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Role of microRNAs in the regulation of blood-brain barrier function in ischemic stroke and under hypoxic conditions *in vitro*

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The blood-brain barrier (BBB) is a highly specialized structure that separates the brain from the blood and allows the exchange of molecules between these two compartments through selective channels. The breakdown of the BBB is implicated in the development of severe neurological diseases, especially stroke and traumatic brain injury. Oxygen-glucose deprivation is used to mimic stroke and traumatic brain injury *in vitro*. Pathways that trigger BBB dysfunction include an imbalance of oxidative stress, excitotoxicity, iron metabolism, cytokine release, cell injury, and cell death. MicroRNAs are small non-coding RNA molecules that regulate gene expression and are emerging as biomarkers for the diagnosis of central nervous system (CNS) injuries. In this review, the regulatory role of potential microRNA biomarkers and related therapeutic targets on the BBB is discussed. A thorough understanding of the potential role of various cellular and linker proteins, among others, in the BBB will open further therapeutic options for the treatment of neurological diseases.

KEYWORDS

blood-brain barrier, microRNA, stroke, traumatic brain injury, tight junctions, transporter

Abbreviations: A, alanine-preferring system; ABC, ATP-binding cassette; APC, activated protein C; ASC, alanine-, serine- and cysteine-preferring system; BCRP, breast cancer resistance protein; EAAT1-3, excitatory amino acid transporters 1–3; EPCR, endothelial protein C receptor; GLUT1, glucose transporter 1; IR, insulin receptors; LAT1, L-type amino acid transporter 1; LRP1, lipoprotein receptor-related protein 1; MCT1, monocarboxylate transporter 1; MRP, multidrug resistance protein; OATP, organic anion transporters/organic anion transporter peptide; PTS1, peptide transport system 1; SLC2A1, solute carrier family 2 facilitated glucose transporter member 1; TFR, transferrin receptors.

1 Introduction

Globally, cerebrovascular diseases, such as ischemic stroke and traumatic brain injury (TBI) are the leading cause of death or long-term disability (Obermeier et al., 2013). Although cerebrovascular diseases of different entity cause different initial events of brain injury, it has been shown that there is an overlap of events downstream of blood-brain barrier (BBB) breakdown after the onset of disorder. It is therefore crucial that mechanisms contributing to BBB disruption reveal new therapeutic targets and strategies that preserve BBB integrity. The role of the BBB is to control homeostasis in the CNS and to protect it from physical, chemical, and biological damage. A highly dynamic and complex structure, the BBB consists of highly specialized endothelial cells (ECs) that form the thin capillaries. Together with pericytes, astrocytes, microglia and neurons, ECs form a cellular unit called the neurovascular unit (NVU) and regulate the transport of substances from blood to brain and *vice versa* (Shi and Liu, 2007; Jiang et al., 2018). Furthermore, the barrier function of brain ECs is tightly regulated by the intricate interactions of tightly linked proteins such as tight junctions (TJs) and adherens junctions (AJs) (Obermeier et al., 2013).

BBB breakdown takes place during the acute phase of ischemic stroke (Jiang et al., 2018). Tissue damage and/or death occurs primarily as a result of hypoxia (oxygen deficiency) and glucose deprivation (hypoglycemia) and is highly dependent on the extent and duration of the disruption in blood supply. Although oxygen levels are restored after reperfusion (reoxygenation), there is an increase in the secretion of various cytokines that exacerbate the ischemic injury and thereby cause further cellular damage (Lambertsen et al., 2012). During ischemia, switching to anaerobic metabolism in cells leads to a decrease in ATP levels and intracellular pH. Disturbances in transport of ions dependent on ATP are responsible for the excess of calcium concentration in the cytoplasm and in the mitochondria, with all consequences including cell death by necrotic, apoptotic or autophagic mechanism (Kalogeris et al., 2012).

MicroRNAs (miRNAs, miRs) are involved in all processes including cell proliferation, cell differentiation and apoptosis, cell metabolism, and cellular responses under physiological and pathological conditions (Chandran et al., 2017; Mitra et al., 2017; Hicks et al., 2018). To date, miRNAs have been studied as biomarkers for a variety of diseases, including stroke and TBI (Borlongan and Emerich, 2003; Abbott et al., 2010; Yin et al., 2014; Keaney et al., 2015; Curtaz et al., 2020a; Curtaz et al., 2022). However, various studies have shown that targeting changes in BBB functions can alleviate the adverse effects of thrombolytic drugs, extend the treatment window and improve patient prognosis. Therefore, a potential benefit of a targeted opening of the BBB, i.e., by miRNAs, would facilitate delivery of therapies to the brain and should be considered when considering treatment strategies (Borlongan and Emerich, 2003).

This article provides an overview of BBB dysfunction after ischemic injury and highlights recent advances in our understanding the underlying regulatory mechanisms, with a particular focus on the role of miRNAs. We provide an overview of changes in BBB-related cellular components and junctional proteins associated with oxygen-glucose deprivation (OGD) and bring new ideas and new targets for a deeper understanding of brain injury arising from cerebrovascular disintegration.

2 Blood-Brain barrier structure and what is known on the BBB dysfunction in the context of ischemia and/or stroke

Under normal physiological conditions, adjacent ECs and their TJs ensure the paracellular impermeability of the BBB (Tietz and Engelhardt, 2015; Liebner et al., 2018). However, after the onset of OGD, ECs respond to ischemia and potentially harmful substances released by the vasculature. This leads to cytoskeletal rearrangements, increased cytosis, and altered TJ proteins, which in turn progressively lead to BBB dysfunction. The BBB forms a barrier at three different levels. First, it is a physical barrier that blocks the paracellular transport of polar substances (including ions) between adjacent ECs (Stamatovic et al., 2016). Second, different types of transporter proteins have a broad affinity for lipophilic substances and pump them out of ECs or transport them specifically (Pardridge, 2012). Third, various enzymes act to metabolize the substances and form the enzymatic barrier (Decleves et al., 2011). Prominent pathological features of ischemic and hemorrhagic stroke are characterized by structural disruption and increased permeability of TJs and are often associated with poor prognosis (Prakash and Carmichael, 2015).

