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Review

The Blood-Brain Barrier in Health and Chronic Neurodegenerative Disorders

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The blood-brain barrier (BBB) is a highly specialized brain endothelial structure of the fully differentiated neurovascular system. In concert with pericytes, astrocytes, and microglia, the BBB separates components of the circulating blood from neurons. Moreover, the BBB maintains the chemical composition of the neuronal "milieu," which is required for proper functioning of neuronal circuits, synaptic transmission, synaptic remodeling, angiogenesis, and neurogenesis in the adult brain. BBB breakdown, due to disruption of the tight junctions, altered transport of molecules between blood and brain and brain and blood, aberrant angiogenesis, vessel regression, brain hypoperfusion, and inflammatory responses, may initiate and/or contribute to a "vicious circle" of the disease process, resulting in progressive synaptic and neuronal dysfunction and loss in disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, and others. These findings support developments of new therapeutic approaches for chronic neurodegenerative disorders directed at the BBB and other nonneuronal cells of the neurovascular unit.

The great British physiologist Hugh Davson, one of the founders of the modern concept of the blood-brain barrier (BBB), discussed in his state-of-the art review some 30 years ago the model and functions of the BBB (Davson, 1976). In Davson's era, and later in the eighties and early nineties, very little, if anything, was known about the role of the BBB in the pathogenesis of brain disorders, a field that has exploded over the past decade, and is the subject of the present review.

Introduction

At the beginning of the last century, Lewandowsky (1900) suggested that the absence of central nervous system (CNS) pharmacological actions of intravenously administered bile acids or ferrocyanide was due to the BBB, a mechanical membrane which separated blood from brain. The failure of the intravenously administered dye, trypan blue, to stain the brain and spinal cord tissue confirmed this view (Goldmann, 1909) (Figure 1A). It was suggested that such a barrier did not exist between the cerebrospinal fluid (CSF) and brain (Goldmann, 1913) (Figures 1B and 1C).

Electron microscopy (EM) studies with ferritin and horseradish peroxidase have since shown that the BBB is localized at the level of tight junctions (TJ) between adjacent brain endothelial cells (BEC) (Reese and Karnovsky, 1967; Brightman and Reese, 1969). The molecular nature of different interendothelial junction proteins, and their roles in mediating the BBB breakdown in various CNS diseases, are discussed below.

Physiological studies in the fifties, sixties, and seventies began to change the concept of the BBB as an impermeable barrier. Owing to the presence of highly specialized and diverse transport systems for chemically well-defined substrates (Ohtsuki and Terasaki, 2007), early transport studies demonstrated that the brain endothelium, a site of the BBB in vivo, regulated active transport of ions (Davson, 1976) and carrier-mediated transport of glucose (Yudilevich and De Rose, 1971) and amino acids (AA) (Oldendorf, 1973; Davson, 1976). The role of different BBB transporters in the normal brain as potential therapeutic targets or effectors in the development of brain pathology is discussed later.

Today, we accept the view that the BBB limits the entry of plasma components, red blood cells, and leukocytes into the brain. If they cross the BBB due to an ischemic injury, intracerebral hemorrhage, trauma, neurodegenerative process, inflammation, or vascular disorder, this typically generates neurotoxic products that can compromise synaptic and neuronal functions (Zlokovic, 2005; Hawkins and Davis, 2005; Abbott et al., 2006).

An intact BBB is also a major obstacle for the development of drugs for CNS disorders. Approximately 98% of small molecule drugs and all large molecule neurotherapeutics, e.g., recombinant peptides, proteins, anti-sense-agents and genetic vectors, are normally excluded from the brain (Pardridge, 2007). When confronted with the BBB, they behave essentially like trypan blue (Figure 1).

Neurovascular System

In humans, the brain receives up to 20% of cardiac output. If cerebral blood flow (CBF) stops, brain functions stop in seconds and damage to neurons may occur in minutes (Girouard and Iadecola, 2006).

The normal neuronal-vascular relationship is critical for normal brain functioning. It has been estimated that nearly every neuron in human brain has its own capillary (Zlokovic, 2005). The total length of capillaries in human brain is about 400 miles, and the capillary surface area available for molecular transport is about 20 m^2 (Begley and Brightman, 2003). The thickness of the cerebral endothelial membrane is 0.2 to 0.3 μm. The length of brain capillaries is reduced in neurodegenerative disorders, as for example in Alzheimer's disease (AD) (Bailey et al., 2004; Wu et al., 2005). These vascular reductions can diminish transport

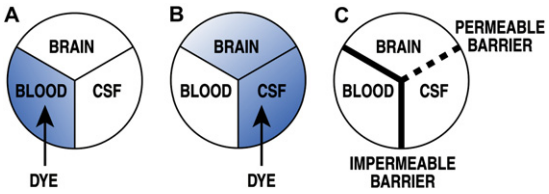

Figure 1. The Blood-Brain Barrier, or BBB, to Trypan Blue and Its Diffusion from the Cerebrospinal Fluid, or CSF, into the Brain
(A) Goldmann's first experiment. Trypan blue (dye) was injected into the blood. Brain and CSF were analyzed.
(B) Goldmann's second experiment. Dye was injected into the CSF. Brain and blood were analyzed.
(C) Conclusions from the two experiments.

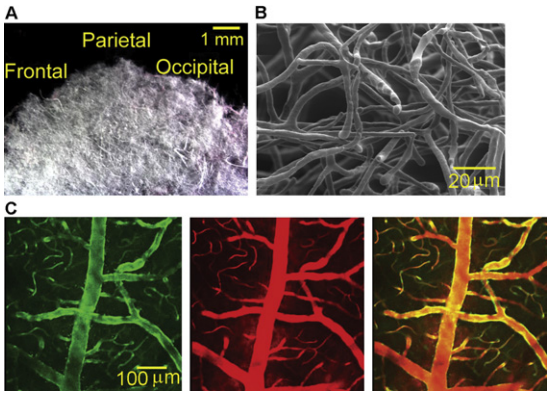

Figure 2. A Cast of the Microvascular Network and Two-Photon In Vivo Imaging of the Mouse Brain Microcirculation
(A) Vascular cast of a mouse cortex. Mouse was infused via carotid artery with methyl metacrylate. To obtain a corrosive cast, brain tissue was dissolved with potassium hydroxide.
(B) Scanning electron micrograph of microvessels from the cast in (A).
(C) Cortical microcirculation in Tie-2-GFP-expressing mice studied in vivo by two-photon in vivo imaging. (Left) GFP (green) is localized exclusively to the brain endothelium. (Middle) Intravenously injected intravascular marker tetra-meta rhodamine (TMR, mw = 70 kDa, red) remains restricted within brain microvessels by the BBB. (Right) Merged left and middle panels indicate that all cortical microvessels were perfused with TMR. A two-photon image of the parietal cortex was taken through the thinned skull at a depth of 50 μm.
(A) and (B), courtesy of Dr. Yaoming Wang, and (C), courtesy of Rachal Love from B.V. Zlokovic laboratory.

of energy substrates and nutrients across the BBB, and reduce the clearance of potential neurotoxins from the brain, as discussed below.

Figure 2A illustrates a vascular cast of the mouse brain demonstrating the rich network of blood vessels that continues at the level of brain capillaries (Figure 2B). In vivo multiphoton imaging of cortical blood flow in mice expressing green fluorescent protein (GFP) in the brain endothelium shows that virtually all brain microvessels are constantly perfused at any time (Figure 2C). The former "capillary recruitment" hypothesis, proposing opening of new capillaries from an increase in the CBF and closing of brain capillaries from a decrease in the CBF (Weiss, 1988), has thus been modified to a "functional recruitment" hypothesis: brain capillaries are perfused all the time, but they transition from low to high blood flow with an increase in the CBF, or from high to low blood flow with a decrease in the CBF (Kuschinsky and Paulson, 1992). As shown in rodent models of brain hypoxemia, the major mechanisms raising the CBF are increased velocity of microvessel perfusion (Bereczki et al., 1993) and recruitment of red blood cells to the capillary networks (Krolo and Hudetz, 2000).

