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Review

The blood-brain barrier in brain homeostasis and neurological diseases

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ABSTRACT

Brain endothelial cells are unique among endothelial cells in that they express apical junctional complexes, including tight junctions, which quite resemble epithelial tight junctions both structurally and functionally. They form the blood-brain-barrier (BBB) which strictly controls the exchanges between the blood and the brain compartments by limiting passive diffusion of blood-borne solutes while actively transporting nutrients to the brain. Accumulating experimental and clinical evidence indicate that BBB dysfunctions are associated with a number of serious CNS diseases with important social impacts, such as multiple sclerosis, stroke, brain tumors, epilepsy or Alzheimer's disease. This review will focus on the implication of brain endothelial tight junctions in BBB architecture and physiology, will discuss the consequences of BBB dysfunction in these CNS diseases and will present some therapeutic strategies for drug delivery to the brain across the BBB.

Contents

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1. Introduction

Apical junctional complexes, notably tight junctions, are present in physiological barriers constituted by a variety of epithelia and in brain endothelium forming the blood-brain-barrier (BBB) which controls cerebral homeostasis and provides the central nervous system (CNS) with a unique protection against the toxicity of many xenobiotics and pathogens. Conversely, BBB dysfunction has been recently proposed to be involved in the pathophysiology of a variety of neurological diseases (inflammatory, infectious, neoplasic and neurodegenerative diseases). This review aims at presenting the most recent knowledge of BBB architecture and function and will present novel therapeutic approaches designed to address these neurological diseases on the basis of this knowledge.

2. Blood-brain barrier in health

2.1. General description

2.1.1. Localization

The BBB is localized at the interface between the blood and the cerebral tissue [1,2], formed by the endothelial cells (ECs) of cerebral blood vessels which display a unique phenotype characterized by the presence of intercellular tight junctions and the polarized expression of numerous transport systems. Another blood-brain interface is localized at the choroid plexus epithelium which controls the exchanges between the blood and the cerebro-spinal-fluid (so-called the blood-CSF barrier): these specialized epithelial cells also express tight junctions and a number of transporters, but will not be described below for space constraints (for reviews, see [3]).

It is worth mentioning at this point that some restricted brain areas encompassing circumventricular organs (area postrema and median eminence, neuro-hypophysis, pineal gland, sub-fornical organ and lamina terminalis) do not present any blood-CNS barrier and constitute specialized sites of physiological cross-talks between the brain and the periphery (for example regarding food intake or control of body temperature) [4]; however, they are very limited in size and will not be described in this review (see [5]).

2.1.2. The neurovascular unit

In close proximity to brain ECs, as shown in Fig. 1, pericytes, glial cells (especially astrocytes), neurons, together with the basal lamina (also called *lamina basalis*) ensheathing cerebral blood vessels, are indirectly involved in the establishment and maintenance of the BBB: these various cell types and basal lamina collectively constitute the 'neurovascular unit' (NVU), a concept recently proposed to highlight the functional interactions which control BBB integrity.

2.1.2.1. Endothelial cells. Brain ECs differ significantly from non-brain ECs by (i) the absence of fenestration correlating with the presence of intercellular tight junctions (TJs), (ii) the low level of non specific transcytosis (pinocytosis) and paracellular diffusion of hydrophilic compounds, (iii) a high number of mitochondria, associated with a strong metabolic activity and (iv) the polarized expression of membrane receptors and transporters which are responsible for the active transport of blood-borne nutrients to the brain or the efflux of potentially toxic compounds from the cerebral to the vascular compartment [6,7]. In brief, the hallmark of brain endothelium in mammals is its highly restricted and controlled permeability to plasmatic compounds and ions, reflected by a very high trans-endothelial electrical resistance [7,8].

2.1.2.2. Basal lamina. The basal lamina of the cerebral endothelium is constituted by 3 apposed layers, one produced by ECs and containing laminin-4 and -5, one being astrocyte-derived, containing laminin-1 and -2 and the collagen IV-containing middle one, contributed by both cell types [9]. All three layers are also made of various types of collagen, glycoproteins and proteoglycans [2,10]. Although its

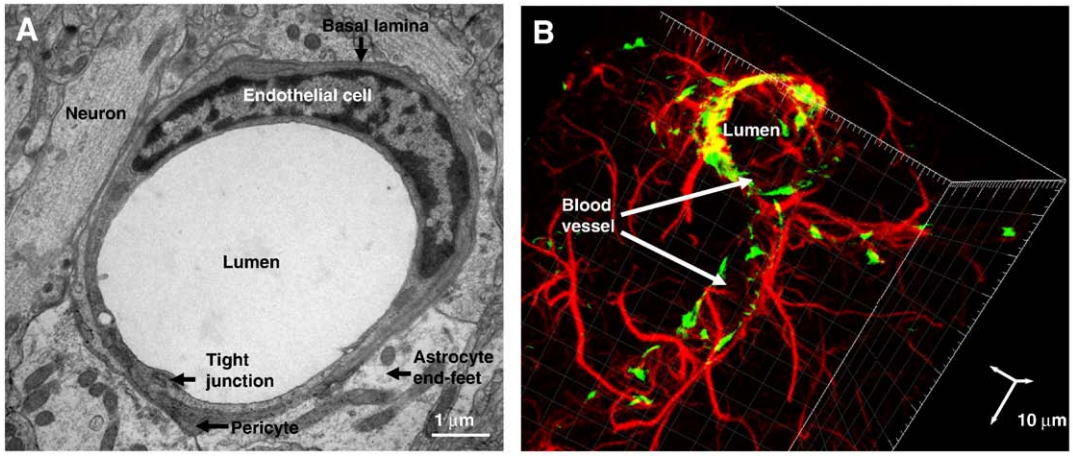

Fig. 1. The neurovascular unit. A. Electron microscopy (TEM) of rat brain section showing a neurovascular unit. This complex includes microvessel endothelial cells, based on basal lamina, pericytes embedded in basal lamina, astrocytes end-feet and in vicinity some neurons. B. Confocal microscopy 3D-reconstruction of rat brain section showing part of cerebral vascular tree: endothelial cells (green) are surrounding with astrocytes (red), which are visualized with von-Willebrand factor and glial fibrillary acidic protein staining respectively.

contribution to BBB integrity has been often underestimated, the basal lamina must now be considered as a key component of the NVU [11]. Multiple basal lamina proteins, matrix metalloproteases (MMPs) and their inhibitors, the Tissue Inhibitor of Metalloproteases (TIMPs), are involved in the dynamic regulation of the BBB in physiological as well as inflammatory conditions [12].

2.1.2.3. Glial cells, astrocytes. Whereas the role of astrocytes in the induction and maintenance of BBB integrity has been well documented for more than two decades [13], the molecular mechanism mediating their action still remains unclear. Indeed, a number of astrocyte-released and more generally glial-released factors have been suggested to contribute to BBB integrity, including glial-derived neurotrophic factor (GDNF), angiopoietin-1 [14,15] and more recently angiotensin II [16].

2.1.2.4. Pericytes. Pericytes are present along brain and non-brain microvessels, within the basal lamina surrounding ECs; interestingly, brain microvessels are notably rich in pericytes and the pericytes/ECs ratio has been correlated with the barrier capacity of the endothelium. Pericytes are actively involved in maintenance of the integrity of the vessel [17], vasoregulation [18] and restricted BBB permeability.

2.1.2.5. Neurons. Brain endothelium, perivascular astrocytes and pericytes are in close contact with neuronal projections, allowing neuronal mediators to affect cerebral blood flow and vessel dynamics. However, the precise physiological or pathophysiological consequences of neuronal input onto the BBB still remain largely unknown.