TJs are composed of transmembrane proteins that close the cell gap (e.g., Claudin-1,-5, -3, -12, occludin, junctional adhesion molecule-A), cytoplasmic scaffolding proteins that physically support the TJs (e.g., *zonula occludens* proteins ZO-1-3) and the actin cytoskeleton (Stamatovic et al., 2016). Ischemia affects TJ proteins at several levels: structure (e.g., through protein phosphorylation), distribution (e.g., through internalization) and expression (e.g., through degradation), resulting in destruction of the TJs. For example, claudin-5, occludin and ZO-1 undergo phosphorylation and other post-translational modifications in response to ischemic and inflammatory conditions. Such modifications affect protein-protein interactions within the TJs (e.g., claudin: claudin and claudin: ZO-1), resulting in instability, internalization of the protein *via* endocytosis, and potential lysosomal degradation. Many, but not all, studies have shown reduced expression of TJ proteins after cerebral ischemia, due to reduced transcription or increased

TABLE 1 MicroRNAs interfering with blood-brain barrier function in brain ischemic injury and *in vitro* model of oxygen-glucose deprivation.

MicroRNA	Molecular target	References
miR-18a	ZO-1	Ren et al. (2021)
miR-22	Cox-2, IL-6, IL-10, iNOS, TNF-α	Yu et al. (2015)
miR-27a-3p	P-gp	Hammad et al. (2022)
miR-29b	AQP4	Sepramaniam et al. (2012); Wang et al. (2015)
miR-33-5p	Abca1, Bax/Bcl2, caspase-3, caspase-9	Sung et al. (2021)
miR-98	CCL2, CCL5	Bernstein et al. (2020)
miR-122	VCAM-1, iNOS, PLA2g2a, ALOX5, ITGA2b, TIMP3, IL-1β, IL-2, MMP8	Yu et al. (2015); Liu da et al. (2016)
miR-124	Iba-1, CD206, MCT-1, IL-1β, IL-6, IL-10, TNF-α	Huang et al. (2018); Xu et al. (2019b)
miR-125a	ZO-1	Wang et al. (2020)
miR-130b	AQP4	Zheng et al. (2017)
miR-135b-5p	Abca1, Bax/Bcl2, caspase-3, caspase-9	Sung et al. (2021)
miR-146a	IRAK1, NFκB p65	Chu et al. (2018)
miR-149-5p	MMP9, S1PR2, SOD1, SOD2, SOD3	Wan et al. (2018); Yan et al. (2021)
miR-182-5p	IL-1β, IL-6, TNF-α, TLR4	Wang et al. (2018)
miR-183 (agomir)	IL-1β, IL6, TNF-α, NFκB p65, IκBα	Xiang et al. (2019)
miR-210	Occludin, Notch1	Lou et al. (2012); Ma et al. (2017)
miR-212/132	Cldn1, Jam3, Tjap1	Burek et al. (2019)
miR-320a	AQP1, AQP4	Sepramaniam et al. (2010)
miR-375-3p	Cldn1	Mao et al. (2021)
miR-424-5p	ZO-1	Lin et al. (2019)
miR-429	ZO-1	Chen et al. (2018)
miR-539	SNAI2	Li et al. (2021)
miR-let-7c-5p	AQP4, Caspase-3	Ni et al. (2015)
anti-miR-15a/16-1	Bcl-2, Bcl-w, IL-6, Mcp-1, TNF-α, Vcam-1	Yang et al. (2017)

protein degradation (Kleinschnitz et al., 2011; Neuhaus et al., 2012; Blecharz-Lang et al., 2018; Burek et al., 2019; Gabbert et al., 2020; Ittner et al., 2020; Rosing et al., 2020).

BBB hyperpermeability that occurs during and after brain ischemia is mainly caused by the mediators of the inflammatory process, which stimulate phosphorylation of TJs proteins. Significant phosphorylation of Tyr, Thr and Ser residues in the ZO-1 protein was observed in brain endothelial cells exposed to various cytokines and chemokines: tumor necrosis factor (TNF)-α, IL-6 and monocyte chemotactic protein 1 (MCP1)/CCL2 (Rochfort and Cummins, 2015; Salvador et al., 2015; Ittner et al., 2020). In concomitant cultures of endothelial cells and monocytes, activation of Rho/ROCK signaling pathway followed by phosphorylation of Ser and Tyr residues in occludin and claudin-5 was observed (Yamamoto et al., 2008); it allows facilitated migration of monocytes across the BBB. The dynamic behavior of TJ proteins in epithelial cells and ECs of peripheral organs has been extensively studied (Stamatovic et al., 2017). Incorporation of proteins that form TJs into cell membranes and subsequent transport followed by their degradation is involved in TJ recycling and modulation of BBB functions (Stamatovic et al., 2017). Diverse studies have shown, i.e., that occludin phosphorylation status of serine/threonine affects BBB permeability. Furthermore, ubiquitination of occludin and interactions with other cytokines such as TNF-α, IL-1ß, IFN-γ, and HGF (Capaldo and Nusrat, 2009; Murakami et al., 2009; Sundstrom et al., 2009) can affect BBB function by affecting the expression of occludin. In the OGD environment, BBB integrity can be affected by the activation of MAPK, PKC, ERK1/2 (Zhao et al., 2019) signaling pathways, and miRNAs such as miR-210 regulating occludin levels (Ma et al., 2017) (Table 1).