The morphometric analysis of the mouse cortical vasculature in vivo based on two-photon imaging indicates that perfused capillaries (~4–8 μm in diameter) and small arterioles and venules (10–60 μm in diameter) occupy between 3%–4% and 4%–6% of the brain volume, respectively. This correlates well with some in vivo measurements of the blood volume in the gray matter in human brain determined by magnetic resonance imaging (MRI) (Rengachary, 2005).

BBB Cellular Junctions

The BBB is composed of a tightly sealed monolayer of BEC, which normally precludes free exchanges of solutes between blood and brain and brain and blood (Ohtsuki and Terasaki, 2007). An exception to this rule are small lipid-soluble molecules <400 Da with fewer than nine hydrogen bonds, which can cross the BBB unassisted, via lipid-mediated diffusion (Pardridge, 2007). All drugs presently in clinical use for CNS therapy are small molecules that have these characteristics. Lipid mediation of small molecules through biological membranes requires molecular movement through channels of a finite size within the lipid bilayer.

BEC are normally connected at a junctional complex by the TJ and adherens junctions (AJ) (Hawkins and Davis, 2005). Gap junctions have also been identified at the BBB (Nagasawa et al., 2006), but their role in the barrier function is not clear. The TJ primarily confer the low paracellular permeability and high electrical resistance of the BBB (Bazzoni and Dejana, 2004). Possible roles of different TJ proteins in the pathogenesis of various brain disorders are discussed below.

Tight Junctions

The molecular biology of the TJ is quite complex (Wolburg, 2006). The TJ proteins and their adaptor molecules, which link the TJ to the cytoskeleton, are often affected during acute and chronic diseases of the brain. Figure 3A shows the molecular organization of the BBB TJ, which form a continuous cellular membrane that restricts transport of molecules between blood and the brain interstitial fluid (ISF), and vice versa.

Occludin. Occludin was the first integral membrane protein discovered within the TJ of endothelial cells, including the BBB. A deletion construct lacking the N terminus and extracellular domains of occludin exerted a dramatic effect on the TJ integrity (Bamforth et al., 1999). Cell monolayers failed to develop an efficient permeability barrier, as demonstrated by low transcellular electrical resistance, an increased paracellular flux to small molecular mass tracers, and the presence of gaps in the P-face associated TJ strands on the freeze-fracture EM analysis. These findings demonstrated that the N-terminal half of occludin had an important role in maintaining a TJ assembly and the barrier function.

Deletion of occludin in mice results in a complex phenotype and postnatal growth retardation (Saitou et al., 2000). Surprisingly, the TJ themselves are not affected by the lack of occludin, as demonstrated by well-developed networks of TJ strands and normal transepithelial electrical resistance. Likely, normal

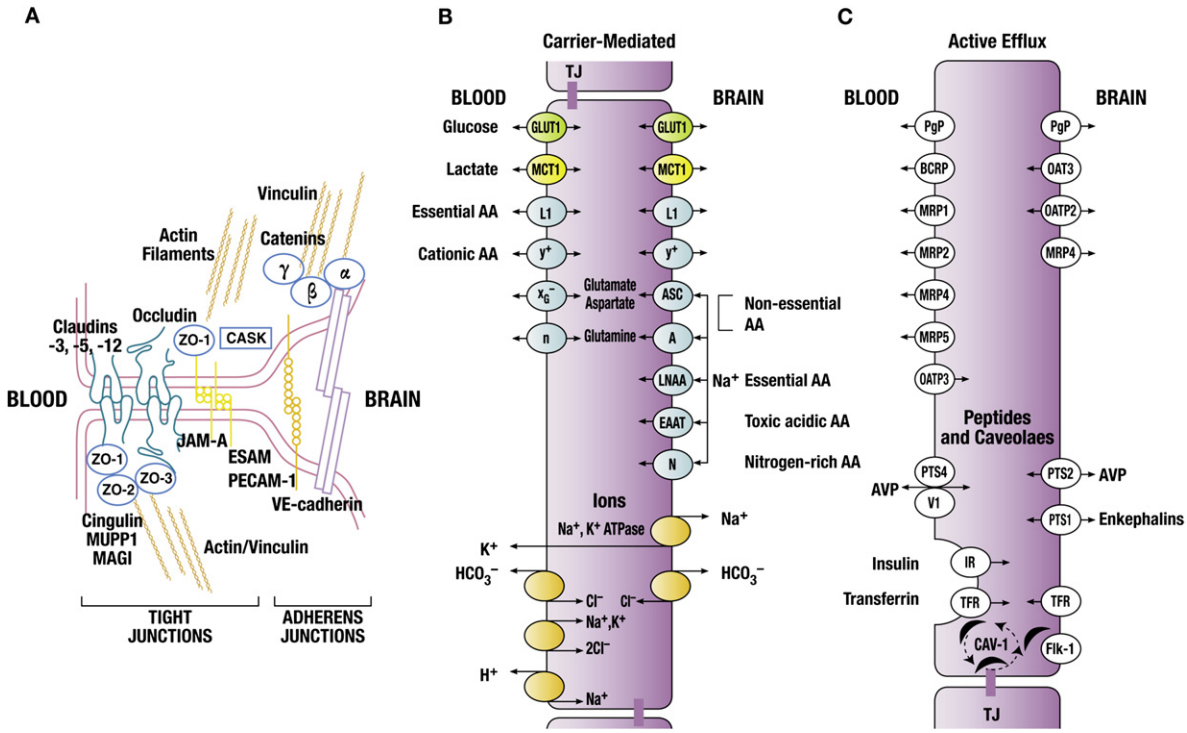

Figure 3. A Simplified Molecular Atlas of the BBB

(A) *Tight junctions.* Claudins (claudin-3, -5, and -12) and occludin have four transmembrane domains with two extracellular loops. The junctional adhesion molecule A (JAM-A) and the endothelial cell-selective adhesion molecule (ESMA) are members of the Ig superfamily. Zonula occludens proteins (ZO-1, ZO-2, and ZO-3) and the calcium-dependent serine protein kinase (CASK) are first-order cytoplasmic adaptor proteins that contain PDZ binding domains for the C terminus of the intramembrane proteins. Cingulin, multi-PDZ protein 1 (MUPP1), and the membrane-associated guanylate kinase with an inverted orientation of protein-protein interaction domain (MAGI) are examples of second-order adaptor molecules. The first- and second-order adaptor molecules together with signaling molecules control the interaction between the intramembrane proteins and actin/vinculin-based cytoskeleton. *Adherens junctions*. The vascular endothelial cadherin (VE-cadherin) is the key molecule. Platelet endothelial cell adhesion molecule 1 (PECAM-1) mediates homophilic adhesion. Catenins (α, β, γ) link adhesion junctions to actin/vinculin-based cytoskeleton.

(B) *Carrier-mediated transporters.* GLUT1, glucose transporter, and monocarboxylate transporter 1 (MCT1) for lactate exist at both the luminal and the abluminal membranes. All essential amino acids (AA) are transported by the L1 and y^+ systems on each membrane. Five Na^+-dependent transport systems mediate elimination of nonessential AA (ASC, A), essential AA (LNAA), the excitatory acidic AA (EAAT) (e.g., glutamate and aspartate), and nitrogen-rich AA (N) (e.g., glutamine) from the brain. Facilitative transporters x_G^- and n on the luminal membrane mediate glutamate, aspartate, and glutamine efflux to blood. *Ion transporters.* The sodium pump (Na^+, K^+-ATPase) on the abluminal membrane controls Na^+ influx and K^+ efflux. Sodium-hydrogen exchanger on the luminal membrane is a key regulator of intracellular pH. Na^+-K^+-$2Cl^-$ cotransporter is on the luminal membrane. The chloride-bicarbonate exchanger exists on each membrane.

