

## Review Article

# Cellular Components of the Blood-Brain Barrier and Their Regulation

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- The blood-brain barrier
- Endothelial cells
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**Abstract**

Human life expectancy has been significantly extended, which poses major challenges to our healthcare and social systems. Aging-associated cognitive impairment is attributed to endothelial dysfunction in the cardiovascular system and neurological dysfunction in the central nervous system. The central nervous system is considered an immune-privileged tissue due to the exquisite protection provided by the blood-brain barrier. The present review provides an overview of the structure and the function of blood-brain barrier, extending the cell components of blood-brain barrier from endothelial cells and pericytes to astrocytes, perivascular macrophages and oligodendrocyte progenitor cells. In particular, the pathological changes in the blood-brain barrier in aging, with special focus on the underlying mechanisms and molecular changes, are presented. Furthermore, the potential preventive/therapeutic strategies against aging-associated blood-brain barrier disruption are discussed.

**ABBREVIATIONS**

20-HETE: 20-Hydroxyeicosatetraenoic Acid; 5-HT: 5-Hydroxytryptamine; A $\beta$ : Amyloid-Beta; ADAMTS: A Disintegrin and Metalloproteinase with Thrombospondin Motifs; AGEs: Advanced Glycation End Products; AKAP12: A-Kinase Anchor Protein 12; AMPK 5: Monophosphate-Activated Protein Kinase 5; Angpt1: Angiopoietin 1; Angpt2: Angiopoietin 2; AQP4: Aquaporin-4; BACE: Beta-Site Amyloid Precursor Protein Cleaving Enzyme; BBB: Blood-Brain Barrier; Cx43: Connexin 43; eNOS: Endothelial Nitric Oxide Synthase; EPCs: Endothelial Progenitor Cells; ER: Endoplasmic Reticulum; ERK: Extracellular Signal-Regulated Kinases; GABA: Gamma-Aminobutyric Acid; GLP-1: Glucagon-Like-Peptide-1; Htra: High-Temperature Requirement a Serine Peptidase; IGF-1: Insulin-Like Growth Factor-1; IGFR: Receptor for Igf-1; IL: Interleukin; Mfsd2a: Major Facilitator Superfamily Domain Containing 2a; NF- $\kappa$ B: Nuclear Factor-Kappa B; NG2: Neuron-Glial Antigen 2; NMDA: N-Methyl-D-Aspartate; MMP: Matrix Metalloproteinases; OPCs: Oligodendrocyte Progenitor Cells; PDGF: Platelet-Derived Growth Factor; PDGFR $\alpha$ : Platelet-Derived Growth Factor Receptor Alpha; PDGFR $\beta$ : Platelet-Derived Growth Factor Receptor Beta; PLVAP: Plasmalemma Vesicle-Associated Protein; POCD: Postoperative Cognitive Disorder; PVM: Perivascular Macrophages; ST2: Suppression of Tumorigenicity 2; TDP-43: Transactive Response Dna-Binding Protein of 43 Kda; TGF- $\beta$ : Transforming Growth Factor-Beta; TNF: Tumor Necrosis Factor; Tregs: Regulatory T

Cells; VCAM: Vascular Cell Adhesion Molecule; VEGF: Vascular Endothelial Growth Factor; VEGFR2: Vascular Endothelial Growth Factor Receptor 2; ZO-1: Zonula Occludens-1

**INTRODUCTION**

In 2020, the World Health Organization reported that till 2050, the proportion of the world's population over 60 years would reach 2.1 billion, which nearly doubles from 12% to 22% (<https://www.who.int/news-room/fact-sheets/detail/ageing-and-health>). Aging is an independent risk factor for cardiovascular and neurological diseases; as such, it poses societal challenges. With aging, endothelial and neurological dysfunction progress, significantly contributing to pathological conditions [1-3].

The central nervous system is considered an immune-privileged tissue due to the exquisite protection provided by the blood-brain barrier (BBB). The BBB is the primary site to protect the central nervous system from pathological stimuli [4]. Vascular endothelial cells, pericytes, as well as the end-feet of astrocytes are major components of the BBB. The study using recently developed single-cell sequencing and flow cytometry techniques reports that the brains of aged mice, compared to those of young counterparts, have a higher prevalence of endothelial cells and microglia, but not neurons, with high senescence gene enrichment scores [5]. In addition, lymphocytes are detected and significantly increased in the brains of 12-month-old mice when compared to younger ones [6], implying that the breakdown of

the BBB occurs during aging [7]. Using an advanced dynamic contrast-enhanced magnetic resonance imaging approach in the living human brain, early vascular leakage during normal aging is observed in the CA1 and dentate gyrus regions of the hippocampus, but not in other parts of the brain regions [8], and is accompanied by an increased level of soluble platelet-derived growth factor receptor  $\beta$  (PDGFR $\beta$ ), a cell surface marker of pericyte, in the cerebrospinal fluid [7,8]. It indicates the loss of BBB integrity in the process of aging in both animal and human studies, and aging-associated cerebromicrovascular dysfunction plays a causal role in the development of cognitive impairment.

Therefore, the present review focuses on the pathological changes in the BBB in aging, including those in endothelial cells and pericytes, as well as in astrocytes, perivascular macrophages and oligodendrocyte progenitor cells of the BBB. Specifically, the molecular changes in each cell types accounting for the pathological outcome are reviewed. Furthermore, potential therapeutic strategies for preserving BBB integrity during aging are discussed.

### Cell components of the BBB and their interaction in the maintenance of the integrity of BBB

It is well-established that the blood-brain barrier is composed of endothelial cells, pericytes and astrocytes. For a long time, the central nervous system is considered to be devoid of immune cells other than microglia. With the technological advances, a group of macrophage-lineage cells surrounding arterioles in the BBB has been identified [9,10], and they are termed perivascular macrophages. The cells forming the BBB interact not only with each other, but also with the neighbouring neuronal cells and oligodendrocyte progenitor cells (OPC), to regulate the function of the BBB. As such, dysfunction of any one type of these cells would have an impact on the integrity of the BBB, as discussed below.

### Vascular endothelial cells

Endothelial cells lie in the innermost layer of vascular wall and play a crucial role in regulating vascular tone, preventing platelet aggregation and leukocyte adhesion, and regulating cell proliferation [11-16]. The BBB endothelial cells are vital for the anatomical structure. They have very tight junctions and are tightly adhered to the extracellular matrix components of the basement membrane. Integrins are transmembrane receptors in the endothelial cells binding to the extracellular matrix [17]. Under hypoxia and pathological conditions, brain endothelial integrin  $\alpha 5 \beta 1$  and its ligand fibronectin, a major component of basement membranes in the BBB, are upregulated [18], and their interaction is important for angiogenesis [19]. An increased fibronectin/ $\alpha 5 \beta 1$ -interaction, which occurs following exposure to the inflammatory cytokine interleukin (IL)-1, modifies the localization of the tight junction protein claudin-5 in endothelial cells, and promotes the transendothelial migration of peripheral blood mononuclear cells in an *in vitro* BBB model [20]. Deletion of endothelial  $\alpha 5 \beta 1$  integrin compromises vascular integrity

and impairs vascular remodeling following immunization [21], although it appears to be neuroprotective against ischemic stroke injury [22]. By specifically deleting the autophagy-related protein Atg-7 in endothelial cells, the expression of fibronectin is downregulated, leading to BBB leakage and reduced adhesion of astrocytes to brain microvessels [23].