As it was shown, ischemia also induces the expression of claudin-1 in BMECs. It occurs long after stroke and is accompanied by TJ instability and BBB leakage. Targeting Claudin-1 with claudin-1 peptides improves brain endothelial barrier permeability and results in a significant neurological recovery after stroke (Zwanziger et al., 2012; Dithmer et al., 2017; Sladojevic et al., 2019). Jam-3 belongs to the superfamily of immunoglobulins found not only in TJs of polarized cells but also on cell membranes of leukocytes (Wyss et al., 2012). Several members of the JAM family interact with scaffolding proteins containing PDZ domains (e.g., ZO-1) to regulate cell-cell contact maturation and the production of junctional complexes, such as TJ and AS junctions (Keiper et al., 2005; Ebnet, 2017). Recent studies have shown that Jam-3 stimulates human BMEC migration *in vitro via* the activation of src, p38, and PI3K

signaling pathways (Rabquer et al., 2010) and its expression is directly regulated by the hypoxia-induced miRNA-212/132 (Burek et al., 2019) (Table 1). ZO-1 plays an important role in maintaining the integrity of the BBB. The functionality of ZO-1 can be regulated by its expression level and phosphorylation status (Halder et al., 2018). Stimulation by factors such as hypoxia can cause a decrease in ZO-1 expression and thus an impairment of BBB maintenance. For example, the pro-inflammatory cytokines TNF-α and IL-6 increase ZO-1 phosphorylation and reduces the integrity of BMECs (Ittner et al., 2020).

Astrocytes and glial cells are crucial components of the BBB that maintain the integrity of the BBB and the tight junctions between endothelial cells (Abbott et al., 2006; Sweeney et al., 2019). Astrocytes form an additional barrier at the endothelium by forming gab junctions and tight junctions between adjacent astrocytic protrusions. The astrocytic end-feet are tightly connected to the endothelium through specific interactions between the basal lamina and ECM (Xu et al., 2019a). Ischemic and/or traumatic brain injuries are responsible for the activation of astrocytes, which transform to their reactive form involved in numerous processes. The role of activated astrocytes varies over time after injury and in different parts of the CNS (Shen et al., 2021). Astrocyte swelling has been found to be one of the earliest responses after cerebral ischemia. Translocation of the water channel protein aquaporin 4 (AQP4) to the cell surface, facilitation of water influx along an osmotic gradient (ionic edema), and increased glutamate and lactate uptake have been proposed as possible mechanisms involved in astrocyte swelling (Larsen and MacAulay, 2017; Patabendige et al., 2021). AQP4 is widely expressed throughout the brain, particularly predominately at the astrocyte end-foot, where it selectively regulates blood-brain water flux and maintains cerebral water balance (Nagelhus and Ottersen, 2013). AQP4-deficient mice have reduced cytotoxic cerebral edema after ischemic stroke (Manley et al., 2000). Interestingly, astrocyte AQP4 is upregulated in the delayed phase after ischemia, which could be an additional mechanism involved in BBB repair (Tourdias et al., 2011). Over 80% of glutamate transporter proteins, particularly excitatory amino acid transporter 2 (EAAT2), are located on astrocytes, making them a major site of NVU glutamate uptake. In cerebral ischemia, glutamate uptake by astrocytes is essential for neuroprotection and for the prevention of extracellular glutamate rise into excitotoxic levels (Rosenberg et al., 1992; Petr et al., 2015). The swelling of astrocytes can compress the blood vessels in the ischemic area and exacerbate the lack of vascular perfusion (Sykova, 2001). Astrocytes may promote BBB disruption after ischemic stroke. In the co-culture of ECs and astrocytes, EC-derived macrovesicles stimulate increased apoptosis in OGD-treated astrocytes, which was accompanied by disruption of BBB and downregulation of occludin and claudin-5 (Pan et al., 2016). In addition, post-ischemic neurons stimulate astrocytes to synthesize VEGF that contributes to an increase in endothelial permeability and the loss of TJs proteins (Gabbert et al., 2020; Ittner et al., 2020). Knocked down VEGF expression in astrocytes suppressed the effects of OGD-treated neurons on BBB integrity (Li et al., 2014). Astrocytes are also a source of matrix metalloproteinases (MMPs), whose increased activity have been associated with the degradation of TJs and extracellular matrix after ischemia (Mun-Bryce and Rosenberg, 1998). Furthermore, an inhibition of MMPs prevented the loss of TJ proteins in the focal ischemia model (Yang et al., 2007). Activation of astrocytes can be accompanied by microglial activation and the secretion of some inflammatory factors such as transforming growth factor-α (TGF-α), IL-6, leukemia inhibitory factor (LIF), and TNF-α (Shen et al., 2021). In addition, post-ischemic neurons and ECs are also involved in astrocyte activation by releasing cytokines to regulate astrocyte activation and proliferation.

Pericytes have been described as capillary multifunctional mural cells with the highest density at cerebral capillaries within the NVU of the BBB (Brown et al., 2019). Pericytes are critical for regulation of cerebral blood flow, expression of TJs, and maintenance of BBB integrity (Liu et al., 2012; Oztop-Cakmak et al., 2017). Besides supporting cells for capillaries, pericytes have been suggested to play an important role in endothelial cell function and cerebral blood flow modulation (Bergers and Song, 2005; Armulik et al., 2010). After cerebral ischemia, pericytes respond rapidly and show protective or deleterious features such as contraction, migration, detachment from the microvascular wall, or even cell death (Hall et al., 2014; Fernandez-Klett and Priller, 2015; Brown et al., 2019). These changes observed in pericytes after stroke appear to be induced by an influx of intracellular calcium and increase of oxidative stress. Similar to ECs or astrocytes, pericytes release MMP9, causing a massive disruption of TJ proteins and BBB permeability (Brown et al., 2019). During middle cerebral artery occlusion (MCAO) in rats, pericytes detach and migrate from the basement membrane just 1 hour after occlusion. Three hours after injury, the gap between the pericytes and the basement membrane was even larger (Duz et al., 2007). In addition to mesenchymal properties of pericytes, post-ischemic pericytes can differentiate into major components of the NVU, supporting the hypothesis that pericytes may contribute to both neurogenesis and vasculogenesis at the site of brain injury (Nakagomi et al., 2015). These morphological changes and microvascular wall detachment were proposed to be the first step in the loss of BBB integrity. After MCAO, capillary reflux can be impaired by inducing pericytes contraction in the microvessels, resulting in obstruction of blood flow. Since the changes in the morphology of the pericytes persisted even after the restoration of the circulation in the cerebral vessels, it may be responsible for the development of brain pathology. Reactive oxygen and nitrogen species in microvessels play a crucial role in pericytes dysfunction in addition to their role in BBB damage (Yemisci et al., 2009; Dalkara et al., 2011). Inhibition of oxidative-