2.1.3. In vitro BBB models

In line with the understanding of the aforementioned cell–cell interactions taking place at the BBB, most *in vitro* BBB models proposed so far are based on co-cultures of brain ECs and astrocytes (or glial cells) [19] in two-chamber cell culture systems (Fig. 2A). In particular, whereas bovine brain endothelial cells alone only partly recapitulate BBB properties, a co-culture system with rat glial cells has been extensively validated as a reference BBB model [20]. Because these cells express TJs and a number of membrane transporters, they constitute a valuable alternative or complement to the epithelial cell lines Caco-2 and MDCK, currently used for drug screening by pharmaceutical industries because of their very high permeability restriction [21]. Alternative BBB models are also available, using pig, mouse, rat or human brain ECs [15,22–28]. In addition, stable immortalized rat EC lines were produced and validated as *in vitro* models of brain endothelium: first the RBE4 cell line [29–31], followed by a number of other cell lines [32–35] have been widely used for biochemical, immunological and toxicological studies. More recently, we produced the human hCMEC/D3 brain EC line which retains most of the morphological and functional characteristics of brain endothelial cells (*i.e.* expression of TJs and the polarized expression of multiple active transporters, receptors and adhesion molecules), even without co-culture with glial cells, thus appearing as a unique *in vitro* model of the human BBB [36–38]. Recently, three dimensional flow conditions (Fig. 2B) [39] were able to further decrease significantly drug permeability [40]. All together, these *in vitro* BBB models will help screening new drugs as well as unraveling the molecular mechanisms of BBB control in physiological conditions and BBB dysfunctions in various CNS diseases.

2.2. Intercellular junctions

Tight junctions between brain endothelial cells are structurally quite similar to epithelial TJs (*this issue*): they are constituted by three major transmembrane proteins (or protein families), occludin, claudins and Junction Associated Molecules (JAMs), and several cytoplasmic proteins including Zonula Occludens (ZO) -1, ZO-2, ZO-3, which interact with these transmembrane proteins in multi-protein complexes linked to the actin cytoskeleton (Fig. 3) [2]. However, EC-

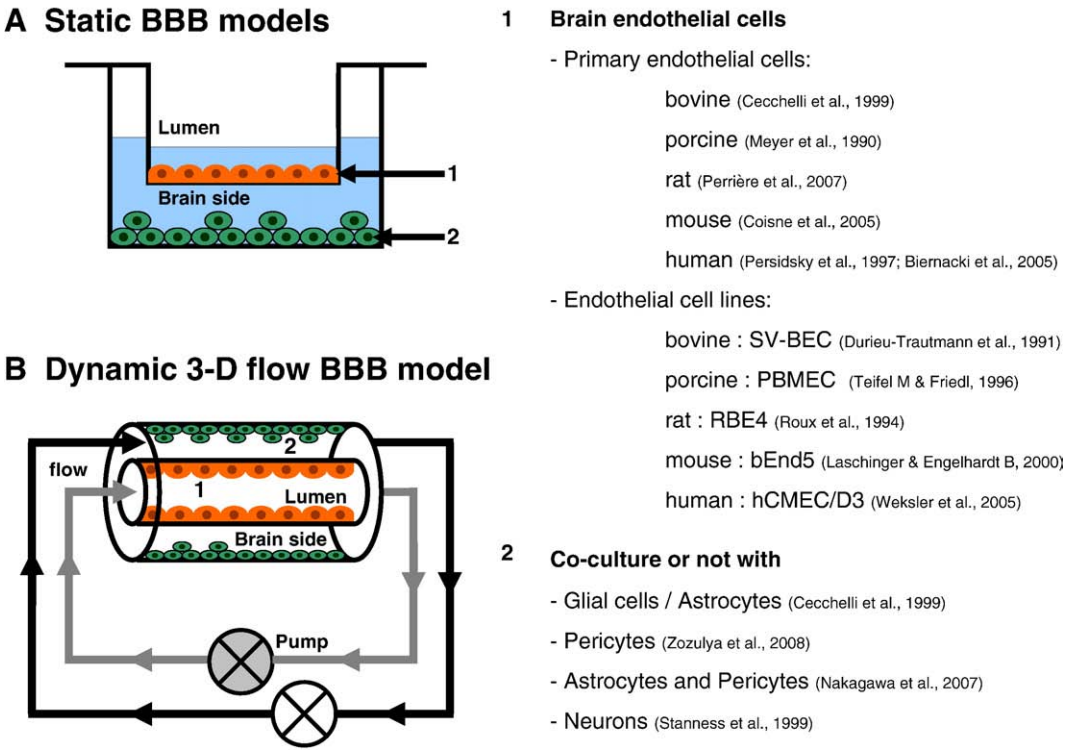

Fig. 2. Modeling the blood-brain-barrier and neurovascular unit *in vitro*. Static BBB models (A) use brain ECs (1), primary brain ECs or EC lines, grown in the upper compartment of Boyden chambers. In order to model the NVU, various other cell types (glial cells, pericytes or neurons) can be co-cultured in the lower compartment (2). Recently described dynamic three-dimensional flow BBB models (B) provide an interesting model to study brain EC properties under flow conditions, based on observations that pulsatile flow induces further differentiation of cultured brain ECs to a BBB phenotype.

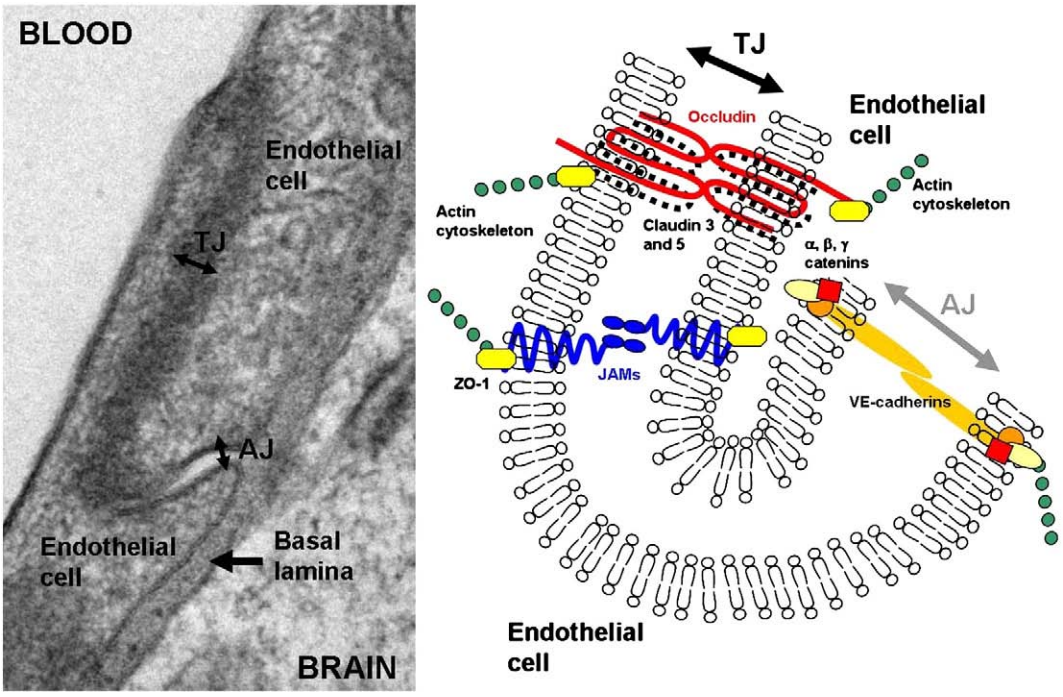

Fig. 3. Tight junctions on the blood-brain-barrier. Left: Electron microscopy of rat brain section showing a tight junction (TJ) between two cerebral endothelial cells. Right: Schematic view of cerebral TJ. Cerebral endothelial cells have close intercellular contacts due to the presence of tight junctions (TJs) constituted by transmembrane proteins: occludin, claudins (claudin-3 and -5) associated with actin cytoskeleton via cytosolic proteins, such as the ZO family. Peripherally to TJs are localized JAMs and proteins of Adherens Junctions, such as VE-cadherin which is also associated with actin cytoskeleton via catenins.

specific accessory proteins were identified in brain endothelium TJs, such as cingulin, AF-6 and 7H6 [41], whereas some epithelial TJ proteins have not been detected so far at the BBB (like the CRUMBS/PALS-1/PAT-J and PAR-3/PAR-6/aPKC complexes) [42,43]. Moreover, whereas epithelial tight junctions, including choroid plexus epithelial cells, mostly contain claudin-1, -2 and -11 [44], brain endothelial TJs express claudin-3 and -5 and possibly claudin-12 [45,46]. In particular, claudin-5 was shown to actively contribute to BBB integrity, inasmuch as claudin-5 knock-out mice display severe BBB dysfunction and permeability increase [47]. Accumulating further evidence indicates that claudin-3 and -5 are involved, together with occludin, in BBB genesis [46,48] and control of paracellular permeability [49–52].