In endothelial cells, glycolysis is the principal energy supply [24]. The glucose transporter Glut1 and hexokinase 2, the first rate-limiting enzyme of glycolysis, are located in the abluminal side of the BBB, creating a concentration gradient of glucose to facilitate the influx of glucose from the blood into the brain [25]. During the conditions requiring increased glycolysis, the Glut1 protein translocates to the luminal side, leading to increased glucose uptake [26]. Single-cell transcriptome analysis demonstrates that the expression of Glut1 in endothelial cells of aged mouse brains is reduced compared to that in young adult mouse brains; in particular, the downregulation of Glut1 expression resembles that found in the brain of patients with Alzheimer's disease [27].

It is well-known that nitric oxide is a major endothelium-derived relaxing factor, which counteracts the effects of vasoconstrictors and inhibits the production of endothelium-derived contracting factors, a major one being endothelin-1 [12-16]. Nitric oxide is also inactivated by superoxide anions. Thus, reduced nitric oxide production and elevated levels of oxygen-derived free radicals are team players in vascular disorders [28,29]. Consistent with macrovascular endothelial dysfunction in aging, reduced vasodilatation has been reported in aged mice [30,31], as well as in human and mice with cognitive impairment [32]. Endothelial dysfunction is associated with reduced nitric oxide production [33,34], increased endothelin-1-mediated vasoconstriction [35,36], elevated reactive oxidative stress [30-37], enhanced inflammation [6-39], cerebro-microvascular rarefaction [37-44], and cerebral microbleeds [45,46]. Reduced expression of endothelial nitric oxide synthase (eNOS), the enzyme responsible for nitric oxide production in endothelial cells, results in spontaneous thrombotic cerebral infarction and cognitive impairments in aged mice [44]. Consistently, lack of redox enzymes, such as p66<sup>Shc</sup> [30], and nicotinamide adenine dinucleotide phosphate oxidase 5 [47], in aged mice improves endothelium-dependent and nitric oxide-mediated relaxations in basilar arteries and prevents the development of memory deficits. In mice with endothelial-selective overexpression of endothelin-1, compared to the wild types, there is increased oxidative stress in the hippocampal region following ischemia/reperfusion challenge of the middle cerebral artery; this is associated with poorer cognitive function and more severe BBB breakdown [36]. Beta-site amyloid precursor protein cleaving enzyme (BACE) is a membrane-bound aspartyl protease [48], and its two isoforms, BACE1 and BACE2, are present in the brain endothelial cells. BACE1 cleaves amyloid precursor protein at the  $\beta$ -secretase site (the amyloidogenesis pathway) and BACE2 promotes the non-amyloidogenic processing of amyloid precursor protein like an  $\alpha$ -secretase [49,50]. The amyloidogenesis pathway results in the release of amyloid- $\beta$

peptide (A $\beta$ ) into the extracellular space, while the  $\alpha$ -secretase-processing of amyloid precursor protein leads to a reduction of A $\beta$  production. Indeed, increased plasma and cerebrospinal fluid levels of BACE1 are correlated to the progression of Alzheimer's disease [51,52]. In addition to processing amyloid precursor protein, endothelial BACE1, the expression of which is increased in cerebral vessels of hypertensive subjects, cleaves occludin in endothelial cells and disrupts tight junctions of the BBB, resulting in memory deficits [53]. Deleting BACE1 improves microvascular, but not macrovascular, endothelial function by upregulating the expression of eNOS [54]. Moreover, BACE1 promotes the association between caveolin-1 and eNOS, resulting in reduced activation of eNOS. By contrast, BACE2, which is highly expressed in cerebrovascular endothelial cells, is downregulated in the endothelium of Alzheimer's patients [55]. BACE2 deficiency impairs the expression and function of eNOS in brain endothelial cells, suggesting that BACE2 is a vascular protective protein [56].

Inflammation in endothelial cells also leads to the disruption of the integrity of the BBB. Intracellular levels of tumor necrosis factor (TNF)  $\alpha$  and IL-1 $\beta$  in cerebrovascular endothelial cells are increased in 24-month-old mice when compared with those in the brains of 3-month-old mice [57]. Expressions of tight junction proteins, occludin-1 or zonula occludens-1 (ZO-1), are negatively correlated to the increased level of TNF $\alpha$  [57]. Complement precursor C3a cleaved from astrocytic C3 interacts with endothelial C3aR at the BBB, promotes upregulation of vascular cell adhesion molecule (VCAM)-1, and drives proinflammatory signaling and chemotaxis, resulting in immune cell infiltration in the aged brain [58,59].

Taken together, with aging, the metabolism and signaling of endothelial cells of the BBB are altered. These changes contribute to BBB leakage, leading to reduced glucose and blood supply to the brain and increased oxidative stress and inflammation, and are associated with impairment of cognitive function.

## Pericytes

Pericytes are perivascular cells enveloped in the vascular basement membrane with extended processes. They are characterized by ovoid cell bodies and long, thin processes with cell surface markers of chondroitin sulfate proteoglycan [CSPG4, also known as neuron-glia antigen 2 (NG2)] and PDGFR $\beta$  [60-63] (Figure 1). Pericytes are in contact with the neighboring pericytes and endothelial cells, but not with the surrounding neurons or glia. They are an important component of the BBB, as demonstrated by the findings that pericyte loss, which occurs during aging, is associated with disruption of BBB integrity.