nitrative stress by a suppression of peroxynitrite attenuates pericyte contraction after MCAO in mice (Yemisci et al., 2009). In addition to oxidative stress, ischemia-induced inflammation can further exacerbate pericyte-mediated BBB dysfunction. In co-cultures of pericyte cells and microvascular-derived fibroblasts, pro-inflammatory stimuli induce widespread changes in the expression of interleukins, chemokines and cell adhesion molecule genes in pericytes (Persidsky et al., 2016). It has been suggested that reducing pericyte-mediated inflammation may be beneficial in restoring BBB function and reducing inflammation in CNS injuries (Yemisci et al., 2009). The lack of a specific indicator of pericytes due to their multipotent (self-renewing) properties limits the study of these cells. Platelet-derived growth factor receptor-beta (PDGFRβ) and neural/glial antigen 2 (NG2), meaning the receptor and co-receptor for PDGF, respectively, are commonly used as relatively specific markers for pericytes (Jiang et al., 2018). The elevated endothelial PDGF-β during ischemia is associated with an increase in microvessel diameter and blood supply due to dilated pericytes (Arimura et al., 2012). Stark et al. (2013) showed the active involvement of NG2+ pericytes in the immune response to inflammatory mediators. Namely, NG2+ pericytes control the pattern and potency of leukocyte infiltration into the CNS through upregulation of Intercellular Adhesion Molecule 1 (ICAM-1) and synthesis of chemotactic macrophage migration inhibitory factor (MIF) (Stark et al., 2013). Recently, post-ischemic brain pericytes have been reported to adopt a microglial phenotype. In human brain tissue obtained from stroke patients, activated pericytes were found to express microglia-specific markers such as ionized calcium-binding adapter molecule 1 (Iba-1), CD11b and galectin-3 (GAL-3) (Ozen et al., 2014). Consistent with the results mentioned above, PDGFRβ+ perivascular cells isolated from areas of ischemia showed upregulation of microglia-specific markers (e.g., Iba1, CD11b) and stem cells markers (e.g., nestin, c-myc, Klf4, and Sox2), suggesting that pericytes can function as microglia-generating stem cells with potential phagocytic capacity (Sakuma et al., 2016). Furthermore, in the adult spinal cord, pericytes have been identified as a source of scar-forming cells in CNS injury. Also, after spinal cord injury, PDGFRβ+ cells co-express stromal cell markers and produce scar tissue distinct from glial scars (Fernandez-Klett et al., 2013).

Microglia are specialized CNS macrophages responsible for orchestration of the innate immune response, tissue development and maintaining homeostasis (Kettenmann et al., 2011). These primary CNS immune cells play a crucial role in brain and retinal vasculature development and are involved in vascular sprouting, migration and anastomosis (Arnold and Betsholtz, 2013; Harry, 2013). As an important component of the NVU, microglia also regulate BBB development actively communicating with ECs (da Fonseca et al., 2014). Microglia are present in vascular junctions and apical cells of the pontine endothelium and synergistically promote cerebrovascular network formation in combination with VEGF-induced vascular sprouting (Fantin et al., 2010). Microglia may increase vascular sprouting by secreting soluble factors rather than by direct contact with EC, as shown by organotypic aortic ring culture (Rymo et al., 2011). Under physiological conditions, microglia are inactive, with small cell bodies and highly ramified branching processes. After injury or invasion by pathogens, microglia transform into active phagocytic microglia, migrate and accumulate at the site of injury (Fu et al., 2014). After ischemic brain injury, microglia are rapidly activated, followed by morphological and genetic changes in these cells (Kettenmann et al., 2011; Liu et al., 2020). Microglia/macrophages are activated in two states of polarization: the pro-inflammatory phenotype (known as M1 microglia), and the anti-inflammatory phenotype (M2 microglia) (Liu et al., 2020; Rodriguez-Gomez et al., 2020). Activated microglia play a dual role in ischemic brain injury as well as in disrupting BBB integrity, possibly due to their phenotypic polarization (Kang et al., 2020). These highly dynamic cells produce large amounts of cytokines and chemokines. Moreover, microglial cells are able to increase the level of adhesion molecules on the surface of endothelial cells, which increases the likelihood of infiltration by leukocytes (da Fonseca et al., 2014). It has been postulated that dynamic changes in microglia and astrocyte phenotype are crucial in determining their deleterious or beneficial character at specific stages of CNS injury (Lan et al., 2017). Beneficial effects of activated microglia have been associated with phagocytosis of cellular debris and suppression of inflammatory responses (Sierra et al., 2010; Herzog et al., 2019). Neuroinflammation induced by MCAO or lipopolysaccharide (LPS) administration *in vitro* modulates the direction of microglia polarization. Microglia are more sensitive to pathogens or damage and therefore impair BBB function. Initially activated pro-inflammatory microglia produce common inflammatory cytokines such as IL-1 and TNF-α, leading to activation of astrocytes and increase in the permeability of BMECs (Nishioku et al., 2010; Huang et al., 2020). During CNS injury, microglia/macrophages are continuously activated, leading to distinct effects on the structure of BBB and NVU as the M1/M2 phenotype is switched and exchanged between the activated microglia and astrocytes (Kirkley et al., 2017). Pro-inflammatory microglia also lead to P-glycoprotein (P-gp) dysfunction in the outer cerebral cortex through activation of NADPH oxidase (Matsumoto et al., 2012) and a concomitant reduction in the efflux of neurotoxic substances from the CNS. However, microglial cells are also involved in the defense mechanisms against neuroinflammation, such as e.g., vascular remodelling that helps restore CNS functions and alleviate neurological symptoms (Yang et al., 2015). These microglia can also promote angiogenesis through the production of VEGF, IL-8 and microtubule-associated protein (MAP9) (Medina et al., 2011; Willenborg et al., 2012; Zajac et al., 2013).