The regulation of TJs appears to be mediated by multiple signaling pathways (for review: [2]). Early *in vitro* experiments pointed to cAMP as a major regulator of TJs in brain ECs [53]. Several evidences suggest that phosphorylation and/or degradation of occludin and ZO-1 regulates TJs [54]. Later, serine/threonine phosphorylation of occludin was associated to a decrease in paracellular permeability, whereas tyrosine phosphorylation of ZO-1 was associated to an increase in paracellular permeability [55,56]; also, VEGF was shown to affect TJ assembly and increase BBB permeability by promoting occludin phosphorylation [57] and/or degradation [58,59]. In addition, TJs are regulated by the activation of small G proteins of the Rho family (Rho GTPases) coupled to various receptors of vasoactive compounds (Bradykinin, angiotensin II) as well as adhesion molecules (ICAM-1) or reactive oxygen species: activation of Rho GTPases leads to actin cytoskeleton rearrangements and increases paracellular permeability as well as trans-endothelial migration of leukocytes [37,60,61].

Interestingly, crosstalk between components of TJs and Adherens junctions was proposed to contribute to TJ regulation: first, extracellular calcium, known to control Adherens junctions integrity via cadherins was also shown to regulate TJs. More recently, the functional relationship between AJs and TJs was further documented by the demonstration that VE-cadherin at AJs upregulates the expression of claudin-5 at TJs, through activation of the transcription factor Fox01 [62].

2.3. Physiological function of the BBB

As mentioned above, the BBB is responsible for strictly controlling the exchanges between the blood and brain compartments, by (i) preventing the paracellular diffusion of hydrophilic solutes, (ii) mediating the active transport of nutrients to the brain, (iii) effluxing hydrophobic molecules and drugs from the brain to the blood and (iv) regulating the trans-endothelial migration of circulating blood cells and pathogens.

2.3.1. Solute permeability

Because of the presence of TJs, BBB permeability to blood borne solutes [63] largely depends on their molecular weight and lipophilic/hydrophilic characteristics. A number of algorithms are available, which help anticipate the BBB permeability of drug candidates on the basis of their structural features [64,65], but they remain insufficiently predictive [66]. Hydrophilic nutrients, such as amino acids, are actively transported through the brain endothelium by a variety of membrane transporters, notably the large family of Solute Carrier transporters (SLC) [67]. In addition, although most proteins are not able to diffuse across TJs, some of them efficiently cross the BBB by receptor-mediated transport, as described for transferrin, insulin, insulin-like growth factor, leptin or LDLs [68–70].

Conversely, efflux transporters of the *ATP Binding Cassette* (ABC) family transport a large panel of lipophilic molecules, in particular xenobiotics, against a concentration gradient by ATP hydrolysis [63,71]. The most active ABC-transporters at the BBB are P-glycoprotein (P-gp) encoded by the multidrug resistance gene (MDR1 or ABCB1), multidrug resistance proteins (MRPs, or ABCC proteins) and the breast cancer resistance protein (BCRP or ABCG2) [72,73].

2.3.2. Trans-endothelial migration of circulating leukocytes

Leukocyte trans-endothelial migration, known as extravasation or diapedesis, involves a complex set of adhesion molecules at the surface of leukocytes and vascular endothelial cells [74] (Fig. 4).

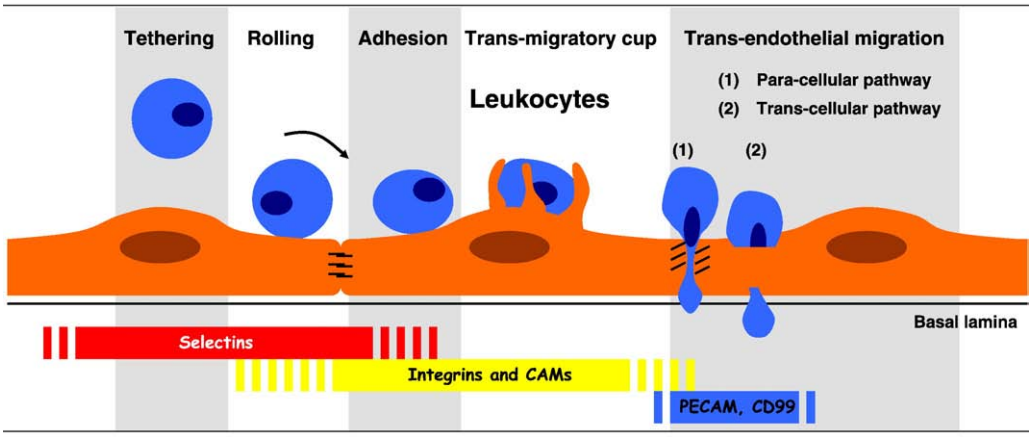

Fig. 4. Trans-endothelial migration through the blood-brain-barrier. Leukocyte trans-endothelial migration proceeds in several steps that are highly controlled by various adhesion molecules (selectins, LFA-1, VLA-4, CD44) and their respective counter-receptors (selectin ligands, ICAM-1, VCAM-1, CD44) expressed by ECs. After tethering and rolling, firm adhesion and trans-endothelial migration of leukocytes are mediated by the formation of apical membrane protrusions, termed transmigratory cups, at the surface of ECs. Whether these structures support paracellular (1) and/or trans-cellular (2) migration *in vivo* is still debated.

Tethering or rolling of leukocytes is followed by their firm adhesion to endothelium which precedes diapedesis. Although tethering is generally mediated by selectins, it appears in brain to be mostly mediated by the integrins VLA-4 ($\alpha4\beta1$) and $\alpha4\beta7$ [34]. The endothelial adhesion molecules ICAM-1, VCAM-1 and PECAM-1, belonging to the superfamily of immunoglobulins, actively contribute to the firm adhesion and/or migration of distinct subsets of leukocytes to the CNS through cytokine-activated brain endothelium [75,76]. Also, CD44, a polymorphic family of related membrane proteoglycans was shown to play a pivotal role in lymphocyte trafficking [77,78]. Recently, it has been shown that leukocytes were able to induce the formation of endothelial membrane protrusions, containing ICAM-1, VCAM-1 and CD44, linked to the actin cytoskeleton: because these structures appear to support leukocyte trans-endothelial migration, they are known as 'transmigratory cups' [79]. After migrating across TJs, in the classical paracellular pathway, leukocytes interact with CD99 and PECAM-1, present in the intercellular endothelial cleft, between TJs and the endothelial basal lamina. In inflammatory conditions, JAM-A is redistributed from intercellular junctions to the endothelial apical membrane where they bind the leukocyte integrin LFA-1 ($\alpha L\beta2$) [80,81], thus possibly contributing to leukocyte diapedesis and migration to the brain [82,83]. More recently, the prion protein PrP^C was unexpectedly identified as an additional junctional adhesion molecule involved in the trans-endothelial migration of monocytes [84].

In addition to the migration of leukocytes across cell-cell junctions (i.e. paracellular migration), the existence of a transcellular migration pathway has recently been highlighted [85–87] (for review see [86,88]). The previously described transmigratory cups are associated to both paracellular and transcellular migration [86]. These structures are in close contact with ICAM-1, caveolin-1 and the vesiculo-vacuolar organ [89]. More recently, some other structures has been described as actin-rich podosome-like membrane protrusions of leukocytes, able to extend transiently through EC and these could be associated to crawling and/or trans-endothelial migration of leukocytes [85,86]. Even if transcellular trans-endothelial migration has been proven in brain EC [90], the relative importance of paracellular versus transcellular endothelial migration in brain EC is still matter of debate [86,89].