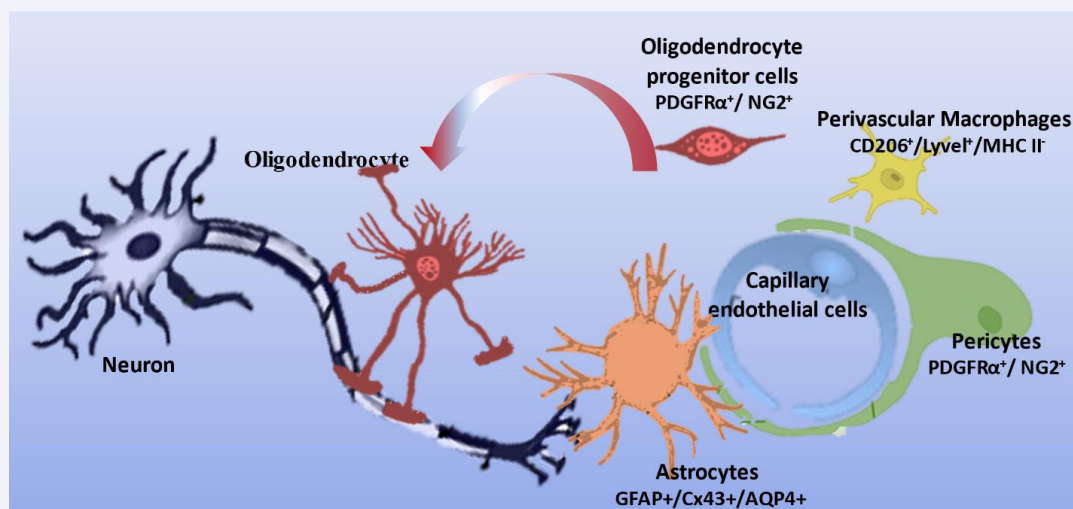
A role of pericytes in regulating cerebral blood flow is suggested by the presence of contractile proteins such as alpha-smooth muscle actin and myosin. Indeed, it is reported that noradrenaline (released by locus coeruleus) [64], reactive oxygen species [63], and optogenetic stimulation of gamma-aminobutyric acid (GABA) interneurons [63] induce pericyte contraction, which in turn constrict cerebellar capillaries. Focal ablation of pericytes results in capillary dilation, thus indicating

that pericytes regulate a basal vasomotor tone on the cerebral vasculature [41-63]. Pericyte contraction, at least following optogenetic stimulation, involves an influx of calcium through L-type calcium channels; and the increased intracellular calcium is taken up by mitochondria leading to reactive oxygen species generation and activation of Rho-kinase [63].

Pericytes relax to the neurotransmitter glutamate, which activates N-methyl-D-aspartate (NMDA) receptor in the neuron and subsequently nitric oxide synthase to release nitric oxide [64,65], leading to capillary dilatation [60]. Notably, glutamate-induced capillary dilatation is attributed to nitric oxide-induced suppression of 20-hydroxyeicosatetraenoic acid (20-HETE) formation, but not through activation of guanylyl cyclase to produce cyclic 3'-5' guanosine monophosphate (the canonical second messenger downstream of nitric oxide) in pericytes [60]. Both glutamate and NMDA stimulate an outward (hyperpolarizing) current, likely due to potassium efflux induced by prostaglandin E $_2$  in pericytes; and cerebellar capillary dilatation, being more prominent in areas where pericytes are present, is inhibited by blockade of EP $_4$  receptors (receptors for prostaglandin E $_2$ ) [60]. The findings thus confirm that pericytes actively relax in response to glutamate to cause capillary dilatation.

Reduced pericyte counts and coverage [37-67], as well as low capillary density [41-61], have been reported in aged rodent brains. By using an optical ablation approach, which damages pericytes but leaves microvascular endothelial cells undestroyed, local loss of pericytes induces capillary dilation. In aged mice, as well as under hypoxic conditions, this focal dilation results in severe flow steal [61-68] and exacerbates flow heterogeneity in capillary networks compared with adult young mice. Some capillaries stall in flow and regress, leading to a loss of capillary connectivity. Furthermore, in aged mice the remodeling of neighboring pericytes is impaired that pericyte coverage and capillary tone cannot be restored [61].

The crosstalk of pericytes with endothelial cells is important for the development and maintenance of the function of the BBB. Communications between pericytes and endothelial cells involve both physical contact through the gap junctions and via paracrine signalings. Through gap junction coupling, the depolarizing signals (leading to contraction) and hyperpolarizing signals (leading to dilatation) propagate from pericytes to endothelial cells [65]. The paracrine signalings between pericytes and endothelial cells appear more complicated. On the one hand, angiogenic endothelial cells release platelet-derived growth factor (PDGF)-B, which binds to PDGFR $\beta$  on pericytes to stimulate pericyte proliferation and attachment to endothelial cells [41-61]; the latter, in turn, supports the cerebrovascular stability [64,65]. On the other hand, pericytes contribute to the regulation of endothelial transcytosis, as demonstrated by the findings that pericyte deficiency results in upregulated endothelial transcytosis and disrupted endothelial tight junctions, and hence increased BBB permeability [69-72]. Focal absence of pericytes is associated with low expression of major facilitator superfamily domain containing 2A (Mfsd2a) and angiopoietin 2 (Angpt2);



**Figure 1** The blood-brain barrier in the central nervous system. The blood-brain barrier is composed of capillary endothelial cells, pericytes, and end feet of astrocytes. In addition, perivascular macrophages and oligodendrocyte progenitor cells are reported involved in the protection of the blood-brain barrier.

as well as the presence of plasmalemma vesicle-associated protein (PLVAP, which is normally found in fenestrated blood vessels) in brain endothelial cells [72]. Moreover, in endothelial cells devoid of pericyte contact, the expression of intercellular adhesion molecules is upregulated; this is coupled with increased extravasation of leukocytes at the site, thus suggesting that pericytes also contribute to limiting neuroinflammation [60-72]. Exposure of pericytes to inflammatory stimuli leads to inflammasome activation, and this further stimulates the release of inflammatory mediators and the loss of tight junctions in cerebral endothelial cells [73].

CD146, also known as the melanoma cell adhesion molecule, is reported as a cell surface marker of endothelial cells [74] and pericytes [75]. In particular, CD146 is present in the endothelial cells of immature brain microvessels without pericyte coverage, and is present in pericytes of the microvessels, but not in pericyte-covered brain endothelial cells [76]. Such observation indicates that CD146 plays an essential role in BBB formation during embryogenesis. Endothelial cell-specific knockout of CD146 downregulates claudin 5, but not ZO-1, protein expression, whereas pericyte-specific knockout of CD146 reduces pericyte recruitment to endothelial cells since the activation of PDGFR $\beta$  requires the direct interaction with CD146 [76]. Furthermore, endothelial expression of CD146 is reduced by pericytes-derived transforming growth factor-beta1 (TGF- $\beta$ 1), leading to reduced adhesion of leukocytes to endothelial cells [76].

As such, the loss of pericyte with aging not only leads to disrupted BBB structure, but also contributes to increased endothelial permeability. It is also an underlying cause of cerebrovascular inflammation and impaired brain capillary flow, both of which are characteristic features of aging-associated dementia.

## Astrocytes

In the central nervous system, astrocytes (GFAP $^+$ /Cx43 $^+$ /AQP4 $^+$ ) are the main type of glial cells, which closely connect with neurons and extend elongated processes (end-feet) covering cerebral capillaries [77,78] (Figure 1). Astrocytes are responsible for secreting trophic factors and extracellular matrix proteins that support the BBB structure [77-79]. Connexin 43 (Cx43) is a major membrane protein of astrocytes, forming gap junctions and hemichannels for cell-cell communication and for exchange with extracellular space [79]. It is reported that the translocation of Cx43 to the cytoplasm in astrocytes challenged with oxygen-glucose deprivation/recovery results in disruption of astrocyte cell-cell adhesion and inhibition of the growth of adjacent neurons, implying that the membrane Cx43 is essential in maintaining the structure and function of astrocytes [80]. Membrane aquaporin-4 (AQP4), localized in the end-feet of astrocytes for regulating ion and water homeostasis in the brain, contributes to the clearance of A $\beta$  [81]. It has been reported that with aging, there is a pathological accumulation of phosphorylated tau protein (a major mediator of the A $\beta$ -neurotoxicity) in astrocytes. In a study of the Austrian elderly subjects, the protein presence of phosphorylated tau is increased, which is associated with the upregulation of Cx43 and AQP4 in astrocytes [82]. Together with the findings that Cx43 is involved in neuronal recovery [80] and AQP4 in A $\beta$  clearance [81], these data suggest that increased Cx43 and AQP4 protein expressions are likely neuroprotective responses of astrocytes preceding pathological changes in the brain [82].