2.1 The effect of miRNA on BBB dysfunction

MiRNAs regulate the function of the BBB in health and disease. Recent studies have shown that miR-98 directly targets the production of the pro-inflammatory cytokines CCL2 and CCL5 (Bernstein et al., 2020) and is implicated in the attraction of leukocyte adhesion and migration across the BBB (Schober, 2008). This is accompanied by an exacerbation of BBB dysfunction due to activation of small Rho GTPases followed by actin cytoskeletal rearrangements and redistribution of TJ proteins. Moreover, miR-375-3p can additionally affect the vascular barrier by targeting claudin-1 to promote metastasis in small cell lung cancer (Mao et al., 2021). The opposing effect can be induced by miR-155, which inhibits OGD-induced barrier dysfunction in human primary BMECs (Pena-Philippides et al., 2018). In addition, miR-429 inhibits the expression of ZO-1, which markedly worsens BBB function (Chen et al., 2018). Under hypoxic circumstances *in vitro*, miR-125a-5p, miR-18a, and miR-424-5p increase BBB permeability by downregulation of ZO-1 expression (Lin et al., 2019; Wang et al., 2020; Ren et al., 2021). In contrast, after OGD exposure, the expression of miR-15a was shown to be significantly increased in brain EC cultures as well in the bloodstream 1 day after ischemic stroke. Additionally, while miR-15a could post-transcriptionally enhance or repress its direct downstream target BCL-2, selective miR-15a transgene overexpression in ECs resulted in decreased brain capillary density, significantly enhanced the cerebral infarction area, and neurological deficits in mice 7 days after MCAO. These findings may indicate that miR-15a has a meaningful impact on post-ischemically-induced cerebral angiogenesis (Yin et al., 2014). Furthermore, the hypoxia-induced miR-210 is significantly upregulated in ischemic cerebral cortex of adult rats, where the Notch1 signaling is hyperactivated. These results could be verified *in vitro* in HUVEC cells. It was observed here, that an overexpression of miR-210 led to Notch1 signaling activation followed by the increase of EC migration and the formation of capillary-like structures. MiR-210 may therefore be responsible for post-ischemic neovascularization (Lou et al., 2012). Moreover, miR-210 was observed to regulate the angiogenesis of brain vessels under physiological conditions (Zeng et al., 2014).

Mechanisms of action of miRNA-based therapeutic are established anti-inflammatory activities such as an inhibition of astrocyte activation, cytokine secretion, and leukocyte extravasation (Sun et al., 2018). For example, altered expression of some miRNAs, either increased (i.e., miR-22 and miR-122) or decreased (miR-15a/16-1), is responsible for decreased expression of some pro-inflammatory factors in the ischemic brain: TNF-α, IL-6, monocyte chemoattractant protein-1 (MCP-1), cytochrome c oxidase (COX-2), inducible nitric oxide synthase (iNOS), and Vascular cell adhesion protein-1 (VCAM-1) in the ischemic brain (Yu et al., 2015; Liu da et al., 2016; Yang et al., 2017). Exosome-mediated delivery of miR-124-3p promoted M2 microglia polarization and suppressed tissue inflammation after TBI (Huang et al., 2018), and reduced lesion core after MCAO in mice (Hamzei Taj et al., 2016a). Interestingly, it was also found that an overexpression of miR-29b can reduce BBB disruption after ischemic stroke by downregulating AQP4 (Wang et al., 2015). *In vitro* experiments identified miR-130 as a strong transcriptional repressor of the *AQP4* M1 isoform. In addition, *in vivo* studies have shown that anti-miR-130a upregulated the *AQP4* M1 transcript (Sepramaniam et al., 2012). AQP4 is also a potential target for the regulation by miR-130b. Thereby, miR-130b accelerates the downregulation of AQP4 in primary astrocytes under normoxic and OGD conditions (Zheng et al., 2017). In contrast, anti-miR-320a treatment upregulates the expression of this water channel protein after stroke, which is associated with a reduction in cerebral edema (Sepramaniam et al., 2010). A few studies have examined the significance of astrocyte-enriched expression of miRNAs after ischemia and stroke. For example, miR-181a is overexpressed in the infarct core (Ouyang et al., 2012a) and associated with increased mitochondrial dysfunction and apoptosis in astrocytes (Ouyang et al., 2012b). Treatment with miR-181a antagomir reduced the loss of hippocampal CA1 neurons after forebrain ischemia (Moon et al., 2013). In addition, inhibition of miR-181a resulted in an improvement of 17β-estradiol-mediated stroke protection in females, in part by increasing estrogen receptor-α production (Stary et al., 2017). Intriguingly, astrocyte-enriched miR-29a changed in the opposite direction compared to miR-181a following transient forebrain ischemia, decreasing in hippocampal CA1 neurons and increasing in DG (Ouyang et al., 2013).