Because immunosurveillance exists in the CNS under physiological conditions with infiltration of activated leukocytes (essentially T lymphocytes and monocytes) across the cerebral endothelium, the former conception that the CNS was an immune privileged site has been recently tempered [91]. This has been recently dramatically highlighted by the occurrence of severe JC virus infection in multiple sclerosis patients treated with anti-VLA-4 monoclonal antibody natalizumab in whom leukocyte patrolling is presumed to be impaired (see below) [92,93].

3. Blood-brain barrier in diseases

In a variety of neurological (inflammatory, infectious, neoplasic and neurodegenerative) diseases, BBB dysfunctions have been described, not only as a late event, but more interestingly as putatively involved in the early steps of disease progression. Two related but distinct types of BBB dysfunction will be documented below: (1) increased permeability, *i.e.* passive diffusion of blood-borne substances through BBB TJs, associated with edema formation and (2) massive cellular infiltration across the BBB.

3.1. BBB dysfunction in multiple sclerosis

Multiple sclerosis (MS) is a neuro-inflammatory, slowly progressing and invalidating disease affecting 0.05%–0.15% of Caucasians [94], mostly young adults, hence with a considerable social and economic impact [95]. A key factor in MS progression appears to be BBB alteration in genetically predisposed individuals, leading to increased vascular permeability and leukocyte infiltration into the brain [96,97].

3.1.1. Alteration of solute permeability in MS

Various animal studies on experimental autoimmune encephalomyelitis (EAE), the most commonly used MS animal model, suggest that disease severity could be correlated with alteration of BBB integrity, whereas limitation of the disease could be achieved by preventing BBB alterations (Fig. 5) [98–100]. At the molecular level, occludin dephosphorylation was shown to be timely associated with the onset of clinical signs [100] and was indeed recently suggested to mediate the permeability increase in response to angiotensin II, acting via its ATI receptor expressed by brain endothelial cells [16].

Additional observations on brain tissue from MS patients further pointed to a correlation between BBB permeability increase and loss or delocalization of brain endothelial junction proteins, like occludin, VE-cadherin or ZO-1 [101,102]. However, interpretation of these data (primary lesions, secondary damage or reparation default) remains a matter of debate [101]. Such an increase in BBB permeability in active MS lesions, commonly referred to as 'BBB opening', is visualized using magnetic resonance imaging (MRI) by monitoring the diffusion of

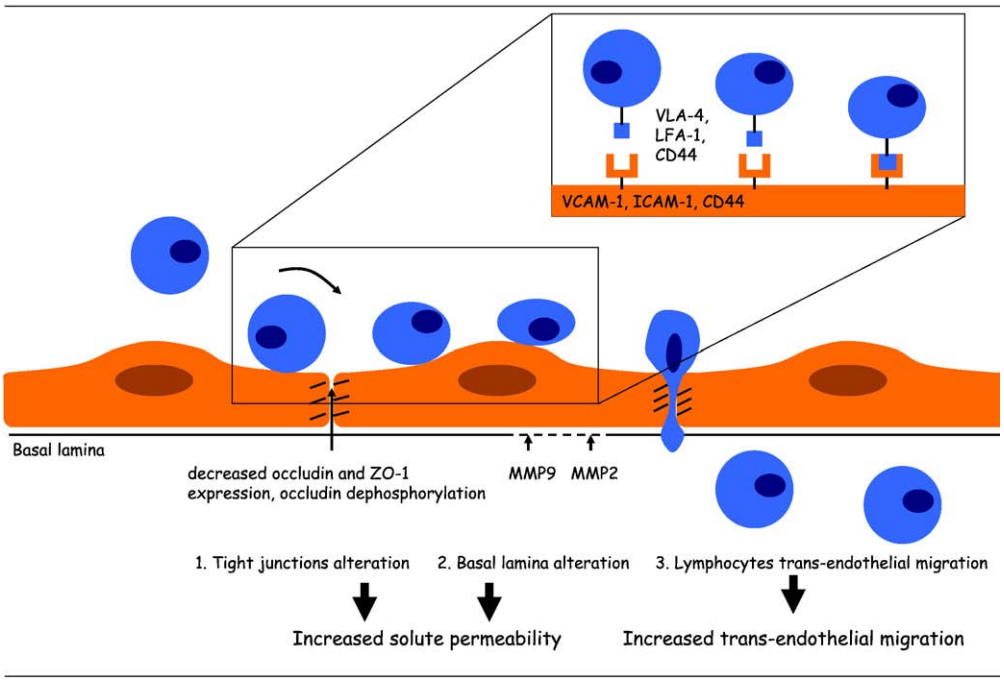

Fig. 5. Blood-brain-barrier alterations in multiple sclerosis. Accumulating data suggest the contribution of BBB dysfunction in the onset and progression of multiple sclerosis (MS). Several mechanisms account for alteration of TJ integrity (1): decreased expression of occludin and ZO-1 and changes in occludin phosphorylation status. In addition, activated MMPs directly contribute to alteration of basal lamina (2). Finally, up-regulation of the expression of several adhesion molecules by brain ECs supports the increased trans-endothelial migration of leukocytes (3) observed in MS.

gadolinium, a contrast agent of small molecular weight, through brain endothelial TJs [103].

BBB permeability has been well documented to be increased by MMPs, in line with early reports showing that intracerebral injection of MMPs increased brain capillary permeability [104]. More recently, various data in animal and clinical studies further highlighted the role of MMPs in the pathophysiology of MS [12,105]. In particular, the serum MMP9 level in MS patients was correlated with the BBB dysfunction index, as measured by MRI using gadolinium as a contrast agent [103,106].

3.1.2. Increased trans-endothelial migration of leukocytes in MS

Although the etiology of the disease still remains elusive, it was hypothesized that systemic infection or inflammation might enhance the expression of adhesion molecules on brain ECs, allowing activated lymphocytes and monocytes to access the CNS (Fig. 5) [95]. Indeed, overexpression of leukocyte adhesion molecules by brain ECs (ICAM-1, VCAM-1, E-selectin) has been extensively documented in brain lesions of MS patients, compared with normal appearing white matter or control tissue [107–109]. Accordingly, it was established that IFN-β, a current treatment in MS known to delay inflammation-associated disease relapses, could prevent the increase in ICAM-1 expression by brain ECs in response to the inflammatory cytokine TNF-α [110] and decrease VLA-4 expression by lymphocytes [111,112]. Moreover, whereas early reports indicated that anti-VLA-4 antibodies could delay or prevent the onset of EAE in rodents [77,113], a similar approach (using the anti-VLA-4 monoclonal antibody natalizumab) was shown to significantly decrease the number of relapses in MS patients [92,93] and has now been approved for clinical use. Interestingly, antibodies against the recently identified adhesion molecule ALCAM (*activated leukocyte cell adhesion molecule*), the expression of which by brain endothelium is up-regulated in MS lesions, were similarly shown to improve the functional outcome of EAE in rodents, indicating that ALCAM, together with ICAM-1 and VCAM-1, are involved in leukocyte trafficking into the CNS in inflammatory conditions [114].

It must be stressed that BBB permeability increase and leukocyte infiltration in the brain are two related, although distinct, consequences of CNS inflammation. Indeed, whereas CD4+ IL-17-producing T lymphocytes (Th17) were recently identified as major actors in disease progression, at least part of their pathological role may be mediated by IL-17 which is able to disrupt brain endothelium TJs [115–117]. In addition, circulating monocytes have been shown, after crossing the BBB, to acquire dendritic cell function and further enhance the infiltration of Th17 lymphocytes by releasing chemokines across the BBB [116,118]. Whereas gadolinium-enhanced MRI allows imaging of increased BBB permeability, recent MRI developments (using ultra small particles of iron oxide, so-called USPIO) aim at tracking infiltrated monocytes/macrophages and may thus provide a valuable complementary imaging strategy in MS [119,120].