Astrocyte mitochondria are responsible for maintaining homeostasis by participating in glutamate and fatty acid metabolism, as well as by regulating calcium signals and reactive oxygen species generation [83]. The transfer of healthy



mitochondria from astrocytes to neurons is an important mechanism protecting neurons against oxidative stress-induced damage [84,85]. However, with aging, astrocytes undergo senescence [79-86], with increased production of reactive oxygen species [87], vulnerability to oxidative stress [86], and enhanced generation of proinflammatory mediators [79].

Inside the astrocyte, the mitochondria form physical contact with endoplasmic reticulum (ER) for metabolic function, oxidative metabolism, and calcium signaling [88]. Mitochondria and ER are clustered in the end-feet of astrocytes, and are further accumulated in the end-feet following neuronal injury, suggesting that perivascular astrocytes are metabolically active and likely responsible for vascular remodeling [89,90]. Indeed, by specifically deleting mitofusin 2 in astrocytes, which disrupts the mitochondria-ER contact, especially in the end-feet, endothelium-mediated angiogenesis, and hence neovascularization in the injured area, following neuronal damage are impaired [89].

Astrocytes are also capable of producing endothelin-1, and the astrocyte-derived endothelin-1 is increased when mice are subjected to stroke, resulting in progressive neurodegeneration [91]. Elevated levels of endothelin-1, in turn, stimulate the production and secretion of A $\beta$ , which further stimulates astrocytes to release soluble factors, including the inflammatory mediators (TNF- $\alpha$ , IL-6, IL-1 $\beta$ , monocyte chemoattractant protein-1 and interferon-inducible protein-10), vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), nitric oxide, and oxygen-derived free radicals. These soluble factors participate in downregulating junction proteins ZO-1 and claudin-5, and impairing angiogenesis by reducing the phosphorylated levels of eNOS (at p1177) and VEGF receptor 2 (VEGFR2) in brain microvascular endothelial cells [92,93]. Therefore, as astrocytes progress to senescent phenotype with aging, their neuroprotective ability of promoting clearance of A $\beta$  and vascular repair is reduced; more importantly, they contribute to neurodegeneration by increasing oxidative stress, A $\beta$  production, inflammatory responses, and breakdown of endothelial tight junctions.

### Perivascular macrophages

The perivascular macrophages (PVM), characterized by CD206<sup>+</sup>/Lyve1<sup>+</sup>/MHC II<sup>+</sup>, take up molecules greater than 10 kDa to limit their passage to the brain [94] and regulate immune responses by recruiting neutrophils during pathogen invasion [95]. They express angiotensin type 1 receptors, the activation of which by angiotensin II, following chronic administration of low concentrations of the peptide for 14 days (due to angiotensin II increasing the BBB permeability), results in increased production of reactive oxygen species by PVM and consequently impaired cerebral blood flow response and cognitive dysfunction [96]. In aging and neurodegenerative diseases, signatures of PVM change and monocyte-derived macrophages emerge [97]. Using a lineage-tracing approach, the presence of CD206<sup>+</sup>/Lyve1<sup>+</sup> PVM cells is increased in a photothrombotic-induced stroke model, probably due to local proliferation rather than replacement by bone

marrow-derived monocytes [97]. Therefore, it indicates that PVM in brains is more heterogeneous than previously reported [10], which calls for further investigation.

### Oligodendrocyte progenitor cells

Oligodendrocyte progenitor cells (OPCs), also known as NG2 glia, are a subtype of glia in the central nervous system with surface markers of PDGFR $\alpha$ <sup>+</sup> and CSPG4<sup>+</sup> [98-100]. PDGFR $\alpha$  is critical for OPC maturation: its activation by low concentrations of its ligand PDGF is coupled to phosphoinositide 3-kinases (PI3K)-extracellular signal-regulated kinases (ERK) signaling for cell migration [101,102], while with high ligand concentrations, phospholipase C gamma signaling is activated for cell proliferation [101,102]. PDGF-A released by astrocytes increases OPCs counts in the adult central nervous system, especially in white matter tracts.

OPCs are located close to endothelial cells, which release VEGF to promote the survival and proliferation of OPCs. In turn, OPCs mediate the function of endothelial cells. When brain endothelial cells are co-cultured with OPCs, endothelial cell permeability is reduced; this decrease is prevented by blockade of PDGFR $\alpha$  in the OPCs, thus suggesting that communication between brain endothelial cells and OPCs play a role in the maintenance of BBB integrity [103]. The obligatory role of OPCs is to replenish damaged oligodendrocytes for the regeneration of myelin sheath and to maintain microglia homeostasis under pathological conditions [104-106]. With aging, OPCs have reduced capacity for cholesterol biosynthesis, leading to impaired differentiation into oligodendrocytes and blunted remyelination [100-107], myelin destruction [107,108], and oligodendrocyte death [109]. OPCs undergo senescence and are shifted to a proinflammatory phenotype, which can be induced by the neurotoxin A $\beta$ ; and removal of senescent OPCs by the senolytic cocktail of dasatinib and quercetin downregulates neuroinflammation and ameliorates cognitive deficits in Alzheimer's disease [110].

Both increased counts of OPCs and decreased counts of mature oligodendrocytes are observed in microvascular brain injury patients, suggesting that myelination disturbances in these patients are related to the disrupted differentiation of OPCs to myelinating oligodendrocytes [32]. In addition to endothelial cells, astrocytes, microglia, and pericytes in the central nervous system also contribute to the regulation of the differentiation of OPCs to oligodendrocytes [111,112]. In the differentiation stage, the expression of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4, the most expressed proteoglycanase among the ADAMTS family in the central nervous system [113], in the OPCs is increased [114]. One of the substrates of ADAMTS-4 is NG2, a surface marker of OPCs [60-63]. Genetic ablation of ADAMTS-4 impedes NG2 proteolysis, leading to elevated PDGFR $\alpha$  signaling and impaired oligodendrocytes differentiation and neuronal myelination [114]. The expression of ADAMTS-4 is increased in cultured human astrocytes and mouse microglia by the inflammatory mediator TNF $\alpha$ , as well as in the brains of human and rodents with stroke [115]. With increased

ADAMTS-4, the expression of NG2 and PDGFR $\alpha$ -activation are reduced, and oligodendrocyte differentiation is accelerated [114]. Aged microglia, as well as microglia activated by TGF- $\beta$  (the circulation of which is increased in aging) diverts the OPCs to differentiate into astrocytes instead of oligodendrocytes [111]. Likewise, the depletion of A-kinase anchor protein 12 (AKAP12) on pericytes results in impaired OPC-to-oligodendrocytes differentiation and reduced myelin density in the central nervous system [112].