(C) *Active efflux transporters*. Multidrug efflux transporters at the luminal membrane limit drug uptake into the brain. Transporters at the abluminal membrane could act in concert with luminal transporters to eliminate drugs from brain ISF. P-gp is expressed on each membrane. Breast cancer resistance protein (BCRP) is on the luminal membrane. Multidrug resistance-associated proteins (MRPs) are expressed mainly on the luminal membrane. Organic anion transporting polypeptide (OATP) 2 and 3 exist on the luminal and abluminal membranes, respectively. Organic anion transporter 3 (OAT3) is on the abluminal membrane. *Peptide transporters and caveolae*. Peptide transport system 1 (PTS-1) on the abluminal membrane mediates efflux of opioid peptides (e.g., enkephalins) from brain. PTS-2 mediates efflux of arginine-vasopressin (AVP). PTS-4 on the luminal membrane requires the vasopressinergic receptor 1 (V1) to transport AVP into the brain. Receptors for insulin (IR) and transferrin (TFR) are found in the caveolar membranes. Caveolin-1 (Cav-1) could be associated with receptors (e.g., TFR), tight junctions (TJ), or growth factor receptors, such as vascular endothelial growth factor receptor (Flk-1).

expression and localization of other junctional proteins such as claudin-3, zonula occludens-1 (ZO-1), ZO-2, vascular endothelial cadherin (VE-cadherin), and α-catenin may compensate well for occludin loss (Saitou et al., 2000). However, hyperplasia of the gastric epithelium, calcifications in the brain, and testicular atrophy found in occluding-deficient mice (Saitou et al., 2000) raise a possibility that occludin has some other important physiological roles beyond its function as a TJ protein. Indeed, recent studies have demonstrated that occludin regulates epithelial cell differentiation (Schulzke et al., 2005). In addition, it controls claudin-2-dependent TJ function, as well as cell apoptosis through inhibition of mitogen-activated protein kinases (MAPK) and Akt signaling pathways (Murata et al., 2005). Whether occludin has a role in the BBB differentiation during normal development, brain vascular repair, or both remains to be explored.

Recent studies of mice with experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (MS), have shown that occludin dephosphorylation precedes visible signs of disease and happens just prior to apparent changes in the BBB permeability (Morgan et al., 2007). These findings suggest that occludin could be a target for signaling processes in EAE and that it may regulate the response of the BBB to the inflammatory environment, as seen in MS.

Treatment of mice with Tat protein results in decreased expression of occludin and ZO-1 (Pu et al., 2007). Tat is normally released from human immunodeficiency virus type 1 (HIV-1)-infected

blood-borne leukocytes. It contributes to the BBB breakdown by providing potential entry for HIV-1 into the brain. Tat-induced loss of occludin and ZO-1, and the resulting loss of the TJ integrity, can be implicated in the pathogenesis of HIV-1-related brain diseases.

Occludin is also vulnerable to attack by matrix metalloproteinases (MMPs) (Rosenberg and Yang, 2007). Reperfusion injury in rodent models of stroke leads to a biphasic opening of the BBB, with the early opening occurring several hours after the onset of reperfusion due to activation of the constitutive enzyme gelatinase A (MMP-2). This initial opening is transient and followed 24 to 48 hr later by more intense damage to the blood vessel, which is associated with the expression and activation of gelatinase B (MMP-9) and stromelysin-1 (MMP-3). MMPs can also degrade basal lamina proteins such as fibronectin, laminin, and heparan sulfate after an ischemic insult, which contributes to the BBB breakdown (Cheng et al., 2006; Zlokovic, 2006).

Accumulation of occludin in neurons, astrocytes, and microglia in AD, frontotemporal dementia, and vascular dementia has also been reported (Romanitan et al., 2007), suggesting possible new functions of occludin and the TJ proteins in the pathogenesis of these dementias. Their exact role in dementia-related pathologies, however, remains to be elucidated, as discussed below.

Claudins. The claudins are a multigene family of more than 20 members that form TJ strands through homophilic claudin-claudin interactions mediated by the extracellular loop 2 of claudins (Piontek et al., 2008). Claudin-5, -3, and -12 are localized at the BBB (Nitta et al., 2003; Wolburg, 2006), whereas the presence of claudin-1 is controversial (Lee et al., 2003). Each claudin regulates the diffusion of a group of molecules of a certain size. For example, mice with claudin-5 deletion die as neonates due to a size-selective loosening of the BBB for molecules <800 Da (Nitta et al., 2003).

Selective downregulation of claudin-8 by kindling epilepsy (Lamas et al., 2002) suggests that selective modulation of claudin expression in response to abnormal neuronal synchronization may lead to BBB breakdown and brain edema, as seen in epilepsy.

Claudin-5 is degraded by MMP-2 and MMP-9 after an ischemic insult, and both claudin-5 and occludin were found in the surrounding astrocytes, but not in the brain endothelium, after the BBB disruption (Yang et al., 2007). It is possible that increased levels of occludin found in astrocytes and neurons in vascular dementia, AD, and frontotemporal dementia (Romanitan et al., 2007) might reflect the autophagy of the TJ proteins by the surrounding cells after the BBB breakdown caused by chronic hypoxia, aberrant angiogenesis, or both (Wu et al., 2005).

As with occludin, exposure of BEC to HIV-1 Tat protein decreases expression and alters distribution of claudin-5, which in turn may contribute to BBB disruption in the course of HIV-1 infection, and the entry of HIV-1 into the brain (Andras et al., 2005).

Other Junctional Proteins. Junctional adhesion molecule A (JAM-A) (Bazzoni et al., 2005) and the endothelial cell-selective adhesion molecule (ESAM) (Nasdala et al., 2002) are members of the immunoglobulin (Ig) supergene family. A recent study in spontaneously hypertensive rats has suggested that JAM-A is upregulated throughout the body compared with the control rats, and that this is not secondary to the hypertension (Waki et al., 2007). When JAM-A is expressed in the nucleus tractus solitarii, it raises arterial pressure, suggesting a prohypertensive role of this TJ protein in the brain stem. It has been suggested that altered expression of JAM-A, in addition to affecting the junctional tightness, may also affect leukocyte trafficking, with implications for immune status within the diseased CNS (Padden et al., 2007). The role of JAM-A and ESAM in different brain pathologies is still relatively poorly understood.

Cytoskeleton Link

ZO-1 Proteins. The integral membrane proteins of the TJ are linked to the cytoskeleton via cytoplasmic multidomain scaffolding proteins of the peripheral membrane-associated guanylate kinase (MAGUK) family, such as ZO-1, ZO-2, and ZO-3 (Hawkins and Davis, 2005). Besides providing the cytoskeletal anchorage for the transmembrane TJ proteins, the MAGUK also control correct spatial distribution of claudins though their PDZ binding domains.

Significant differences in the incidences of TJ abnormalities related to reduced ZO-1 expression have been detected between different types of lesions in MS, and between MS and control white matter (Kirk et al., 2003). It has been shown that about 42% of vessel segments in active MS plaques have reduced ZO-1 expression with severe plasma leakage, while about 23% of vessels in inactive plaques also demonstrate reduced ZO-1 protein levels. It has been suggested that persistent endothelial abnormalities associated with BBB leakage may contribute to MS progression and have prognostic implications, and should be considered when planning disease-modifying therapy (Leech et al., 2007).

In a model of experimental diabetes in rats, the BBB permeability to ^{14}C-sucrose increases concurrently with decreased production of ZO-1 and occludin at the BBB (Hawkins et al., 2007). Degradation of these two TJ proteins has been related to an increased plasma MMP activity, suggesting that peripheral MMPs, in addition to central MMPs, could be targets for stabilizing BBB dysfunction. Since MMPs also participate in regulating neurogenesis and angiogenesis, as for example during a repair phase after stroke (Zhao et al., 2006), more work is needed to clarify the therapeutic potential of MMP inhibition in different brain pathologies.