In rat pericytes after MCAO, miR149-5p increased expression of sphingosine-1-phosphate receptor 2 (S1PR2), superoxide dismutase (SOD) 1-3 and significantly decreased the expression of MMP9, thereby alleviating BBB permeability and playing a protective role for neurons (Yan et al., 2021). Furthermore, ICV injection of antagomir-149-5p attenuated BBB permeability and improved MCAO outcomes in rats (Wan et al., 2018). In BMECs, miR-539 bound to the zink-finger transcription factor SNAI2 and was involved in the regulation of EC permeability by affecting the SNAI2/MMP9 signaling pathway, suggesting that circulating miR-539 is a potential marker for predicting BBB integrity after ischemic stroke (Li et al., 2021).

Modulation of miRNAs has been reported to contribute to post-stoke pathology, including the regulation of neuroinflammation. Numerous miRNAs are involved in the molecular action of different microglia phenotypes (Lian et al., 2020). Differential expressions levels of miRNAs (e.g., miR-210, miR-155, miR-373, miR-689, and miR-182-5p) have been previously reported after LPS stimulation of microglial cells *in vitro* (Freilich et al., 2013; Wang et al., 2018). Similarly, different expression of certain miRNAs (such as miR-183, miR-146a) has been reported in the plasma and brain of animals exposed to experimental stroke (Chu et al., 2018;

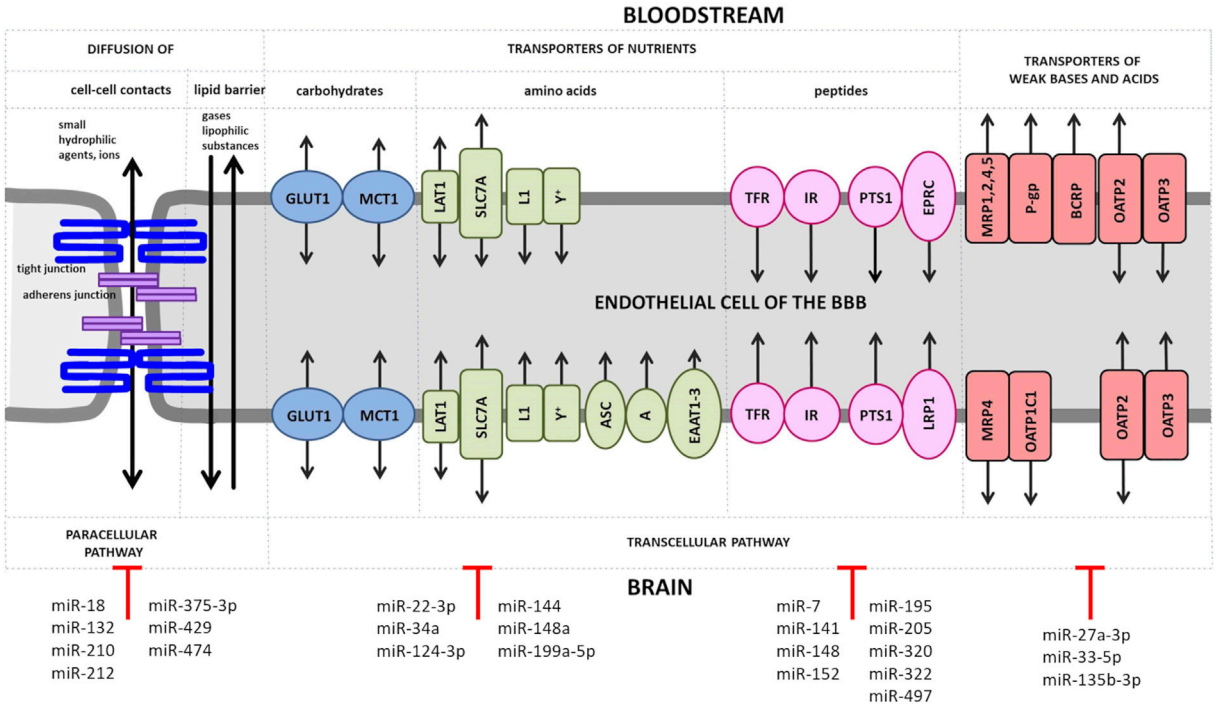

FIGURE 1
Transport of solutes across the BBB. Small ions and hydrophilic substances up to 800 Da permeate the endothelial layer of the BBB in both directions through tight junctions such as claudins by the paracellular pathway. At the basolateral site of these substances have also to pass the adherens junction. Small lipophilic compounds, such as drugs, and gases, like oxygen or carbon dioxide, diffuse transcellularly across the lipid monolayer. Transporters for carbohydrates are expressed at both, luminal and abluminal membrane of the BBB endothelium and include the following: glucose transporter 1 (GLUT1), lactate transporter monocarboxylate transporter 1 (MCT1), L-type amino acid transporter 1 (LAT1), solute carrier family 2 facilitated glucose transporter member 1 (SLC2A1) and L1 and y + transporters for large neutral and cationic essential amino acids. Non-essential amino acid transporters (the alanine-, serine- and cysteine-preferring system (ASC), the alanine-preferring system (A)) and the excitatory amino acid transporters 1–3 (EAAT1-3) are located at the abluminal side. Transporters of peptides or proteins including the transferrin receptors (TFRs), the insulin receptors (IRs) and the peptide transport system 1 (PTS1) for encephalins are expressed at both EC membranes, while the endothelial protein C receptor (EPCR) for activated protein C (APC) and the low density lipoprotein receptor-related protein 1 (LRP1) for amyloid-β are found at the luminal or abluminal side, respectively. The ATP-binding cassette (ABC) efflux transporters include multidrug resistance protein 1 (MRP1, 2, 4, 5, of which only MRP4 is present at both membrane sides of BBB ECs) and further the solute carrier organic anion transporter family member 1C1 (OATP1C1), the breast cancer resistance protein (BRCP) and the organic anion transporters/organic anion transporter peptides (OAT/OATPs). MicroRNAs (miRs), which have been shown to directly regulate genes encoding tight and adherens junction proteins and various transporter proteins, are shown.