### Pathological changes and the mechanism leading to aging-associated cognitive dysfunction

From the above discussion, it appears that during aging, the cells compositing the BBB have increased oxidative stress and inflammatory responses, which lead to the disruption of the integrity of the BBB and hence impaired cognitive function (Figure 2). These changes are partly related to the alteration of the cell function, and partly due to the responses of the cells to changes in the systemic soluble factors which circulates to and subsequently increases the permeability of the BBB. The latter can be demonstrated by the phenomenon known as postoperative cognitive disorder (POCD), a condition describing the cognitive disorder [impaired learning capacity, memory loss, confusion, anxiety, and personality changes] after major surgery and is attributed to exposure to anesthetics and/or surgical procedures [116]. Propofol, isoflurane, and sevoflurane are commonly used anesthetics in clinical practice, and several animal and clinical studies have suggested that their use is associated with the occurrence of POCD [117-120]. Propofol increases the BBB permeability, and this effect is enhanced by advanced glycation end products (AGEs), which are accumulated under the condition of diabetes, as demonstrated by the increased bacterial traversal in an *in vitro* BBB model [117]. Exposure to isoflurane leads to the disruption of BBB through the upregulation of VEGF in the hippocampus of aged mice [121,122]. The neurotoxicity associated with sevoflurane involves an enhanced inflammatory response associated with microglial activation and upregulation of a major proinflammatory transcription factor, nuclear factor-kappa B (NF- $\kappa$ B) [123,124].

### Protein expression changes in the cells of the BBB during aging

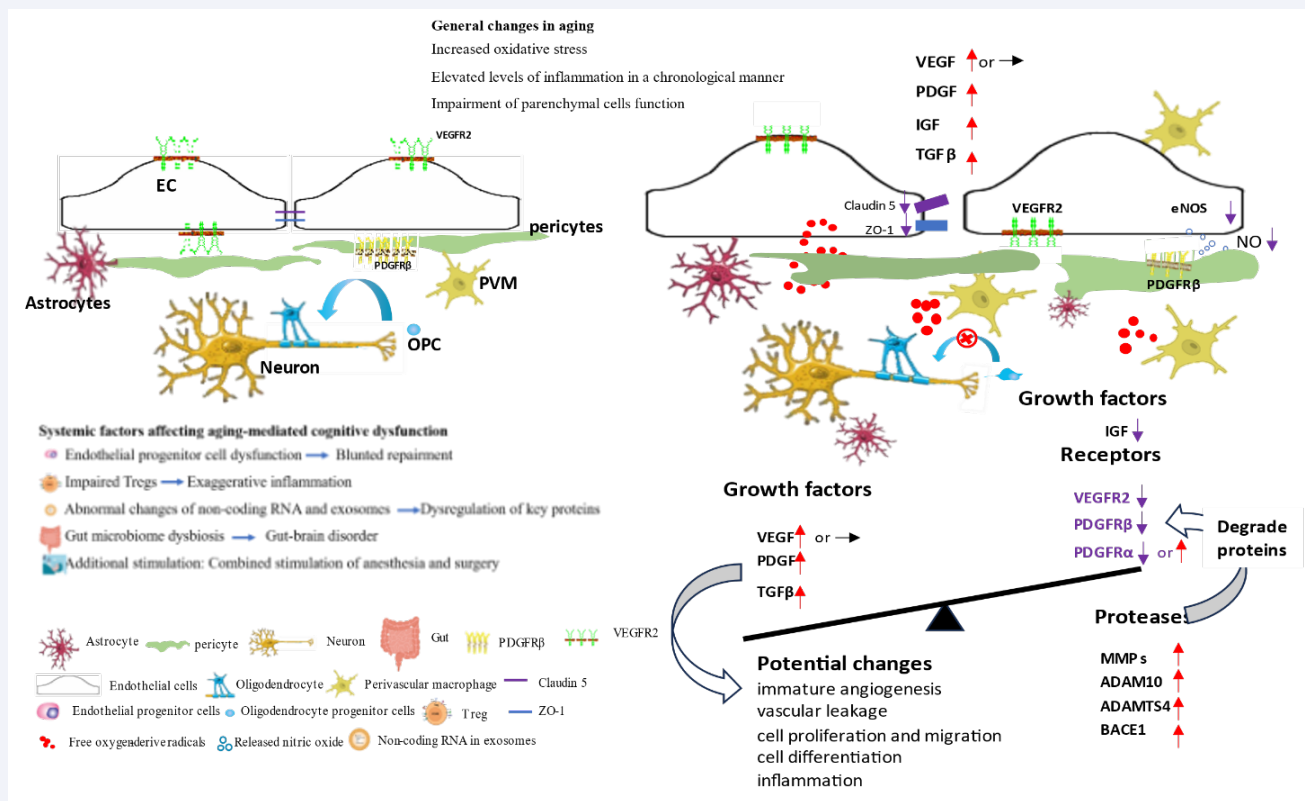
With aging, the expressions and/or activities of several proteins in astrocytes, endothelial cells and pericytes are altered. Among them, changes in the expressions/activities of the cleavage enzymes, MMPs and caspases, as well as the inflammatory mediators, IL-33 and transactive response DNA-binding protein of 43 kDa (TDP-43), appear to be involved in the development of aging-associated cognitive dysfunction.

MMPs, belonging to a large family of proteases, play an essential role in degrading extracellular matrix proteins. In mice with ischemia stroke [34-125], exposed to anesthesia and surgery [126], or fed with high-salt diet [93], MMPs are activated leading to the breakdown of the BBB [127]. In particular, tight