It has been demonstrated that caveolin-1 regulates expression of occludin and ZO-1 in BEC monolayers, a model of an in vitro BBB (Song et al., 2007a). Loss of caveolin-1 facilitates the ability of the chemokine CCL2, formerly called monocyte chemoattractant protein-1 (MCP-1), to permeablize the BBB. Thus, caveolin-1 may be critical in regulating inflammation at the BBB, and therefore could represent a novel therapeutic target for stabilizing the BBB.

Actin. The importance of the cytoskeleton in establishing and maintaining the BBB has become evident from studies in mice lacking the actin-binding protein dystrophin, i.e., the mdx mice (Nico et al., 2003). These mice exhibit an increase in the brain vascular permeability due to disorganized α-actin cytoskeleton in endothelial cells and astrocytes, as well as altered subcellular localization of junctional proteins in the endothelium and the water channel aquaporin-4 in the astrocytic endfeet. These findings demonstrate that properly arranged actin filaments and their

binding to the TJ and/or AJ proteins are critical for normal barrier function.

HIV-1 gp120 and alcohol can reorganize the cytoskeleton and induce stress fiber actin formation, causing increased permeability of the human BBB endothelium (Shiu et al., 2007). It has been suggested that alcohol-mediated changes in the BEC monolayers may increase diffusion of plasma components and viral penetration across the BBB, and therefore, especially at levels attained in heavy drinkers, accelerate HIV-1 penetration into the brain.

Adherens Junctions

The AJ are typically found intermingled with the TJ.

VE-cadherin. Within the AJ, the endothelial-specific integral membrane protein VE-cadherin is linked to the cytoskeleton via catenins, which belong to the family of armadillo proteins (Bazzoni and Dejana, 2004). At the BBB, expression and localization of β-catenin, χ-catenin, and p120^{cas} is crucial for the functional state of the AJ.

Caveolin-1-induced reductions in ZO-1 and occludin expression are associated with comparable alterations in the AJ proteins, VE-cadherin, and β-catenin, which may enhance CCL2-medited stimulation of transendothelial migration of monocytes (Song et al., 2007a). Therefore, changes in the AJ proteins may potentially contribute to increased paracellular BBB permeability and leukocyte trafficking in the CNS. On the other hand, β-catenin expression is not altered in MS lesions (Padden et al., 2007), which would argue against the role of AJ in MS pathogenesis.

Recent in vitro and in vivo data show that VE-cadherin is required for endothelial integrity in quiescent vessels and for the correct organization of new vessels (Lampugnani and Dejana, 2007). Several mechanisms by which VE-cadherin may regulate endothelial functions have been proposed, such as (1) direct activation of signaling molecules with a role in survival and organization of the actin cytoskeleton (e.g., PI3 kinase and Rac); (2) regulation of gene transcription cofactors (e.g., β-catenin and p120); and (3) formation of complexes with growth factor receptors, as, for example, with the vascular endothelial growth factor (VEGF) receptor 2 (VEGFR-2) (also called Flk-1), and modulation of VEGFR-2 signaling. The role of AJ in chronic neurodegenerative disorders with documented functional and morphological changes at the BBB, such as AD, Parkinson's disease (PD), MS, and others, remains mainly unexplored.

PECAM-1. Platelet endothelial cell adhesion molecule 1 (PECAM-1), also known as CD31, is localized in the endothelial cell contacts outside of the TJ. PECAM-1 is a major participant in the migration of leukocytes across endothelium.

A chimeric, soluble form of PECAM-1 fused to human IgG-Fc fragment (sPECAM-Fc) impairs migration of lymphocytes across the brain endothelial monolayers and diminishes the severity of EAE in a mouse model (Reinke et al., 2007). These findings suggest a therapeutic potential of the sPECAM-Fc construct for short-term treatments of diseases like MS.

A recombinant construct targeting a single-chain variable fragment (scFv) for urokinase-type plasminogen activator (uPA) to stably expressed PECAM-1 (anti-PECAM-1 scFv-uPA) accumulates in the brain after intravascular injection, while unconjugated uPA does not (Danielyan et al., 2007). It lyses clots in the cerebral arterial vasculature without hemorrhagic complications and provides rapid and stable cerebral reperfusion. It has been suggested that effective and safe thromboprophylaxis in the cerebral arterial circulation by anti-PECAM-1 scFv-uPA represents a prototype of a new heuristic to prevent recurrent cerebrovascular thrombosis by using molecules expressed at the BBB as therapeutic targets. More research is needed to explore the full therapeutic potential of this novel approach.

PECAM-1 and the receptor for advanced glycation end products (RAGE) are required for AD amyloid β-peptide (Aβ)-mediated migration of monocytes across human BEC monolayers (Giri et al., 2000). Whether blocking PECAM-1 would have a potential therapeutic benefit in AD, and in other brain pathologies associated with peripheral leukocyte infiltration, remains to be determined.

BBB Transport Systems

The TJ-controlled paracellular impermeability of the brain capillary endothelium implies that the hydrophilic molecules must cross the endothelial wall transcellularly to reach their neuronal targets or leave the brain (Deane and Zlokovic, 2007). In general, transcellular bidirectional transport across the BBB can be classified into five main categories: carrier-mediated transport, ion transport, active efflux transport, receptor-mediated transport, and caveolae-mediated transport.

Carrier-Mediated Transport

Specific, carrier-mediated transport systems facilitate transport of nutrients such as hexoses (glucose, galactose); neutral, basic, and acidic AA and monocarboxylic acids (lactate, pyruvate, ketone bodies); nucleosides (adenosine, guanosine, uridine); purines (adenine, guanine); amines (choline); and vitamins (Hawkins et al., 2006; Simpson et al., 2007; Ohtsuki and Terasaki, 2007; Deeken and Loscher, 2007; Spector and Johanson, 2007). The concentration gradients for nutrients are generally in the direction from blood to brain. These are regulated by brain metabolic needs, and by the concentrations of substrates in plasma. GLUT1 glucose transporter, the L1 large neutral amino acid transporter, the CNT2 adenosine transporter, and the monocarboxylate transporter 1 (MCT1) have been cloned from BBB-specific cDNA libraries (Pardridge, 2005).

GLUT1. The glucose transporter GLUT1 is of special importance because glucose is the main energy source for the brain (Qutub and Hunt, 2005; Simpson et al., 2007). GLUT1 (mw = 55 kDa) is a member of a gene family of sodium-independent glucose transporters, which is expressed exclusively at the BBB. GLUT1 transports glucose and other hexoses across the BBB (Figure 3B). The density of GLUT1 transporters at the abluminal membrane is higher than at the luminal (Simpson et al., 2007). The asymmetrical distribution of GLUT1 at the BBB provides a homeostatic control for glucose influx into the brain by preventing glucose accumulation in the brain ISF at levels higher than those in the blood.

Heterozygous mutations or hemizygosity of the GLUT1 gene cause GLUT1 deficiency syndrome. GLUT1 deficiency syndrome in humans is characterized by infantile seizures, developmental delay, and acquired microcephaly. It is caused by haploinsufficiency of the BBB hexose carrier. GLUT1$^{+/-}$ mice that have been generated by the targeted disruption of the promoter and exon 1 regions of the mouse GLUT1 gene have epileptiform

discharges on electroencephalography, impaired motor activity, incoordination, microencephaly, decreased brain glucose uptake, and substantially decreased brain GLUT1 expression in brain capillaries (Wang et al., 2006). Human GLUT1 deficiency syndrome and the mouse model are perfect examples of brain disorders triggered by dysfunctional BBB transporters.

The expression of GLUT1 is controlled by hypoxia-inducible factor-1 (HIF-1), a transcription factor which regulates the adaptive responses to hypoxia. HIF-1α accumulates in the rat cerebral cortex after transient global ischemia, which is associated with corresponding increases in GLUT1 and other HIF-1α target genes (Chavez and LaManna, 2002). The adult rat brain adapts to prolonged moderate hypoxia with increased vascularity and increased GLUT1 density at the BBB (Harik et al., 1996).

