/β-catenin signaling pathway ↑ TEER ↑	Ji et al., 2021
Omarigliptin	Mouse	-	LPS treatment	Toll-like receptor 4/myeloid differentiation factor 88/ NF-κB signaling pathway ↓ Permeability (FITC-Dextran) ↓ Claudin-1 expression ↑ Claudin-5 expression ↑	Du and Wang, 2020
Propofol	Mouse	Astrocytes and microglia	Oxygen deprivation	TEER ↑ (Heat shock protein (HSP) 32 expressions and nuclear translocation of nuclear factor-E2-related factor 2 in microglia ↑) (HSP27 expressions and nuclear translocation of heat shock factor 1 in astrocytes ↑)	Sun et al., 2019
5′-azacytidine	Mouse	-	Homocysteine treatment	miR-29b expression ↓ Permeability (FITC-albumin) ↓	Kalani et al., 2014

expression of claudin-5 in a protein kinase A (PKA)-independent manner and phosphorylated claudin-5 *via* the cAMP/PKA pathway to strengthen barrier tightness (Ishizaki et al., 2003). Other research groups found that activation of an exchange protein directly activated by cAMP1 (Epac1), a downstream effector of cAMP, protected barrier integrity in peripheral vascular endothelial cells (Fukuhara et al., 2005; Lorenowicz et al., 2008). Therefore, the cAMP/PKA and cAMP/Epac1 signaling pathways are considered to independently impact on barrier integrity in endothelial cells. Importantly, a deterioration effect of cAMP on barrier function was reported by several research groups, indicating that cAMP produced in the cytosol by activation of soluble adenylyl cyclases plays a role in the disruption of barrier function (Sayner et al., 2004; Prasain et al., 2009; Sayner, 2011). Therefore, cAMP produced in the cytosol and cAMP produced by membrane-associated enzymes seem to have opposite effects on endothelial barrier stabilization. Although the roles of cAMP in BMECs, such as reduction of intracellular cAMP and site-specific production of cAMP, have been not fully elucidated, cAMP signaling is one of the important pathways for modulation of BBB functions (see **Table 2**).

Wnt/β-Catenin

Wnt/β-catenin signaling is important for brain vascularization and BBB differentiation. Dysregulation of the Wnt/β-catenin pathway is observed in various CNS diseases associated with BBB dysfunction. Wnt can activate both the canonical Wnt/β-catenin signaling pathway and the non-canonical Wnt pathway, which is divided into Wnt/planar cell polarity and Wnt/Ca^{2+} utilization pathways. BMECs expressed Wnt receptor (Frizzled), co-receptors (LRP5, LRP6, ROR2, and RYK), Wnt ligands, and Wnt modulator peptides (DKK, sFRP, and WIF) (Laksitorini et al., 2019). Activation of Wnt/β-catenin signaling in BMECs following treatment with Wnt ligand Wnt3a, which is not expressed in BMECs, or LiCl, increased β-catenin and mRNA expression of claudin-5 and occludin to tighten the paracellular barrier. LiCl is a GSK3β inhibitor that inhibits β-catenin phosphorylation and degradation (Paolinelli et al., 2013). Although Wnt ligands are also expressed in perivascular cells, pericytes, and astrocytes to regulate and maintain the BBB, modulation of brain endothelial Wnt activation is a possible pathway for restoration of BBB functions (see **Table 2**).

CONCLUSION AND PERSPECTIVES

In this review, we focused on the understanding of cellular events in BMECs that lead to BBB dysfunction because protection of the BBB would be a therapeutic target for the development of neurodegenerative diseases involving neuroinflammation. Although a large body of evidence from *in vivo* studies suggests that various inflammatory mediators released by neurovascular unit cells surrounding BMECs induce BBB dysfunction, only a limited number of *in vitro* studies have addressed the direct BBB-impairing effects of inflammatory mediators derived from neurovascular unit cells using co-culture systems with BMECs. More work is needed to identify secreted inflammatory mediators including non-coding RNAs and their related intracellular signaling pathways involved in BBB dysfunction.

In this context, therapeutic drugs for BBB protection need to possess the following abilities: (1) strengthening of brain endothelial barrier properties directly, (2) blockage of intracellular signaling pathways in BMECs leading to BBB dysfunction, and (3) inhibition of release of inflammatory mediators from neurovascular unit cells, particularly pericytes, astrocytes and microglia. Many efforts have been made to discover BBB protective compounds including pharmacological inhibitors of signaling pathways and natural compounds. However, none of these are currently approved therapeutic drugs. Therefore, drug repositioning is an effective approach to discover BBB protective drugs. **Table 3** shows a list of possible candidates from *in vitro* studies demonstrating an improved and/or strengthening effect on barrier properties of BMECs (**Table 3**). *In vivo* studies were excluded because it is unclear whether administrated drugs directly affect neurovascular units. Metformin (Takata et al., 2013), pitavastatin (Morofuji et al., 2010), siponimod (Spampinato et al., 2021), and cilostazol (Horai et al., 2013) possess the ability to strengthen brain endothelial barrier properties through modulating intracellular signaling in physiological conditions. Minocycline (Yang et al., 2015), alogliptin (Hao et al., 2019), zafirlukast (Zeng et al., 2020), siponimod (Spampinato et al., 2021), memantine (Zhu et al., 2019), cilostazol (Horai et al., 2013; Takeshita et al., 2014), candesartan (So et al., 2015), perampanel (Chen et al., 2021), lithium (Ji et al., 2021), omarigliptin (Du and Wang, 2020) and 5′-azacytidine (Kalani et al., 2014) restore the barrier function dependently of specific pathological conditions (e.g., hypoxia, etc.). Ruxolitinib (Takata et al., 2019) inhibits activation of pericytes to release inflammatory mediators, resulting in restoration of the BBB. Propofol (Sun et al., 2019) protects hypoxia-mediated BBB impairment through regulating microglia and astrocytes. BMECs are the interface between the circulating blood and the brain parenchyma. This location is advantageous for pharmacological interventions targeting BMECs because it is not necessary to consider the ability of therapeutic compounds to cross the BBB. Therefore, to navigate and accelerate drug repositioning by high-throughput screening, further studies are needed to determine the intracellular signaling pathways in BMECs that lead to BBB dysfunction and/or strengthening of BBB integrity. New supportive approaches to treat neurodegenerative diseases with neuroinflammation could potentially be developed by targeting (1) maintenance of BBB integrity by blockade of intracellular signaling pathways in BMECs that lead to BBB dysfunction and (2) repair of a leaky BBB by stimulating BMECs to activate signaling pathways that strengthen the barrier integrity.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

The authors would like to thank Alison Sherwin and Benjamin Knight from Edanz Group (https://jp.edanz.com/ac) for editing a draft of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2021.661838/full#supplementary-material

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