junction proteins of BBB, such as ZO-1, claudin-5, and occludin, are sensitive to degradation by MMP-2 and MMP-9, which are expressed and released by activated astrocytes and microglia [126-129]. In aged mice [130], and aged mice with POCD [126], inhibition of MMP-2/-9 increases tight junction protein expression, inactivates microglia and astrocytes, and restores cognition. MMP-2 is present in astrocytic end feet surrounding cerebral blood vessels while the expression of MMP-9 and MMP-12 are upregulated with aging [131]. Unlike that of MMP-2, the promoter gene sequence of MMP-9 and MMP-12 contain the binding sites for the transcription factors NF- $\kappa$ B and activator protein-1; as such, MMP-9 and MMP-12 are upregulated during inflammatory responses and contribute to neuroinflammation by promoting the infiltration of T-lymphocytes and bone marrow-derived microglia in the brain [129-131]. By proteolytic cleavage, MMP-2/-9 activates TGF- $\beta$ , increases chemokines secreted by astrocytes, and enhances the production of chemokines [132]. The expression of MMP-9 in microglia exposed to the bacterial endotoxin, lipopolysaccharide, is reduced by ADAMTS-4, which is upregulated by the inflammatory mediator TNF $\alpha$ , thus suggesting a role of ADAMTS-4 in regulating BBB permeability during neuroinflammation [115]. Besides the tissue inhibitors of metalloproteinase, caveolin-1 in brain microvascular endothelial cells also contributes to the regulation of MMP activity [133]. It has been reported that caveolin-1 negatively regulates nitric oxide production by physically interacting with nitric oxide synthase (all three isoforms) via the caveolin scaffold domain [134], which is also present in the gelatin-binding and hemopexin-like domains at the carboxyl-terminal of both MMP-2 and MMP-9 [135]. In line, the presence of caveolin-1 reduces nitric oxide production and MMP activity; since nitric oxide upregulates caveolin-1 expression, it contributes to regulating the BBB permeability by indirectly inhibiting MMP activity [133].

Caspases are a family of protease enzymes playing essential roles in programmed cell death. A total of 14 caspases have been reported, among which caspase-6 is activated in patients with age-dependent cognitive impairment and Alzheimer's disease, and its overexpression leads to cognitive deficits and astrocyte activation in aged mice, while its inhibition by methylene blue restores cognition function after the onset of cognitive deficits [136]. The activation of caspase-6 in astrocytes is reduced by deletion of caspase-9 in retinal endothelial cells; as a result, astrocyte activation is inhibited and the level of inflammatory cytokines is downregulated, thereby preventing neuronal function decline in a mouse retinal vein occlusion model [137]. Caspase-9 plays a role in autophagosome [138], cytokines activation [137], myocyte differentiation and proliferation [139], and insulin-like growth factor (IGF)-2 receptor retrieval [140], which are independent of its role in apoptosis. Activation of caspase-9 and its downstream effector caspase-7 in endothelial cells is observed in retinas subjected to retinal vein occlusion, resulting in endothelial barrier leakage and prolonged vascular occlusion with the consequence of neuronal injury [141].

In the aged population, the incidence of POCD is higher [142]. The higher occurrence of POCD in aged animal models [143,144],



**Figure 2** The organization of the blood-brain barrier under normal (Left) and pathological conditions (Right). Aging is an unavoidable physiological event, causing structural and functional changes in the blood-brain barrier, resembling many pathological changes under disease conditions. In the process of aging, oxidative stress and serum inflammatory levels are increased in a chronological manner, accompanied by parenchymal cell dysfunction. Impaired endothelial progenitor cells blunt microvascular repairment, aged Tregs exaggerate proinflammation, and non-coding RNAs dysregulate key proteins in the blood-brain barrier. External impacts, including anesthetic exposure, surgery, and emotional breakdown further disrupt the defense of the blood-brain barrier. Indeed, eNOS expression and its product NO are reduced. Tight junction protein presence is downregulated. Pericyte counts and coverage are decreased. Thus, oxidative stress is increased in the central nervous system, leading to increased presence of perivascular macrophages and activated astrocytes. Of importance, growth factors PDGF, VEGF, and TGFβ are increased in the central nervous system. However, their corresponding receptors are reduced, which is attributed to increased expressions of proteases in aging, including MMPs, and ADAM10, ADAMTS4, and BACE1.

contributes to chronic systemic inflammation [144,145], disruption of the BBB [126-145], enhanced inflammation in the central nervous system [143], and impairment of neuronal function. Downregulation of IL-33 and its corresponding receptor, suppression of tumorigenicity 2 (ST2), is observed in the hippocampus of mice subjected to anesthesia and surgery. IL-33 is a member of IL-1 family with a high expression in the central nervous system compared to other organs and functions to induce immune cells to produce type 2 cytokines (including IL-4, IL-5, IL-6, IL-10, and IL-13) [146]; as such, activation of ST2 by IL-33 results in protective effects against cognitive impairment caused by POCD [147] and stroke [148] in mice. Indeed, IL-33 treatment reduces inflammatory responses in the central nervous system, shifts microglia polarization towards M2 (anti-inflammatory), and increases the excitatory (glutamatergic) synapse density in the hippocampus, resulting in cognitive improvement in mice subjected to anesthesia and surgery [147-149]. In a small group of human study, among the patients with amnesic mild cognitive impairment and Alzheimer's disease, those expressing IL-33

preserved their cognitive function over a year compared with IL-33 non-expressing patients, while the levels of Aβ, tau, and apolipoprotein E-ε4 were comparable between the two groups [150]. By contrast, it is also reported that increased expression of IL-33 in mice, either by exogenous administration or induced by experimental cerebral malaria, results in microglial activation and IL-1β production, depicting a harmful effect of IL-33/ST2 axis in cognitive function [185,193]. In endothelial cells challenged with hypoxia, IL-33 is upregulated and triggers pathological neovascularization through activating NF-κB-mediated Jagged1 [151] and VCAM-1 [152] signalings leading to deubiquitination of Notch1. Thus, the role of IL-33 in cognitive dysfunction/preservation warrants further investigation.

TDP-43, a transcriptional repressor, is a structural hallmark of neurodegenerative diseases. Elevated neuronal TDP-43 induces activation of microglia, astrocytes, pericytes, and endothelial cells, resulting in T cell infiltration (without changes in the tight junction protein level) and impaired cognition [153].



## Circulating factors affecting the function of the BBB during aging

PDGF is the ligand to PDGFR $\alpha$  and PDGFR $\beta$ , characteristic markers of OPCs and pericytes, respectively [98-100]. In aged mice, the serum level of PDGF increases; this is associated with the shedding of PDGFR $\beta$  in pericytes, leading to pericyte loss and impairment of hippocampal vasculature [62]. By contrast, in cultured human brain pericytes, PDGF-BB reduces cell apoptosis and stimulates cell proliferation [154] and intravenous treatment with PDGF-BB peptide promotes pericyte coverage and restores capillary tone and flow rate in a mouse epilepsy model [155]. Therefore, it appears that aged pericytes have altered response to PDGF.

VEGF is a family of growth factors specifically affecting vascular endothelial cells due to their expression of VEGFR2, including increasing vascular permeability, promoting cell proliferation, migration, angiogenesis, and inhibiting cell apoptosis [156]. Increased level of VEGF, which are released in the central nervous system by OPCs [157], astrocytes [93], and neurons [158], lead to the destruction of tight junctions in the endothelial cells [93] and immature angiogenesis [159]. In a longitudinal monitoring study in mice, VEGF levels in the plasma and major peripheral organs are not significantly decreased with aging [160]. However, the activation of the VEGFR2, reflected by its phosphorylated levels at tyrosine 1175 residue, is reduced, due to the trapping of VEGF by the soluble VEGF receptor 1 (sFlt1), a decoy receptor [160]. These data indicate that VEGF signaling is compromised in aging with the resultant capillary rarefaction and hypoxia in tissues. The finding thus likely explains that VEGF treatment attenuates age-related neurogenic decline [161].