GLUT1 protein expression in brain capillaries is reduced in AD, although this is not associated with changes in the GLUT1 mRNA structure (Mooradian et al., 1997) or the levels of GLUT1 mRNA transcripts (Wu et al., 2005). The surface area at the BBB available for glucose transport is substantially reduced in AD (Bailey et al., 2004; Wu et al., 2005). These findings suggest that the AD brain is subjected to a continuous shortage in energy metabolites due to GLUT1 deficiency at the BBB.

Indeed, recent PET studies with $^{(18)}$F-2-fluoro-2-deoxy-D-glucose (FDG) have demonstrated that individuals diagnosed with aging-associated cognitive decline have significantly reduced glucose uptake in the right precuneus, posterior cingulate, right angular gyrus, and bilateral middle temporal cortices prior to conversion into AD (Hunt et al., 2007). These respective deficits are more pronounced in AD patients and involve the frontal cortices. Thus, individuals with mild cognitive impairment (MCI) have reduced glucose transport across the BBB prior to neurodegeneration and brain atrophy.

Subsequent FDG-PET studies have confirmed that the reduced FDG uptake by the posterior cingulate gyri and parietotemporal lobes in AD patients is not due to brain atrophy (Samuraki et al., 2007). A longitudinal study using FDG-PET imaging with the follow-up PET exams has suggested that reductions of glucose utilization by the hippocampus during normal aging can predict cognitive decline years in advance of the clinical diagnosis (Mosconi et al., 2008). Consistent with this concept, presymptomatic early-onset autosomal dominant familial AD (FAD) individuals carrying mutations in the presenilin-1 gene show widespread AD-like reductions in FDG utilization in the absence of structural brain atrophy (Mosconi et al., 2006).

In sum, the FGD-PET measures may serve as early biomarkers for the preclinical diagnosis of AD. But more than that, these studies suggest the reductions in glucose uptake across the BBB may precede the neurodegenerative process and brain atrophy in the MCI cases converting to AD. Whether GLUT1 at the BBB can be manipulated therapeutically to prevent the development of dementia and control a chronic neurodegenerative process remains to be explored. Future studies are likewise needed to increase preclinical specificity in differentiating the types of dementias based on the reduced glucose uptake across the BBB.

MCT1. Ketone bodies, such as R-beta-hydroxybutyrate and acetoacetate, are energy sources for the brain. As with glucose metabolism, monocarboxylate uptake by the brain is dependent on the function and regulation of its own transporter system. MCT1 is expressed at each membrane of the BBB (Simpson et al., 2007). MCT1 mediates transport of lactate and other monocarboxylates in and out of the brain (Figure 3B). Recent studies in diet-induced ketotic rats have demonstrated a substantial upregulation of MCT1 and GLUT1 at the BBB, associated with an increased extraction of plasma ketone bodies by the brain, but no changes in CBF (Puchowicz et al., 2007). These findings suggest that upregulation of the BBB transporters (e.g., MCT1 and GLUT1), but not an increase in CBF, is critical for adapting brain metabolism to energy metabolites available in the plasma.

Amino Acid Transporters. The existence of two facilitative transporters for AA, i.e., L1 and y^+, on luminal and abluminal membranes, provides the brain access to all essential AA (Hawkins et al., 2006). The sodium-independent L1 facilitative transporter mediates transport of large neutral essential AA (e.g., leucine, isoleucine, valine, tryptophan, tyrosine, phenylalanine, threonine, and methionine). The y^+ system mediates transport of cationic AA, some of which are essential in the brain (i.e., lysine), and some of which are nonessential in the adult brain (e.g., arginine and ornithine) but are essential during a juvenile period, as for example L-arginine, a precursor of nitric oxide (NO) (Figure 3B).

Five sodium-dependent transporters for AA exist at the abluminal membrane (Figure 3B). The abluminal systems have the capability to actively transfer every naturally occurring AA from the brain ISF to endothelial cells, and from there, into the circulation. This provides a mechanism by which AA concentrations in the brain ISF are maintained at approximately 10% of those in plasma. For example, the ASC and A systems at the BBB transport nonessential AA out of the brain.

The sodium-dependent system for the excitatory acidic AA (EAAT), e.g., glutamate and aspartate, provides a mechanism for net removal of potentially neurotoxic AA from the brain. It also accounts for the low penetration of glutamate into the brain. Excitotoxicity is defined as excessive exposure to the neurotransmitter glutamate or overstimulation of its membrane receptors, leading to neuronal injury or death (Lipton, 2005). Excess glutamate in brain fluids characterizes acute brain insults such as traumatic brain injury and stroke. In addition, it has been suggested that glutamate excitotoxcity is implicated in the neurodegenerative process in epilepsy (Alexander and Godwin, 2006), amyotrophic lateral sclerosis (ALS) (Van Damme et al., 2005), MS (Vallejo-Illarramendi et al., 2006), Huntington's disease (HD) (Cowan and Raymond, 2006), and AD (Lipton, 2005).

Glutamate transporters EAAT1, EAAT2, and EAAT3 determine the levels of extracellular glutamate and are essential to prevent excitotoxicity (Lipton, 2005). It has been demonstrated that scavenging glutamate in the blood increases the efflux of excess glutamate from the brain. For example, systemic treatment of rats with oxalocaetate, a glutamate scavenging agent, prior to or early after closed brain injury can afford brain neuroprotection by reducing the level of glutamate in the blood, which promotes efflux of glutamate from the brain (Zlotnik et al., 2007). Whether the BBB EAAT glutamate transporter can be targeted therapeutically to reduce glutamate levels in acute and chronic neurodegenerative disorders remains to be explored.

The sodium-dependent system for nitrogen-rich AA removes glutamine and other nitrogen-rich AA (e.g., histidine and

asparagine) from the brain. The facilitative transporters at the luminal side, x_G^- and n, mediate transport of acidic AA and nitrogen-rich AA from the endothelium to blood, respectively (Figure 3B).

Vitamins. The vitamins are transported in most cases by separate carriers through the BBB or choroid plexus, as, for example, vitamins B1, B3, B5, or E (Spector and Johanson, 2007). The exception is the sodium-dependent multivitamin transporter for biotin, pantothenic acid, and lipoic acid.

Ion Transporters

The BBB has a high density of mitochondria, which reflects high energy demands for active ATP-dependent transporters such as the sodium pump (Na^+, K^+-ATPase). The sodium pump is localized on the abluminal membrane (Vorbrodt, 1988) (Figure 3B). It regulates sodium influx into the brain ISF in exchange for potassium. Na^+, K^+-ATPase maintains the high concentration gradient for Na^+ at the BBB (extracellular > > intracellular), so that Na^+-dependent transport can occur. Sodium-potassium-two chloride (Na^+-K^+-$2Cl^-$) cotransporter resides predominantly in the luminal BBB membrane (O'Donnell et al., 2006). Na^+-K^+-$2Cl^-$ cotransporter transports sodium, potassium, and chloride from blood into the brain endothelium. Sodium-hydrogen exchanger is expressed on the luminal membrane, whereas chloride-bicarbonate exchanger is expressed at each side (Taylor et al., 2006). These two transporters play critical roles in regulating intracellular pH in the endothelium. The chloride-bicarbonate exchanger also regulates active secretion of bicarbonate across the BBB. The sodium-calcium exchanger is also present at the BBB. In the forward mode (Na^+ entry/Ca^{2+} extrusion), this exchanger mediates Ca^{2+} efflux from the endothelium. In the presence of altered Na^+ gradients or under pathological circumstances, it may transport calcium into the endothelium.