3.2. BBB dysfunction in stroke

Acute obstruction of cerebral blood vessel by clot formation triggers a complex series of cellular and molecular events in the brain parenchyma (membrane depolarization of neurons and astrocytes, release of excitatory amino acids and K^+ ions in interstitial fluids, increase of intracellular Ca^{2+} levels), ultimately leading to dramatic cell damage within hours [121]. While administration of neuroprotective agents has generally failed as a potential treatment, thrombolysis by tissue plasminogen activator (tPA) within hours after the onset of the symptoms so far remains the only efficient therapy in stroke [122]. However, it soon appeared that the ischemia/reperfusion sequence of events initiates perivascular inflammation and BBB permeability increase which largely contribute to brain damage, thus leading to the concept that stroke is primarily a cerebrovascular disorder [123].

3.2.1. Altered BBB permeability in stroke

Early reports already pointed to rapid alterations of brain endothelium after cerebral ischemia and reperfusion, an early phase occurring minutes after reperfusion, followed by a second phase

several hours after ischemia [124–126]. Release of oxidants, proteolytic enzymes and inflammatory cytokines were shown to alter the BBB permeability properties, leading to brain edema formation [121]. Accordingly, MMPs released by activated leukocytes affect the integrity of the NVU, degrading the basal lamina and TJ-associated protein complexes (Fig. 6) [12,127–129]. In particular, MMP9 seems to play a critical role, as suggested by the absence of BBB disruption after transient ischemia in MMP9 gene deficient mice and the demonstration of its contribution to hemorrhages after stroke and thrombolysis by t-PA treatment [127,130]. Whereas tPA permeability across the BBB is physiologically controlled by an LDL receptor-dependent mechanism, BBB breakdown after stroke will lead to passive diffusion of tPA into the brain which may account for its reported neurotoxic side effects [131]. New thrombolytic agents, with no apparent neurotoxic effect, may be useful for designing alternative therapeutic strategies [132].

In conclusion, the complex cellular and molecular changes leading to BBB breakdown after an ischemia/reperfusion event remain to be further understood, before novel treatments of brain edema and stroke prevention can be proposed.

3.2.2. Increased trans-endothelial migration of leukocytes in stroke

Some recent data suggest that leukocyte infiltration into the brain parenchyma might contribute to cerebral ischemia/reperfusion injury [133,134]. Following stroke, circulating activated leukocytes will migrate towards the ischemic lesion, release inflammatory cytokines and activate the various NVU cell types [135]. In particular, increased expression of ICAM-1 by brain ECs facilitate the trans-endothelial migration of leukocytes [136,137], in line with the experimental evidence that treatment with anti-ICAM-1 blocking antibodies reduced brain injury in a rat model of cerebral ischemia [138]. Also, transgenic mice deficient in ICAM-1 or in its integrin receptor LFA-1 failed to present cerebral infiltration of leukocytes following cerebral ischemia as compared with wild-type animals and had a significantly better functional outcome [139]. Based on these preclinical encouraging data, a randomized, double-blind, placebo-controlled, clinical trial using injection of a monoclonal anti-ICAM-1 antibody was performed, but failed for reasons which remain to be clarified [140,141].

3.3. BBB dysfunction in brain tumors

Neovascularisation in primary brain tumors is correlated with tumor progression and morbidity phenotypes [142]. Angiogenesis-related BBB alterations have been well documented both in terms of structural changes of the cerebral vasculature and related permeability increase. In addition, it was recently emphasized that brain ECs may closely interact with brain tumor stem cells such as gliobastoma stem cells [143] and maintain their stemness [144].

3.3.1. Increased BBB permeability in brain tumors

Newly formed blood vessels in primary brain tumors were shown to present an abnormal overall structure, being more tortuous than normal brain vessels, lined with an altered basal lamina [145]. In addition, loss of expression of the TJ proteins claudin-3 and occludin were reported in primary experimental and human brain tumors [145,146]. Recent data showed that VEGF, an angiogenic growth factor known to be produced at a high level in brain tumors [147,148], promoted endocytosis of the endothelial cell adhesion molecule VE-cadherin, leading to disruption of the endothelial barrier function and increase in endothelial permeability [149].

Moreover, the level of occludin expression was proposed to be inversely correlated with brain tumor grading and with the degree of enhancement in CT scans, corresponding to the blood-to-brain diffusion of contrast products [146]. All together, these observations provide compelling evidence that primary brain tumor-associated angiogenesis induces structural and functional alterations of the BBB.

3.3.2. Increased trans-endothelial migration of metastatic tumor cells in the brain

Cells originating from peripheral primary tumors, such as breast cancer or melanoma, can circulate in the blood flow to the brain and form cerebral metastases, invariably associated with bad prognosis. Studies on the mechanisms involved in the trans-endothelial migration of these metastatic cells across the BBB further highlighted the crucial contribution of VEGF, which is highly expressed by breast carcinoma cells [150], and MMPs [151].

In addition, the leukocyte adhesion molecule CD44 was shown to contribute to the adhesion of metastatic breast carcinoma cells to brain vascular endothelium. Regarding the mechanisms of their subsequent trans-endothelial migration, data suggest a major role of the chemokine CXCL12 (or SDF-1α) expressed in the brain and its counter-receptor CXCR4 present at the surface of metastatic tumor cells [152,153]. Indeed, blocking the CXCR4-dependent intracellular pathways (PI3Kinase activation, intracellular Ca^{2+} increase) prevented the trans-endothelial migration of a breast cancer metastatic cell line through cultured human brain ECs [152]. Moreover, preliminary results indicated that adhesion of breast carcinoma cells on cultured brain ECs induced the formation of actin-rich apical membrane protrusions (unpublished observations), similar to the

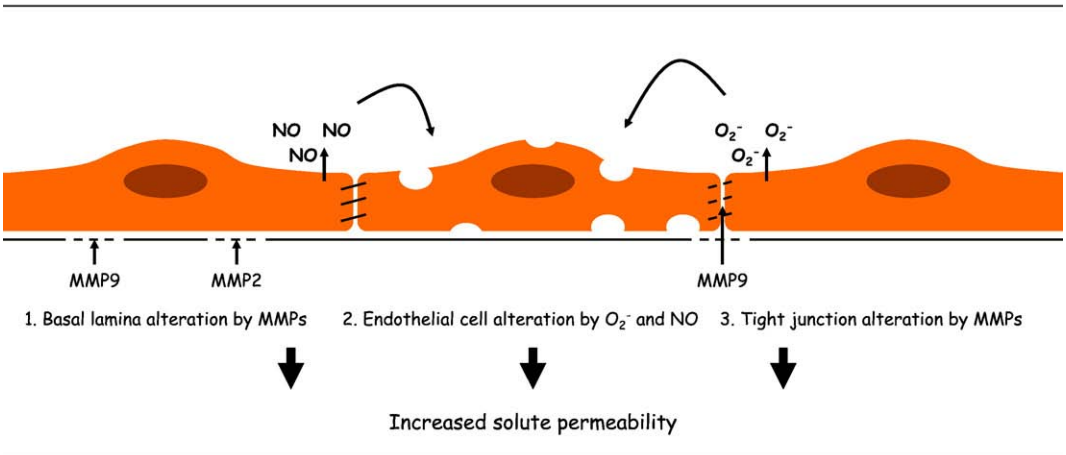

Fig. 6. Blood-brain-barrier alteration in stroke. Within hours following clot formation in brain microvessels, oxygen deprivation leads to an imbalance between MMPs and their inhibitors in favor of MMPs, leading to alteration of the basal lamina (1) and TJs (3). Brain ECs release large amounts of reactive radicals, NO and O_2^- (2), which diffuse and affect adjacent endothelial cells.

trans-migratory cups underneath leukocytes known to mediate leukocyte diapedesis [154]. In the same line, small-cell lung cancer cells were reported to cross brain endothelium by inducing a reorganization of the endothelial actin cytoskeleton through activation of Rho GTPase [155]. In conclusion, some metastatic tumor cells appear to share with activated leukocytes the expression of multiple adhesion molecules and chemokine receptors which provide them with the capacity to adhere to brain endothelium, to activate it and finally to cross the BBB. Following trans-endothelial migration, these tumor cells will receive multiple local inputs which will sustain their survival, migration and proliferation towards the formation of brain metastases [156].