Xiang et al., 2019). In the work by Zhao et al. (2013) the authors showed that overexpression of miR-424 reduced brain injury after ischemic stroke in the mouse MCAO model by suppressing microglial activity. In addition, miR-let-7c-5p has been associated with anti-inflammatory properties promoting neuroprotection after ischemic stroke by inhibiting microglial activation and translational repression of caspase-3 (Zhao et al., 2013; Ni et al., 2015). One of the miRNAs almost exclusively expressed in the CNS is miR-124. Intracerebral injection of miR-124 in focal cerebral ischemia in mice modulated microglia/macrophage activation of the M2 phenotype, thus contributing to the recovery of brain cell function (Hamzei Taj et al., 2016b). Stimulation of mouse microglial cell line BV-2 with IL-4 in an ischemic conditions resulted in the subsequent release of miRNA-26 containing exosomes (Tian et al., 2019). It caused the formation of the tubes by endothelial cells in culture as well as angiogenesis in the MCAO model of stroke. The authors suggest that miRNA-26a, which has already been implicated in angiogenesis, may be responsible for new vessel formation after stroke and that such stimulation of a pre-existing pool of microglia in brain could be a promising therapeutic approach.

3 Transport pathways and their regulation by ischemia and/or stroke

The transporter barrier of the BBB tightly regulates the transport of substances in and out of the brain. Some

substances (e.g., oxygen, carbon dioxide, nitrogen monoxide and lipophilic substances such as hormones) can pass through the BBB unhindered by diffusion. Other substances depend on transporter proteins that facilitate their passage (Figure 1). An alteration of the expression level of BBB transport proteins or the modulation of their activity are observed in CNS ischemic injury and act as an important element of the cellular response to oxygen and nutrient deprivation (Nilles et al., 2022).

There are multiple proteins expressed in BMECs to ensure the supply of the brain with nutrients (Figure 1). Glucose is passively transported by the glucose transporter 1 (GLUT1/*SLC2A1*) in a concentration-dependent manner (Blecharz et al., 2015). GLUT1 is upregulated by ischemia and hypoglycemia and contributes to edema formation in stroke by co-transporting water (Loike et al., 1992; Wright and Turk, 2004; Vemula et al., 2009; MacAulay and Zeuthen, 2010). The presence of a second glucose transporter at the BBB, the sodium-dependent glucose transporter (SGLT1 encoded by the *SLC5A1* gene), is controversial (Elfeber et al., 2004; Vemula et al., 2009). Sglt1 has been shown to be markedly upregulated after OGD in BMECs (Neuhaus et al., 2012).

ECs possess a number of ATP-binding cassette proteins (ABC), which represent a large group of membrane pumps that actively efflux molecules from the cells (Helms et al., 2016; Tornabene and Brodin, 2016). Efflux pumps therefore play a key role in the absorption, distribution and elimination of endo- and xenobiotics or in reducing the permeability of blood-tissue barriers (Chen et al., 2016; Du et al., 2018). Depending on the location of the efflux pump, they can promote transport into the brain and protect the brain from potentially neurotoxic endogenous or lipophilic xenobiotic substances (Loscher and Potschka, 2005). The neuroprotective role of ABC transporters concurrently determines the difficulty for delivery of some drugs to the CNS. Namely, increased expression of transporters enhances neuroprotection, but at the expense of drug distribution in CNS; and conversely—reduced activity of ABC transporters decreases neuroprotection and provides improved drug access to the brain (Miller, 2015). The dominant ABC transporters at the BBB is P-gp, also known as multidrug resistance protein 1 - MDR1/ABCB1), breast cancer resistance protein (BCRPP/ABCG2), and the multidrug resistance-associated proteins (MRPs) (Demeule et al., 2002; Miller, 2015).

An alternated expression and functional changes of ABC transporters were observed after hypoxia or ischemia (Tornabene and Brodin, 2016; Burek et al., 2020). In a model of focal cerebral ischemia, the activity and expression of the brain luminal transporter P-gp was increased in mice (Spudich et al., 2006) as well in rats (DeMars et al., 2017) being consistent with *in vitro* studies showing that OGD treatment was able to upregulate P-gp both at mRNA and protein level (Neuhaus et al., 2014; DeMars et al., 2017). At the same time, the abluminal transporter ABCC1, which carries its substrates in the opposite direction, from blood to the brain, was found to be decreased in capillary extracts in response to MCAO (Kilic et al., 2008). In contrast to the *in vitro* study mentioned above, no changes in P-gp expression under hypoxia were observed in two independent studies (Patak et al., 2011; Lindner et al., 2012) using the immortalized human BMECs (hCMEC/D3). According to the authors, the lack of changes in P-gp expression may be due to the lost ability of hCMEC/D3 to respond to hypoxia (Lindner et al., 2012), or this cell line may not be suitable model for studying the EC response to ischemia *in vitro* (Patak et al., 2011).

Receptor-mediated endocytosis and exocytosis are responsible for the passage of macromolecules such as insulin, leptin, low-density lipoproteins and transferrin through the intact BBB (Blecharz et al., 2015). The transferrin receptor (TfR) is an iron-binding receptor that delivers iron to cells. TfR-mediated brain iron uptake remains partially functional after onset of ischemia and shows complete recovery after reperfusion (Hao and Bickel, 2013). In addition, expression of TfR was upregulated in hypoxic BMECs (Tornabene et al., 2019).