Active Efflux

Efflux of molecules from the brain endothelium can be initiated at the luminal membrane, as in the case of the ATP-binding cassette (ABC) transporters (Hermann and Bassetti, 2007). The multidrug resistance transporter P-glycoportein (P-gp) is an ATP-dependent efflux pump which mediates rapid removal of ingested toxic lipophilic metabolites, such as many amphipatic cationic drugs (Loscher and Potschka, 2005; Hermann and Bassetti, 2007). P-gp is encoded in humans by the *MDR1* gene and in rodents by the *mdr1a* and *mdr1b* genes. In addition to P-gp, several multidrug resistance-associated proteins (MRPs) are expressed in the brain microvessels. The MRPs, including the breast cancer resistance protein (BCRP) and members of the organic anion transporting plypeptide (OATP) family and the organic anion transporter (OAT) family, mediate mainly the efflux of anionic compounds (Figure 3C). These transporters have the potential to work together to reduce penetration of many drugs into the brain and increase their efflux from the brain.

Recent immunogold cytochemistry EM studies in rat and human brain tissue revealed that P-gp is expressed at the luminal and abluminal membrane, as well as in pericytes and astrocytes (Bendayan et al., 2006). Subcellularly, P-gp is distributed along the nuclear envelope, in caveolae, cytoplasmic vesicles, Golgi complex, and rough endoplasmic reticulum.

The possible role of the ABC transporters in the pathogenesis and treatment of different brain disorders such as epilepsy or PD is increasingly recognized. For example, one-third of patients with epilepsy have drug-resistant epilepsy, which is associated with an increased risk of death and debilitating psychosocial consequences. A positive association between the polymorphism in the *MDR1* gene encoding P-gp (or ABCB1) and multidrug-resistant epilepsy has been reported in a subset of epilepsy patients (Siddiqui et al., 2003). However, the follow-up association genetics studies did not support a major role for this polymorphism, as recently reviewed (Tate and Sisodiya, 2007). Future association genetics studies are needed to understand better whether P-gp or some other members of the multidrug transporter gene family at the BBB are involved in multidrug-resistant epilepsy and other epilepsy phenotypes. Finally, as discussed below, it has been suggested that mutation of the *MDR1* gene may predispose carriers to damaging effects of pesticides and possibly other toxic xenobiotics transported by P-gp, leading to a PD phenotype (Droździk et al., 2003).

Peptide and Protein Transport

The peptide bond prevents dipeptides from using the L1 amino acid facilitative transport system (Zlokovic et al., 1983). However, the endothelial cells at the BBB express several transport systems for neuroactive peptides, such as arginine-vasopressin (AVP) (Zlokovic et al., 1990), enkephalins (Zlokovic et al., 1987, 1989), tyrosine melanocyte-stimulating inhibitory factor 1 (Tyr-MIF-1), delta-sleep inducing peptide (DSIP), luteinizing-hormone releasing hormone (LHRH), and some cytokines and chemokines (Zlokovic, 1995; Banks, 2006). Peptide transport system 1 (PTS-1) and 2 (PTS-2) at the abluminal membrane mediate efflux of enkephalins/Tyr-MIF-1 and AVP, respectively, from the brain to the blood (Banks, 2006) (Figure 3C). PTS-3, on the luminal membrane, transports peptide T into the brain. PTS-4 transports LHRH bidirectionally. The V1-vasporessinergic receptor is required for transport of AVP from blood to the brain (Zlokovic et al., 1990). Since enkephalins are transported in the liver by the OATP transporters, and since transport of endorphins is impaired in P-gp null mice, it would be of interest to determine the overlap between PTS-1 and OATP at the BBB, and the role of P-gp in the efflux of enkephalins across the BBB.

Large proteins, such as transferrin (Jefferies et al., 1984), low-density lipoproteins (LDL) (Meresse et al., 1989), leptin (Zlokovic et al., 2000a), immunoglobulin G (IgG) (Deane et al., 2005), insulin, and insulin-like growth factor (Pardridge, 2005) use receptor-mediated transport systems to cross the BBB. To date, the leptin BBB receptor (OBR) and the transferrin BBB receptor have been cloned (Pardridge, 2005) (Figure 3C). Receptor-mediated transport systems at the BBB have been used as targets for drug delivery to the brain via a strategy known as Trojan horses: different growth factors or anti-sense agents that normally do not cross the BBB, or immunoliposomes carrying naked DNA, can be conjugated to monoclonal antibodies against one of the BBB receptors (e.g., insulin and transferrin). The monoclonal antibodies act as surrogate ligands and can be used to carry conjugated neurotherapeutics across the BBB (Pardridge, 2007).

The rates of carrier-mediated or receptor-mediated transcytosis of peptides and proteins across the BBB are typically three orders of magnitude lower compared with large neutral AA (Zlokovic et al., 1985). It is of note that several neuroactive peptides and proteins are active in the brain at low concentrations. Thus, slow transport rates from blood to brain may act to limit the

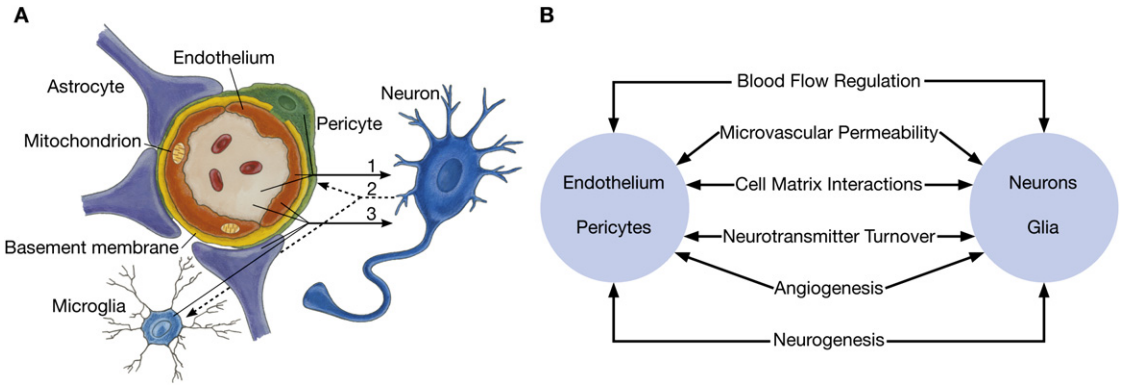

Figure 4. Schematic of the Neurovascular Unit

(A) Endothelial cells and pericytes are separated by the basement membrane. Pericyte processes sheathe most of the outer side of the basement membrane. At points of contact, pericytes communicate directly with endothelial cells through the synapse-like peg-socket contacts. Astrocytic endfoot processes unsheathe the microvessel wall, which is made up of endothelial cells and pericytes. Resting microglia have a "ramified" shape. In cases of neuronal disorders that have a primary vascular origin, circulating neurotoxins may cross the BBB to reach their neuronal targets, or proinflammatory signals from the vascular cells or reduced capillary blood flow may disrupt normal synaptic transmission and trigger neuronal injury (arrow 1). Microglia recruited from the blood or within the brain and the vessel wall can sense signals from neurons (arrow 2). Activated endothelium, microglia, and astrocytes signal back to neurons, which in most cases aggravates the neuronal injury (arrow 3). In the case of a primary neuronal disorder, signals from neurons are sent to the vascular cells and microglia (arrow 2), which activate the vasculo-glial unit and contributes to the progression of the disease (arrow 3).

(B) Coordinated regulation of normal neurovascular functions depends on the vascular cells (endothelium and pericytes), neurons, and astrocytes.

accumulation of neuropeptides in the brain ISF. Uptake of most circulating peptides by the brain can be compared with uptake of drugs such as acetaminophen, which exerts analgesic activity with an uptake of only 0.2%/g brain, or morphine, which has an uptake of <0.02%/g.