IGF-1 is critical for brain development and neurogenesis. Both astrocytes and endothelial cells of the BBB express IGF-1 receptors (IGFR). IGF-1 reaches its highest production in the pubertal stage and decreases in aging [162,163]. With the reduction of circulating IGF-1 level during aging, the structure and function of the neuro-glia-vascular unit are impaired [164]. Exposure of cultured microvascular endothelial cells to IGF-1 promotes angiogenesis and tight-junction formation. The impairment of cerebral blood flow in aged brains is related to blunted IGF-signaling since it can be mimicked by IGFR deletion in endothelial cells [165]. Blockade of IGFR reduces the presence of glutamate transporter in astrocytes [166], leading to impaired glutamate cycling and increased glutamate excitotoxicity in neurons [167].

TGF- $\beta$  belongs to the transforming growth factor superfamily, including TGF- $\beta$ 1 to - $\beta$ 3, among which TGF- $\beta$ 1 level in the brain is increased in vascular dementia and Alzheimer's disease [168]. A possible source of TGF- $\beta$  in the brain is the deposition of fibrinogen carrying the latent TGF- $\beta$  from the systemic circulation under pathological conditions [169]. The released TGF- $\beta$  is activated by the astrocytes in the brain, leading to Smad2 protein phosphorylation in astrocytes and neurite outgrowth arrestment [169]. The brain pericytes also release TGF- $\beta$ 1 and the release

is increased following exposure to AGEs [170]. TGF- $\beta$ 1, in turn, stimulates fibronectin production in pericytes, leading to basement membrane hypertrophy and BBB disruption [170]. Moreover, TGF- $\beta$ 1 upregulates the expression of mitogen-activated protein kinase phosphatase-1 in cerebrovascular smooth muscle cells, thereby impairing endothelin-mediated vasoconstriction by inhibiting the signaling of p38 mitogen-activated protein kinase [171]. TGF- $\beta$  also prevents the differentiation of OPCs to oligodendrocytes, resulting in myelination disturbance in the central nervous system [107]. On the other hand, deletion of TGF- $\beta$ 1 and its downstream signaling molecules leads to blood vessel malformation and embryonic lethality due to an improper attachment between endothelial and mesothelial cells [172]. Deletion of TGF- $\beta$  signaling in brain endothelial cells results in impaired interaction between endothelial cells and pericytes and hence BBB breakdown [172]. These findings, therefore, suggest that proper TGF- $\beta$  signaling is important in the maintenance of BBB. TGF- $\beta$  signaling is regulated by high-temperature requirement A serine peptidase (Htra) 1, the gene expression of which is downregulated in an age-dependent manner [46]. There is no consensus on how Htra1 regulates TGF- $\beta$  signaling: Htra1 suppresses TGF- $\beta$  signaling by cleaving TGF- $\beta$  precursor/receptors [173,174] or activates it by cleaving the latent binding protein of TGF- $\beta$  [175]. Thus, mechanisms underlying the interaction between Htra1 expression and TGF- $\beta$  signaling in cerebral endothelial cells need to be further investigated.

Gut bacteria release many bioactive compounds into circulation, including short-chain fatty acids and bile acid metabolites, and these compounds have been shown to affect the BBB function and neuroinflammation [176,177]. As such, gut microbiome dysbiosis has significant impacts on cognitive function [176,177], as well as on intestinal hemostasis [178], medicine bioavailability [179,180], and immune function [181]. In particular, the plasma level of the gut microbiome-derived metabolite trimethylamine N-oxide is increased with aging and is negatively correlated with cognitive function in the aged healthy population [182]. Further study in mice indicates that trimethylamine N-oxide induces inflammation in the brain by activating astrocytes [182].

Regulatory T cells (Tregs; CD4<sup>+</sup>/CD25<sup>+</sup>Foxp3<sup>+</sup>) play an obligatory role in immune homeostasis by suppressing excessive immune responses [183]. Tregs increase IL-6 and fibroblast growth factor (FGF)- $\beta$  and promote myelin production in OPCs exposed to oxygen-glucose deprivation and reoxygenation *in vitro* [184]. By contrast, in aged mice subjected to surgery, Tregs aggravate the disruption of BBB, resulting in increased production of TNF $\alpha$  in the hippocampus; such effects are partly attributed to the aging of the Tregs with impaired function [144].

Endothelial progenitor cells (EPCs) are an important player in the regeneration of the endothelial lining of blood vessels [185]. They are defined as circulating cells expressing surface markers similar to those expressed in endothelial cells. Compared with young people, circulating EPCs are decreased in aged subjects [186], accompanied by reduced sirtuin 1 protein and increased



acetylation of p53 protein in EPCs [187]. Aged EPCs exhibit impaired migration and reduced secretion of pro-angiogenic factors [187].

### Potential therapeutic strategy for aging-mediated cognitive impairment

There is no doubt that cognitive impairment develops with aging. Therefore, approaches to improve general health during aging, including healthy lifestyle, physical exercise, and diets rich in resveratrol, such as peanuts, pistachios, grapes, and wine, are likely to be beneficial in retarding the progression of cognitive impairment [1-16]. Other more specific approaches have been proposed, and some of them are based on the understanding of the pathological factors leading to cognitive dysfunction relating to the cell components of the BBB, as indicated below.

### Cell-based therapies

Given the importance of brain endothelial cells in the maintenance of BBB integrity, cell therapy with EPCs has been proposed as a potential barrier repair strategy [188-190]. As an analogy of the BBB, the effects on the blood-spinal cord barrier after transplanting bone marrow EPCs (CD15<sup>+</sup>/CD90<sup>+</sup>CD105<sup>+</sup>/CD106<sup>+</sup>/CD117<sup>+</sup>/CD309<sup>+</sup>) have been examined in mice with symptomatic amyotrophic lateral sclerosis. Following intravenous administration, bone marrow EPCs are engrafted into capillaries replacing the damaged endothelial cells, resulting in upregulated junction protein expressions and reduced capillary leakage. Their differentiation into endothelial cells is caused by the increased secretion of the angiogenic factors, VEGFA and angiogenin-1 (Angpt1). It further leads to the re-establishment of the pericyte coverage and perivascular astrocyte end-feet in the damaged blood-spinal cord barrier, as well as the promotion of spinal cord motor neuron survival [188-190]. Adiponectin is derived from adipocytes, exerting protective effects on vascular endothelium [191] and neurons [192,193], and its low plasma level is associated with cognitive impairment [192,193]. Consistently, treatment of adiponectin-transfected EPCs increases microvessel density, reduces proinflammatory cytokine levels, and improves cognitive function in aged rats [194].