3.4. CNS infection

3.4.1. Bacterial meningitis

Because of the existence and function of the BBB, most blood-borne pathogens are excluded from the brain. Only a few bacteria are able to enter the brain and evoke inflammation and disease. Among them, *Neisseiria meningitidis* (also known as meningococcus), *Streptococcus pneumoniae* and *Haemophilus influenzae* which multiply extracellularly, as well as *Group B Streptococcus* and *Escherichia coli K1* in newborn, and *Listeria monocytogenes* which multiplies within macrophages can invade the meninges, causing meningitis, or the cerebrospinal fluid (CSF) after entering the bloodstream.

3.4.1.1. Increased BBB permeability by bacterial secretions. Whereas *L. monocytogenes* and other intracellular pathogens appear to cross the BBB within leukocytes as 'Trojan horses', the extracellular pathogens mentioned above have developed individual strategies which allow them to adhere to and cross brain endothelium, thus exhibiting brain tropism (Fig. 7). These bacteria induce, by releasing lipopolysaccharide (LPS) and/or toxins, the secretion of multiple cytokines and other inflammatory components (TNF-α, IL1β, PAF, MMPs, TGFβ1, caspases) which are all able to increase microvascular permeability, including in the brain [157]. Indeed, the CSF level of some of them (TNFα, IL1β, TGFβ1) in children was correlated with the clinical outcome [158,159], whereas the CSF level of MMP9 was correlated with neurological impairment [160,161]. These inflammatory cytokines are known to increase the trans-endothelial migration of leukocytes which, when infiltrated in the brain compartment, can locally release cytokines and MMPs, thus amplifying the activation of brain endothelium and the inflammation within the CNS [162]. In addition, direct lesion of brain ECs may affect BBB integrity and facilitate brain infection as in the case of *S. pneumoniae* which secretes pneumolysin, a hemolysin forming transmembrane pores in brain ECs [163], and LPS inducing EC apoptosis [164].

3.4.1.2. Trans-endothelial bacterial entry in the brain. Recent experimental data led to a better understanding of the mechanisms mediating the trans-endothelial migration of *E. coli K1*, *N. meningitidis* and *S. pneumoniae* across the BBB [165,166]. These bacteria display individual attributes which allow them to adhere to brain endothelium by interactions with endothelial receptors, as shown in Fig. 7. In the case of *N. meningitidis*, bacterial adhesion to ECs is mediated by elongated multimeric structures known as type IV pili. The formation of actin-rich membrane processes involved in bacteria internalization is highly reminiscent of the leukocyte trans-migratory cups; moreover, the signaling pathways activated in ECs in response to the adhesion of *N. meningitidis* are similar to those induced by activated leukocytes, indicating that bacteria may interfere with leukocyte–EC interaction and hamper host inflammatory response [166,167].

3.4.2. Viral infection of the CNS

Infection by HIV and other neurotropic viruses such as West-Nile or Chikungunia virus highlighted the pathophysiological importance

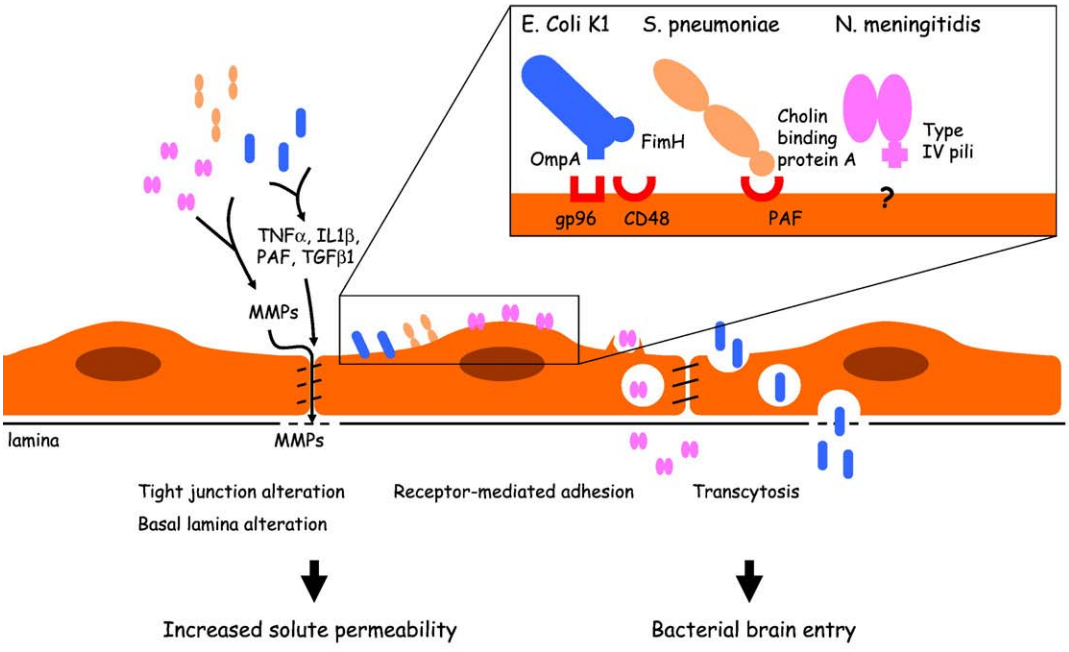

Fig. 7. Pathogens crossing the blood-brain-barrier. Circulating bacteria release active factors (LPS, toxins, etc.) inducing the local production by the host of various inflammatory cytokines (TNFα, IL1β, PAF, TGFβ1) and MMPs which in turn alter both brain endothelial TJs and the basal lamina and ultimately increase BBB permeability. In addition, bacteria use various membrane proteins expressed by cerebral ECs as receptors to cross the BBB by transcytosis. *Escherichia coli K1* strain mainly responsible for meningitis in newborn interacts by the external membrane proteins OmpA and FimH with their respective receptors gp96 and CD48 on brain ECs. This interaction triggers transcytosis through brain ECs. *Streptococcus pneumoniae* interacts by the cholin binding protein A to PAF on ECs. In addition to secreting multiple cytokines and other inflammatory components (TNF-α, IL1β, PAF, MMPs, TGFβ1, caspases) which are able to increase trans-endothelial migration, *S. pneumoniae* secretes pneumolysin, a hemolysin forming transmembrane pores in brain ECs and LPS inducing EC apoptosis. *Neisseiria meningitidis* (also known as meningococcus) interacts by his type IV pili with a yet not identified brain endothelial receptor. Once they have adhered on EC, they are able to induce the formation of actin-rich membrane processes highly reminiscent of the leukocyte trans-migratory cups which support their trans-endothelial migration.

of BBB crossing for viral spreading and, conversely, the role of the BBB as an obstacle for pharmacological treatment.

3.4.2.1. Altered BBB permeability in viral CNS infection. Various immunohistological studies of post-mortem brain tissue from patients with HIV encephalitis revealed a reduced expression of claudin-5 and occludin by brain endothelium [168]; accordingly, *in vitro* interaction of HIV-infected human lymphocytes with primary human brain ECs also induced a loss of these TJ proteins [168]. In the same line, a recent report demonstrated that HTLV-I-infected lymphocytes increased brain endothelial permeability *in vitro* via IL-1α and TNF-α secretion and that BBB breakdown was associated with endothelial TJ disorganization and alteration in the expression pattern of TJ proteins such as ZO-1 [36].

3.4.2.2. Trans-endothelial migration of virus-infected leukocytes. Some neurotropic viruses can cross brain endothelium by transcytosis, *i.e.* HIV, HTLV-1 or cytomegalovirus (CMV) [169,170], likely after macropinocytosis of free viral particles.