Caveolae

Raft-dependent endocytosis is cholesterol sensitive, clathrin independent internalization of ligands and receptors from the plasma membrane (Lajoie and Nabi, 2007). It encompasses endocytosis of caveolae, smooth plasmalemmal vesicles that form subdomains of cholesterol and sphingolipid-rich rafts that are enriched in caveolin-1. The caveolae control transcellular permeability by regulating endocytosis, transcytosis, and signaling in lipid-based microdomains of the BBB (Parton and Richards, 2003). The caveolar membranes contain receptors for transferrin, insulin, albumin, ceruloplasmin, RAGE, LDL, HDL, interleukin-1, and vesicle-associated membrane protein-2 (Wolburg, 2006) (Figure 3C). Signaling complexes at caveolin-1 include heterotrimeric G proteins, members of the MAPK pathway, *src* tyrosine kinase, protein kinase C, and the endothelial NO synthase. The involvement of caveolin-1 in NO and calcium signaling has been demonstrated in caveolin-1-deficient mice (Drab et al., 2001). VEGFR-2 (Labrecque et al., 2003) and P-gp (Jodoin et al., 2003) are also closely associated with caveolin-1. Caveolin-1 can also influence the levels of TJ proteins in BEC (Song et al., 2007a). The role of caveolae in BBB functions in health and disease remains to be explored.

Enzymatic BBB

Endothelial cells of the BBB provide a metabolic barrier by expressing a number of enzymes that modify endogenous and exogenous molecules, which otherwise could bypass the physical barrier and negatively affect neuronal function (Pardridge, 2005). The capillary endothelium, pericytes, and astrocytes express a variety of ectoenzymes on the plasma membranes, including aminopeptidases, endopeptidases, cholinesterase, and others.

Passive Transport by the Brain Fluids

Brain ISF-CSF "bulk flow" mediates transport of molecules into the CSF at a slow rate, irrespective of their size (Davson, 1976; Zlokovic, 2005). The CSF acts as a sink for potentially toxic molecules and metabolic waste products. Toxic molecules and metabolic waste products are removed from the CSF back into the circulation by active transport or facilitated diffusion across the choroid plexus epithelium, or by vacuolar transport across the epithelial arachnoid granulations.

Neurovascular Unit

Endothelium, the site of anatomical BBB, neurons, and non-neuronal cells (e.g., pericytes, astrocytes, and microglia) together form a functional unit, often referred to as a neurovascular unit (Figure 4A) (Lo et al., 2003; Iadecola, 2004; Hawkins and Davis, 2005; Zlokovic, 2005). The close proximity of different nonneuronal cell types with each other and with neurons allows for effective paracrine regulations that are critical for normal CNS functioning and disease processes (Boillée et al., 2006a; Deane and Zlokovic, 2007; Lok et al., 2007). These include regulation of hemodynamic neurovascular coupling, microvascular permeability, matrix interactions, neurotransmitter inactivation, neurotrophic coupling, and angiogenic and neurogenic coupling (Figure 4B).

Vascular versus Neuronal Origin of Brain Disorders

Brain disorders may have a vascular origin (Figure 4A, arrow 1). Vascular cells, i.e., endothelium and pericytes, can directly affect neuronal and synaptic functions through changes in the blood flow, the BBB permeability, nutrient supply, faulty clearance of toxic molecules, failure of enzymatic functions, the altered secretion of trophic factors and matrix molecules, abnormal expression of vascular receptors, or induction of ectoenzymes.

Examples include, but are not limited to, cerebrovascular disorder (stroke), vascular dementia, hypertension, diabetes, apolipoprotein ε4 (apoE4) genotype, hyperlipidemias, homocysteinemia, hypercoagulant blood profile, familial cerebrovascular forms of AD (e.g, Dutch, Flemish, and Iowa Aβ-precursor protein [APP] mutations) and vascular forms of late-onset AD (Iadecola, 2004; Zlokovic, 2005). In response to a vascular insult, signals from neurons and astrocytes (Figure 4A, arrow 2) recruit microglia, which, when activated, secrete several proinflammatory cytokines (Man et al., 2007) (Figure 4A, arrow 3). This further aggravates the neuronal injury and synaptic dysfunction. In the case of a primary neuronal disorder (Figure 4A, arrow 2), vasculo-glial activation follows (Figure 4A, arrow 3), and may critically modify progression of the disease (Boillée et al., 2006a; Lok et al., 2007).

Pericytes

Pericytes were originally discovered by Rouget in 1870 as perivascular cells adjacent to capillaries. They belong to the vascular smooth muscle cell (VSMC) lineage (Allt and Lawrenson, 2001). Pericytes share a common basement membrane with endothelial cells (Figure 4A), and have many cytoplasmic processes that encircle capillaries. There may be up to 90 processes with a width of 300 to 800 nm per 100 μm of capillary length, suggesting that pericytes might encircle 30% to 70% of the capillary wall. Pericyte-to-endothelia ratio in the brain is high, i.e., 1:3 compared with 1:100 in striated muscles. The coverage of BEC by pericytes varies considerably between different microvessel types (Allt and Lawrenson, 2001).

The location of pericytes on the microvessel appears to be functionally determined. The distance between pericytes and BEC is estimated to be only 20 nm. Through long cytoplasmic processes that extend along and encircle the endothelial tube, pericytes make focal contacts with BEC through specialized junctions (von Tell et al., 2006). At points of contact, communicating gap junctions, TJ and AJ have been identified (Allt and Lawrenson, 2001). Given such a close relationship with BEC, and the occurrence of synapse-like peg-socket contacts, pericytes are ideally suited to have many influences on brain microcirculation.

In the CNS, pericytes contribute to stability of microvessels and cover a major part of the abluminal endothelial surface (von Tell et al., 2006). In addition to providing mechanical stability, pericytes predominantly influence vessel stability by matrix deposition and by the releae and activation of signals that promote BEC differentiation and quiescence (Armulik et al., 2005). The molecular mechanisms by which pericytes mediate vascular stability are not understood. The literature is conflicting regarding whether pericytes protect vessels from regression.

Perivascular pericytes release a large number of growth factors and angiogenic molecules which regulate microvascular permeability, remodeling, and angiogenesis (Dore-Duffy and La Manna, 2007). Several ligand-receptor systems have been implicated in regulating vessel maturation and stability through interactions between pericytes and BEC (von Tell et al., 2006). These pathways involve transforming growth factor (TGF)-β and its receptor system, angiopoetins 1 and 2 and their receptor Tie2, platelet-derived growth factor (PDGF)-B and its receptor PDGF receptor beta (PDGF-β), and sphingosine-1-phosphate (S1P) and its receptor S1P1. For example, endothelial-specific ablation of PDGF-B in mice results in mutants that survive into adulthood and exhibit persistent pathological changes, including brain microhemorrhages, focal astrogliosis, and kidney glomerulus abnormalities (Bjarnegard et al., 2004).

Localization studies of pericytes and BEC during angiogenesis suggest that growing microvessels of the human telencephalon are formed by a pericyte-driven angiogenesis (Virgintino et al., 2007). BEC are preceded by and guided by migrating pericytes during organization of the growing vessel wall. In vitro studies with cultured pericytes and BEC have suggested that pericytes strengthen the BBB permeability and maintain the vascular integrity and maturation (Nakagawa et al., 2007).

Recent studies have shown that pericytes are contractile cells which regulate brain capillary blood flow through contraction and relaxation (Peppiatt et al., 2006). In this regard, pericytes may function similarly to VSMC in arterioles and small pial arteries in the brain, which regulate CBF responses (Chow et al., 2007).

It has been speculated that pericytes and VSMC have roles in the development of neuropathology in hypertension, diabetes, MS, CNS tumor formation, and AD (Wyss-Coray et al., 2000; Allt and Lawrenson, 2001; von Tell et al., 2006). Recently, it has been shown that cerebral VSMC in AD individuals have a hypercontractile phenotype that leads to arterial hypercontractility and aberrant responses to vasoactive stimuli (Chow et al., 2007). Still, much remains to be learned about the role of pericytes in chronic neurodegenerative disorders. This exciting field has just begun to open up.