Mesenchymal stem cells, a subgroup of non-hematopoietic stem cells in bone marrow, support the differentiation of hematopoietic stem cells in the bone marrow and facilitate the growth and movement of stem cells in other tissues [195]. Unlike the intravenous transplantation of EPCs, mesenchymal stem cells barely trespass the BBB. Nevertheless, intracranial injection of mesenchymal stem cells restores the central nervous system function, protects against BBB disruption, and downregulates inflammation in the central nervous system in ischemia-induced stroke in rodents [196,197]. Mesenchymal stem cells overexpressing fibroblast growth factor 21 are more effective than those without the overexpression in protecting against ischemia stroke-induced BBB disruption, shown as greater upregulation of tight junction proteins, occludin, claudin-5, and

ZO-1, as well as greater downregulation of AQP4 and MMP-9 [197]. Furthermore, mesenchymal stem cells exert their protection on the BBB through the release of TNF- $\alpha$  stimulated gene/protein 6, which suppresses the NF- $\kappa$ B signaling pathway and reduces the production of oxygen-derived free radicals in the brain subjected to intracerebral hemorrhage [198].

### Cell-free therapies

Micro-RNA (miRNA), belonging to the non-coding RNA family, regulates target genes by binding to 3'-untranslated regions of mRNA sequences to induce gene silencing. Therefore, miRNA therapy offers protection against BBB dysfunction by preventing the expression of pathological signals involved in the damage. Pericyte-derived miRNA-210-5p regulates endothelial barrier function by inhibiting Janus kinase 1/ signal transducer and activator of transcription 3 signaling, promotes the recovery of motor function, and protects the blood-spinal cord barrier in mice after spinal cord injury [199]. MiRNA-181a, by inhibiting the FOXO1 expression, reduces pericyte loss and BBB breakdown with improved cognitive function in aged mice with Alzheimer's disease pathology [200]. MiRNA-671-5p and miRNA-23a-5p preserve BBB integrity following ischemic stroke by downregulating brain endothelial NF- $\kappa$ B/MMP-9 [125] and astrocyte TNF/MMP-3 pathways [201], respectively. MiRNA-195, which is present in extracellular vesicles secreted from astrocytes, protects against age-related BBB leakage through the autophagic-lysosomal pathway by downregulating the expression of thrombospondin-1 in endothelial cells [202].

Extracellular vesicles are lipid-bound vesicles containing cellular signals, including proteins and mRNA, and are released by many cells for cell-cell communication. In an *in vitro* study, the nanosized vesicles derived from EPCs are taken up by mouse brain endothelial cells, protecting them from the damage induced by the serum from amyotrophic lateral sclerosis mice [203]. By using mass spectrometry proteomic analyses, microvesicles derived from mesenchymal stem cells are highly enriched for proteins involved with exosome biogenesis, vesicle trafficking, Rab-related GTPases, anti-apoptosis/programmed cell death, as well as neuron differentiation/neurogenesis. Therefore, it is reasonable to predict that mesenchymal stem cell-derived microvesicles exert protective effects in the central nervous system as the cells [196].

Probiotic treatment of *Lactobacillus* has also been proposed as a potential therapy to preserve BBB integrity with aging, based on the report of a negative correlation between the plasma level of gut microbiome-derived metabolite and cognition function in the aged population [135]. In aged mice subjected to anesthesia and surgery, administering *Lactobacillus* mix or the corresponding metabolite sodium butyrate increases junction protein and reduces BBB permeability [204].

### Commercially available medicinal compounds

In addition to the above innovative approaches awaiting clearance from safety concerns and scientific evidence for

effectiveness in human studies, some currently available medications have been shown to have protective effects on BBB and cognitive function.

Metformin, a first-line pharmacological treatment of diabetes, has the pleiotropic effect of protecting against cognitive dysfunction in individuals with and without diabetes [205]. Both 5'-monophosphate-activated protein kinase (AMPK)-dependent [206,207] and -independent [208] mechanisms are involved in metformin-exerted beneficial effects in the neuro-glia-vascular unit. Upregulation of AMPK restores junction protein occludin and claudin-5 expression by reducing oxidative stress in cultured brain microvascular endothelial cells exposed to lipopolysaccharide [209]. Metformin enhances remyelination by restoring the differentiating potential of aged OPCs [210], and inhibits neuronal apoptosis by inhibiting the mitochondrial fission protein Drp1 in neurons [208].

Glucagon-like-peptide-1 (GLP-1), a meal-stimulated hormone by ileum neuroendocrine L-cells, stimulates insulin release and inhibits glucagon release, and is another class of drugs for diabetic treatment. GLP-1 has direct protective effects on endothelial cells [211-213]. In aged mice, the brain endothelial cells' transcriptomic changes and the BBB leakage are reversed by exenatide, a short-acting GLP-1 agonist [27].

Fluoxetine is an antidepressant and belongs to a group of medicines known as selective serotonin [(also known as 5-hydroxytryptamine (5-HT)] reuptake inhibitors. Fluoxetine has been shown to improve cognition and memory in patients with psychiatric symptoms and Alzheimer's disease [214], through inactivating glycogen synthase kinase 3 $\beta$  in OPCs/oligodendrocytes, preventing cell senescence and promoting the formation of myelin [215,216]. Nevertheless, the use of fluoxetine in healthy aging people would not be advocated due to its antidepressant effects.

In addition, health supplements have become an attractive alternative to clinically used medication for preventive approach. One of the nutrient supplements, huperzine A, which is a natural alkaloid compound, has been used for neurological disorders. Huperzine A is a reversible and selective acetylcholinesterase inhibitor. In a study using rats with experimental aging, long-term administration of huperzine A alleviates age-associated neurovascular damage by inhibiting NF- $\kappa$ B activation, thus preventing the activation of endothelial cells, microglia, and astrocytes in the BBB [217]. Since the activity of acetylcholinesterase is enhanced in the experimental aged rats [217], the characteristic of huperzine A may be beneficial.

## CONCLUSION

The BBB is the anatomic and biological defense for the central nervous system. Aging-induced endothelial dysfunction and pertinent molecular changes affect other structural cells in the BBB and parenchymal cells in the central nervous system (Figure 2). Given the complexity of the BBB involving the interaction among the different cell types, the full picture of

how the BBB integrity are maintained remains elusive. Current strategies against aging-mediated cognitive impairment are mainly for the general anti-aging approaches. The innovative concepts of replacing damaged cells in BBB using progenitor cells and/or stem cells and protecting BBB cells from developing into senescent/apoptotic phenotypes by non-coding miRNAs require further investigation, and the challenges of safe and non-invasive delivery of these cells/miRNAs to the BBB would need to be addressed. Some current medications appear to have the pleiotropic effects to preserve BBB function, and their 'therapeutic' effects may become a hindrance since these may pose adverse effects in healthy aging population. Currently, the BBB cell-derived extracellular vesicles and natural products, including healthy gut microbiota and plant-derived alkaloids, seem to be promising. Further studies are needed for better understanding the interplay among different BBB cell types and the pathological cause of BBB disruption to shed some light on preventing aging-mediated cognitive dysfunction.

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