3.1 The effect of miRNA on transport pathways

Several studies have described the direct regulation of glucose transporters by miRNAs in different tissues, e.g., miR-144 upregulated GLUT1 and glucose uptake (Liu et al., 2016), while miR-148a, -199a-5p, -34a, downregulated GLUT 1 and GLUT1/*SLC2A1* was confirmed to be a direct target of these miRNAs (Qiang et al., 2020; Tiemin et al., 2020; Xu et al., 2022). Monocarboxylate transporter 1 (MCT1/*SLC16A1*) being responsible for the transport of a broad range of monocarboxylates is expressed in BMECs (Hoshi et al., 2013; Curtaz et al., 2020b). Its expression was found to be significantly increased in BMECs after MCAO (Zhang et al., 2005). *SLC16A1* is a direct target of miR-22-3p and miR-124-3p (Xu et al., 2019b; Xue et al., 2022). Interestingly, in the rat model of cerebral ischemia, miR-124-3p showed a time-dependent biphasic effects on MCT-1 expression and protective effects against ischemic brain injury (Xu et al., 2019b).

Studies have repeatedly shown that the ABC transporter family of efflux pumps may be regulated by miRNAs (Haenisch et al., 2014). P-gp is the most studied member within the ABC transporter family. In addition, P-gp expression is tightly regulated by differential molecules including miRNAs. Moreover, P-gp can regulate the intracellular expression of miRNAs (Lopes-Rodrigues et al., 2014). In a very recent study, Hammad et al. (2022) showed that the expression and activity of P-gp in hCMEC/D3 is modulated by miR-27a-3p. In addition, complex interactions

between Pg-p, miRNAs and other cellular proteins have been characterized in several tumor cell lines describing the key mechanism of drug resistance within tumors (Haenisch et al., 2014; Lopes-Rodrigues et al., 2014).

There is accumulating evidence that miRNAs may contribute to the pathogenesis of cerebral ischemia and that the ischemic preconditioning (IPC) paradigm may exert neuroprotective properties by acting as both positive and negative regulators of cell survival (Bell et al., 2017; Vasudeva and Munshi, 2020). In the mouse model of ischemic stroke, miR-33-5p and miR-135b-5b were downregulated by earlier exposure to IPC. Neuroprotective effects of inhibition of these two miRNAs was found under *in vitro* OGD conditions. In addition, inhibition of miR-33-5p and miR-135b-5b increased the ABCA1 level (Sung et al., 2021). Additionally, it was recently reported that ABCA1 knockout mice showed increased BBB leakage, white matter/axonal damage and functional deficits after MCAO compared to healthy wild type mice (Cui et al., 2015).

TFRC encoding TfR is directly regulated by miR-7, -141, -148a, -152, and -320 in various type of cancers, where high iron levels are required for cell proliferation (Schaar et al., 2009; Kindrat et al., 2016; Miyazawa et al., 2018; Babu and Muckenthaler, 2019). The low-density lipoprotein receptor (LDLR) family is responsible for the transport of low-density lipoproteins (LDLs) such as apolipoprotein (Apo) B and E *via* endocytosis (Hladky and Barrand, 2018). Low-density lipoprotein receptor-related protein 1 (LRP1) is direct target of miR-205 (Song and Bu, 2009). Another protein ensuring receptor-mediated endocytosis and being highly expressed at the BBB and other tissues is the insulin receptor (*INSR*). It guarantees insulin signaling to modulate cellular functions either in health or disease (Tashima, 2022). *INSR* is a direct target of miRNA-195, -322, -497 (Wei et al., 2016; Geng et al., 2020). The direct regulation of cellular receptors at the BBB by miRNA can be used for the development of therapeutics or the generation of new tools for drug delivery to the CNS.

4 Discussion

In summarizing the existing literature, we were able to depict the current knowledge and improve the understanding of the impact of miRs on the normal BBB and the whole NVU function as well as on the role of these molecules under ischemic conditions. To maintain the BBB integrity and ensure a permanent homeostasis inside the brain appear to be essential in the treatment of numerous cerebrovascular disorders including ischemic stroke. Functional changes in the cellular architecture of the BBB result in the disruption of the BBB. At the cellular level, ECs, pericytes, astrocytes and microglia composing the NVU are affected by ischemia and influence each other. Structurally, alterations in the expression and distribution of TJ and AJ proteins, then, the local microenvironment, transport systems, enzymes and finally, the extracellular matrix, can ultimately lead to the passage of harmful serum components and immune cells through the BBB, disrupting CNS homeostasis and damaging the surrounding brain parenchyma. The role of miRNAs and their specific direct targets at the BBB needs however to be further explored in future studies as the full picture of miRNA-mediated BBB regulation is still insufficiently described. In addition, ischemia-induced miRNA changes in all NVU components should be intensively studied to identify further useful and specific biomarkers, including therapeutic miRNA. In future BBB studies, more effort should be put into studying proteins and signaling pathways specifically associated with certain cell types composing NVU, hence they may provide further useful indications and insights into the miRNA-mediated regulation of all BBB functions, including their involvement in pharmacokinetics of CNS drugs. Cell-specific proteins and signaling pathways involved in cerebral ischemia and their influence on the EC barrier should be also considered in the context of miRNAs in future BBB studies, hence they may provide further useful indications and insights into the miRNA-mediated regulation of BBB permeability and facilitation of drug delivery to the CNS. Finally, there is an urgent need to identify further precise strategies facilitating the recovery and repair of the BBB after ischemic stroke giving the functional recovery of the affected patients and improving their prognosis. As depicted in this review article, there is an objective evidence suggesting that miRNAs may serve as highly potent clinical therapeutics in the treatment of stroke and its respective consequential damages in future. Further studies to test the clinical effectiveness of miRNAs in the context of cerebrovascular diseases are therefore essential to extend our knowledge on these molecules.

Author contributions

AS, KB-L, MN-C, and MB wrote the manuscript and critically revised the manuscript, AM, PM critically revised the manuscript. All author have read and approved the last version of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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