In addition, trans-endothelial migration of infected leukocytes constitutes an alternative route for entry into the CNS [171,172]. Infection was shown to increase the expression of adhesion molecules (integrins and selectins), in infected monocytes, lymphocytes and macrophages, favoring paracellular trans-endothelial migration of infected cells [170,173,174]. Moreover, this process is dependent upon several chemokines expressed in the brain in response to HIV infection and in inflammatory conditions, like CXCL12 (SDF-1α), CCL2 (MCP-1) and CX3CL1 (fractalkine): indeed, elevated levels of CXCL12 were detected in CSF and in brain tissue of HIV-infected patients [175,176], whereas high levels of the receptors of CXCL12 and CX3CL1 (CXCR4 and CX3CR1, respectively) were observed in HIV-infected monocytes [177,178]. Finally, HIV- and HTLV-I-infected cells were shown to produce high levels of MMPs which likely affect basal lamina integrity at the BBB and contribute to enhance their trans-endothelial migration to the brain [179,180].

3.5. Neurodegenerative diseases and BBB dysfunction

Age-related neurodegenerative diseases, such as Parkinson's and Alzheimer's disease (AD), constitute a major public health problem in western countries. Although BBB has long been dismissed in this type of CNS disease, recent data strongly suggested that BBB dysfunction might contribute to disease progression [181–183].

3.5.1. Altered BBB permeability in neurodegenerative diseases

Early observations of post-mortem brain tissue from AD patients pointed to a number of brain endothelium alterations: decreased number of mitochondria, increased number of pinocytosis vesicles, collagen accumulation in basal lamina and necrosis [184]. More recently, various clinical investigations of AD patients using gadolinium-enhanced MRI or biochemical methods, such as assessment of the CSF/blood albumin ratio, detected functional alterations of the BBB by measuring permeability increase compared to age-matched controls [185]. More recently, identification and functional characterization of peptides and proteins transport through the BBB [186–188], brought up a comprehensive view of the transport of Aβ through cerebral endothelium. Aβ peptide influx into the brain is mediated by the receptor for advanced glycosylation products (RAGE) [189] and is dependent on Aβ chaperones, apoE and apoJ [190]: apoE2 and apoE3, but not ApoE4, block the transport of Aβ to the brain [191]. The clearance of Aβ out of the brain is mediated by the lipoprotein receptor protein (LRP)-1 [192]. This transport activity of Aβ is altered in AD patients (Fig. 8). Indeed, the AD characteristic Aβ amyloid peptide deposits observed in senile plaques and perivascular spaces were suggested as a consequence of age-dependent decrease of Aβ clearance (due to lower expression of LRP-1) and increase in Aβ influx to the brain (due to increased expression of RAGE [189,193–195]). In addition, soluble forms of LRP-1 that normally sequester blood Aβ peptide and reduce its influx into the brain are detected in lower amounts, which may contribute to further increase brain Aβ levels [196]. The role of LRP-2 in Aβ efflux and in AD is still debated [196]. Altogether these observations strongly suggest that age-dependent alteration of Aβ transport across the BBB may significantly contribute to AD progression.

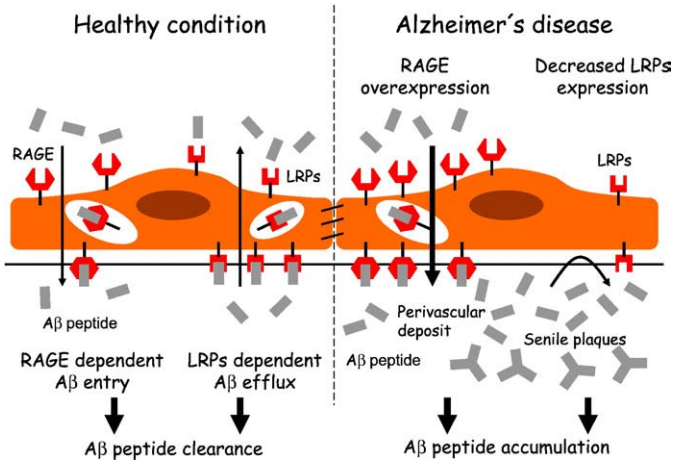

Fig. 8. The blood-brain-barrier in Alzheimer disease. In healthy condition (right), Aβ amyloid peptide is transported to brain by the receptor for advanced glycosylation products (RAGE) and cleared from the brain to the blood by LDL-Receptor-Proteins (LRPs). In Alzheimer's disease (left), these transport systems are impaired. RAGE is overexpressed and the expression of LRPs is decreased, leading to the accumulation of Aβ in the brain.

In the same line, increased BBB permeability associated with a loss of TJ integrity was also demonstrated in animal models of temporal epilepsy or status epilepticus [197].

3.5.2. Increased trans-endothelial migration in Alzheimer's disease

Increased expression of ICAM-1 in senile plaques was reported in human and primate brain tissues, suggesting an inflammatory component in their formation and involvement in AD progression [198,199]. In addition, cerebral infiltration of T lymphocytes has been recently described in AD patients, in line with previous reports in Parkinson's disease [199–203]. Furthermore, Aβ is able to induce monocytes infiltration through the BBB by a mechanism that implicates RAGE and PECAM-1 [204].

In conclusion, accumulating experimental as well as clinical evidence support the concept that BBB dysfunctions significantly contribute to the progression of a number of inflammatory, infectious or neoplasic diseases of the CNS. Furthermore, additional data, based on a better understanding of the molecular mechanisms of BBB function, point to a putative and so far unsuspected role of the BBB in the initiation of various neurodegenerative diseases, in particular following age-dependent alterations of its transport properties, as suggested in AD patients. Besides, even when altered in these situations, the BBB has long been considered as a major obstacle for drug delivery to the CNS. Although still not resolved so far, this key question has been addressed by various strategies which will be briefly considered below.

4. Therapeutic approaches

Because of the existence of the BBB, the first approach ever used to target drugs to the CNS was to try to circumvent it by direct intra-CSF or intra-cerebroventricular injections, in particular to target chemotherapy to some brain tumors. However, drug diffusion from the CSF to the brain parenchyma being usually poorly efficient [205],

convection-enhanced delivery of drugs into brain tumors via implanted catheters, or by post-surgery implantation of drug-impregnated wafers within the resection cavity, have been used as alternative strategies to treat patients with aggressive gliomas [206].

More recently, progresses in the understanding of the specific structural and functional characteristics of the BBB have helped design alternative therapeutic approaches for drug targeting to the brain by (i) increasing BBB permeability, (ii) modulating transporter expression and/or activity, (iii) taking advantage of receptor-mediated transport across the BBB, (iv) designing viral, chemical and cellular vectors with the capacity to cross the BBB (Fig. 9).

4.1. Increase BBB permeability

Local drug administration via intracerebral implants offers only little benefit to patients with brain tumors due to limited diffusion of the drug in brain tissue [207]. Hence, temporary disruption of the BBB was considered and achieved by intracarotid infusions of hyperosmotic solutions of chemical agents, mannitol or alkyl glycerol [208]. Among them, mannitol has been the most widely investigated [209]: mannitol causes reversible shrinkage of brain ECs by water efflux, leading to TJ disruption and increased drug diffusion to the brain. This strategy was proposed for enhanced delivery of anti-cancer drugs, viral vectors or nanoparticles [102,209,210]. However, the risk of toxic side effects may limit this strategy to highly aggressive and life-threatening diseases.

Transient BBB opening could also be performed by using ultrasound with or without contrast products, *e.g.* microbubbles [211,212]. This alternative strategy may limit neuronal damage by targeting a restricted brain area for BBB opening and drug delivery, even if the molecular mechanisms of permeability changes remain largely unknown [211–216]. Interestingly, this new approach was recently proposed for thrombolysis after acute ischemic stroke [213] and might be of interest for delivery of a plethora of substances such as immunoglobulins, viral vectors, plasmid DNA, siRNA, mRNA or high molecular weight drugs [217].

Besides, pharmacological approaches to increase BBB permeability are based on our knowledge that vasoactive mediators affect endothelial permeability by binding to apical membrane receptors and triggering intracellular signal transduction leading to changes in junctional complexes [218]. Indeed, bradykinin being known to increase the permeability of intra-tumoral blood microvessels, RMP-7, a bradykinin analog, was tentatively used to increase drug delivery to brain tumors; however a randomized, double-blind, placebo-controlled, phase II clinical study in recurrent malignant glioma failed to confirm previous preclinical data [219].

4.2. Modulate efflux transport activity at the BBB

It has been mentioned above that the BBB permeability properties are not only dependent upon the presence of endothelial TJs, but also upon the polarized expression of a number of ABC-transporters which protect the brain by effluxing into the blood a wide variety of hydrophobic, potentially neurotoxic compounds. These transporters are also responsible for poor delivery and poor overall efficacy of a number of active drugs, including anti-cancer and anti-viral drugs. It is worth mentioning that the anti-viral drugs successfully used in anti-HIV therapy (abacavir, zidovudine, efavirenz, tenofovir, emtricitabine) are ABC-transporter substrates, which cannot efficiently get access to virus-infected brain cells, leading to the concept of CNS as a viral reservoir for HIV. As a consequence, blocking ABC-transporter activity at the BBB was proposed as a strategy to increase cerebral delivery of anti-HIV drugs [71,220,221]. Experimental evidence strongly supports this hypothesis, by demonstrating that intracerebral concentrations of several HIV protease inhibitors are largely increased in brains of P-gp knock-out mice compared with wild-type animals, or after administration of P-gp blockers in wild type mice [222,223].

4.3. Control receptor-mediated transport through the BBB

Multiple active transport systems are present at the BBB, which control nutrient access to the CNS across the BBB [205]. Taking advantage of these various receptor-mediated transport systems expressed at the luminal side of brain ECs has long been a tempting approach to deliver drugs to the brain compartment. The most studied and best characterized receptors in this field are the transferrin and insulin receptors [224,225] which are known to be internalized after ligand binding and to mediate transcytosis of their ligand to the

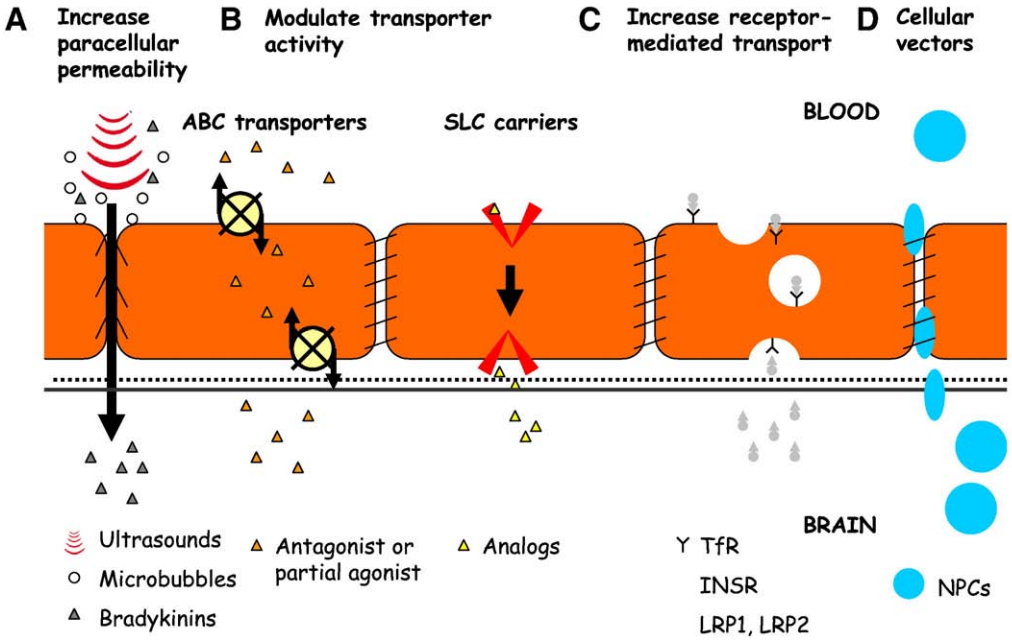

Fig. 9. Drug targeting across the blood-brain-barrier. The BBB is a major obstacle for drug delivery to the brain. Several approaches are currently proposed in order to circumvent these limitations: the route of delivery is showed at the top whereas examples of strategies are indicated at the bottom (for further details and abbreviations, see the text).

abluminal side of the BBB. Multiple proofs of principle for the feasibility of such approaches have been provided by various investigators for more than two decades, using drugs of interest covalently linked to these ligands or to ligand-mimicking anti-receptor antibodies, or incorporated in targeted liposomes or nanoparticles [68,226–229]. However, it must be emphasized that, to our knowledge, no successful clinical trials have been reported yet, suggesting that the drug delivery efficacy attainable by this strategy remains very low at the present time. More recently, similar strategies have been proposed using the LRP-1/LRP-2 receptors or the diphteria toxin receptor [230] and validation of these alternative approaches is in progress. Conversely, it is worth mentioning that inhibition of RAGE, which as mentioned above, mediate Aβ peptide influx into the brain, is currently tested as a potential therapeutic strategy in AD [231].

4.4. Cellular vectors

Based on our current knowledge of the mechanisms of leukocyte migration across the BBB, several studies were recently focused on the capacity of bone-marrow-derived cells or neural precursor cells (NPCs) to migrate across brain ECs. It has been shown in rodents that genetically engineered bone-marrow-derived cells were able to cross the BBB, which might be of great interest for the cerebral delivery of genes, enzymes, growth factors in neurological diseases [232]. It must be stressed however that, although strategies of allogenic bone marrow cell transplantation have been proposed in MS, their efficiency is still debated [233,234].

Neural precursor cells, when injected intravenously in several animal models of neurological diseases (MS, Parkinson's or neurodegenerative metabolic diseases), were recently documented to cross the BBB and to significantly improve the outcome of the corresponding disease [235–242]. Recent evidence suggests that these cells may mimic leukocytes in interacting with brain microvascular ECs and cross the BBB using an active mechanism [243]. Although technical or ethical concerns have to be considered, it is then tempting to speculate that systemic delivery of neural precursor cells might constitute a clinical alternative in the future.

5. Conclusion

The BBB is present at the interface between the brain and the periphery. It strictly controls the exchanges between both compartments, by actively transporting nutrients to the brain and protecting the CNS from various xenobiotics and pathogens. Its extremely low permeability is due to inter-endothelial TJs and the activity of multiple efflux transport systems. In addition, migration of circulating cells, notably activated leukocytes, across the BBB is a finely regulated dynamic process, responsible for the low-level but significant immunological survey of the brain in physiological conditions.

BBB dysfunction, often referred to as “BBB opening”, has long been known to constitute a key feature of the progression of several CNS diseases, as a consequence of neuro-inflammation. A closer observation of these pathological situations, based on a better knowledge of the biology of the BBB, led to the understanding that increased BBB permeability, clinically detected by gadolinium diffusion in MRI analysis, may or may not be accompanied by increased leukocyte migration across the BBB, these two events being clearly distinct on a molecular basis. In addition, experimental data strongly suggest that impaired clearance of the amyloid Aβ peptide across the BBB might largely contribute to the formation of Aβ brain deposits and AD progression, thus suggesting a causative role of BBB dysfunction in a neurodegenerative disease.

It is likely that the recent progresses in our current knowledge of TJ regulation, transport activity and leukocyte transmigration at the BBB will pave the way to novel therapeutic strategies for CNS diseases. Just like anti-VLA-4 antibody treatment has recently appeared as a beneficial approach to limit leukocyte infiltration to the brain in MS, the identification of various active transport systems or other membrane proteins at the BBB may allow for the development of new brain-targeted strategies to efficiently deliver drug to the CNS. Finally, systemic delivery of hematopoietic stem cells or neural precursor cells, based on the ability of these cells to cross the BBB, might constitute in the future alternative approaches for drug delivery to the brain in the context of aggressive neurodegenerative diseases.

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