Basement Membrane and Matrix

The basement membrane separates BEC from its neighboring cells, pericytes and astrocytes (Figure 4A). BEC, pericytes, and astrocytes cooperate to generate and maintain the basement membrane and the unique barrier properties of the BBB. The basement membrane is composed of different extracellular matrix (ECM) structural proteins (e.g., collagen and laminin). Matrix adhesion receptors are expressed in the vascular cells, neurons, and their supporting glial cells (i.e., microglia, oligodendroglia, and astrocyte end-feet) (del Zoppo et al., 2006). Cells within cerebral microvessels express the integrin and dystroglycan families of matrix adhesion receptors. The functional significance of these receptors is only now being explored.

Integrins play a key role in mediating endothelial signaling, cell migration, and brain capillary tube formation during angiogenesis (del Zoppo and Milner, 2006). Matrix adhesion receptors are essential for the maintenance of the integrity of the BBB. Modulation of these receptors contributes to alterations in the barrier in the disease state. Growth factors, such as VEGF, are bound to ECM proteins and can be activated in situ by MMPs (Zlokovic, 2006). In turn, this can regulate postischemic angiogenic and neurogenic repair responses (Zhao et al., 2006). Significant alterations in cellular adhesion receptors and their matrix ligands occur during focal cerebral ischemia, MS, EAE, certain tumors of the CNS, and arteriovenous malformations, which support their functional significance in the normal state.

Astrocytes

Astrocytes are positioned between neurons, pericytes, and capillary BEC, and communicate with these cells via their numerous "foot processes" (Figure 4A). Astrocyte-BEC interactions have a major role in regulating brain water and electrolyte metabolism

under normal and pathological conditions (Abbott et al., 2006). Astrocytes contribute to brain communication pathways by modulating synaptic transmission (Newman, 2003) and neuronal firing thresholds and plasticity (Nedergaard et al., 2003). In the subfornical organ, astrocytes act as a "salt sensor," and, using lactate as a signal, control local activity of neurons involved in neural, hormonal, and behavioral responses underlying sodium homeostasis (Shimizu et al., 2007).

VSMC in small arteries (Iadecola, 2004) and astrocytes contribute to neurovascular coupling, which synchronizes neuronal metabolic demands to local CBF regulation (Anderson and Nedergaard, 2003). In brain slices, astrocytes detect glutamate-dependent synaptic activity, which causes vasodilation by a mechanism that involves prostanoids (Zonta et al., 2003). In vivo, photolysis of caged Ca^{2+} in astrocytic endfeet in the somatosensory cortex of mice results in an increase in CBF (Takano et al., 2006). Abnormal astrocytic activity coupled to vascular instability has been observed in AD models (Takano et al., 2007).

Early studies with EM markers injected into the CSF demonstrated that astrocytes did not structurally contribute to the BBB (Brightman and Reese, 1969). However, the *src*-suppressed C-kinase substrate (SSeCKS) in astrocytes is responsible for the decreased expression of VEGF and increased release of the antipermeability factor angiopotein-1 (Lee et al., 2003). It has been suggested that SSeCKS overexpression can increase the expression of the TJ molecules and decrease paracellular permeability in endothelial cells, suggesting that astrocytes may regulate the microvascular permeability. However, conflicting data have been reported regarding possible roles of astrocytes in controlling BBB differentiation and permeability. More studies are needed to clarify these issues.

Microglia

Microglia were first described in 1932 by del Rio-Hortega as a distinct class of glial cells. Microglia play critical roles in innate and adaptive immune responses of the CNS. Microglia are derived from leptomeningeal mesenchymal cells, which enter the brain and transform into microglia (Bechmann et al., 2007). The process involves (1) passage across the postcapillary venules into the Virchow-Robin spaces, and (2) subsequent progression across the glia limitans into the neuropil. This second step involves perivascular antigen recognition and the induction of MMPs. Circulating monocytes provide another important source of microglia in the brain (Bechmann et al., 2005). The infiltration of blood-derived monocytic cells and their morphologic transformation into microglia in zones of acute, anterograde (Wallerian) axonal degeneration have been demonstrated.

In the absence of pathology, the "resting" microglia are cells with small bodies and long, thin processes. Brain pathology is associated with activation of microglia. Activated microglia lose the long extensions typical of the resting microglia, and show stubby processes. During activation, microglia transform from "ramified" to an "ameboid" form, and finally to a phagocytic form. This evolution is associated with changes in surface antigen expression and cytokine release.

Studies with rat bone marrow chimeras have demonstrated that a subset of endogenous CNS cells, commonly termed "perivascular microglial cells," is bone marrow derived (Hickey and Kimura, 1988). These perivascular cells are fully competent to present antigens to lymphocytes in an appropriately restricted manner. Trafficking signals that guide the transmigration of leukocytes into the brain, as well as leukocyte migratory routes, have been well defined (Man et al., 2007). The connection of microglia in the brain with circulating monocytes and bone marrow cells has changed our concept of the brain as an immune privileged site that separates central microglia from its peripheral precursor pool.

In addition to being involved in MS, mononuclear phagocytes from blood are also recruited in other neurodegenerative diseases, such as AD, via transport across the BBB. Chemokines in the brain can recruit immune cells from the blood or from within the brain (Britschgi and Wyss-Coray, 2007). Disrupting this line of communication exacerbates the disease process in a mouse model of AD, as shown in AD mice deficient in Ccr2, a chemokine receptor on microglia that normally mediates the accumulation of mononuclear phagocytes at sites of inflammation, but is linked to more rapid disease progression when absent (El Khoury et al., 2007).

Angiogenesis and Neurogenesis

The mechanisms involved in wiring the neural and vascular networks share many similarities (Carmeliet and Tessier-Lavigne, 2005). These include shared growth factors and receptors, similar signaling cues for new cell formation and migration, and shared physical space, as a result of parallel anatomic patterning and development. For example, VEGF and its receptor VEGR2 regulate axonal growth, neuronal survival, and new vessel formation (Greenberg and Jin, 2005). Neuropilin receptor (Nrp1) binds VEGF 164/5 on vascular cells and is a coreceptor for the axon guidance molecule semaphorin 3A. Fibroblast growth factor-2 (FGF-2), TGF-β, and PDGF are angiogenic factors that induce proliferation of neural precursors. The four major families of neuronal guidance cues, ephrins, semaphorins, slits, and netrins, direct patterning of the vascular system.

In the adult brain, there are several discrete foci of persistent angiogenesis (Greenberg and Jin, 2005). Angiogenesis is tightly coupled to neurogenesis in the adult mammalian brain (Palmer et al., 2000) and the avian brain (Louissaint et al., 2002). Gonadal steroid-mediated induction of VEGF in higher vocal control (HVC) nucleus in the adult avian brain occurs concurrently with VEGFR2 expression in HVC endothelial cells (Louissaint et al., 2002). This leads to sprouting angiogenesis and release of brain-derived nerve growth factor (BDNF) from steroid-stimulated HVC endothelium, which, in turn, recruits neurons in the adult HVC.

The major progenitor pools of the adult human brain include ventricular zone neuronal progenitor cells, hippocampal neuronal progenitors, and parenchymal glial progenitor cells (Goldman, 2007). Newly generated cells in the adult mouse hippocampus have neuronal morphology, membrane properties, action potentials, and functional synaptic inputs similar to those found in mature dentate granule cells (van Praag et al., 2002). New neurons, similar to mature granule neurons, form contacts by axosomatic, axodendritic, and axospinous synapses (Toni et al., 2007). New dendritic spines primarily synapse on multiple-synapse boutons, suggesting that initial contacts are preferentially made with preexisting boutons already involved in a synapse.

In sprouting angiogenesis, specialized endothelial tip cells lead the outgrowth of blood-vessel sprouts toward gradients of

VEGF (Gerhardt et al., 2003). Recent work has demonstrated that inhibition of Notch signaling with γ-secretase inhibitors or genetic inactivation of Notch ligand delta-like 4 (Dll4) signaling promotes increased numbers of tip cells, which control vessel sprouting in the mouse retina (Hellstrom et al., 2007). This work has suggested that modulators of Dll4 or Notch signaling, as for example γ-secretase inhibitors developed for AD, might be used as pharmacological regulators of angiogenesis.

The importance of angiogenesis and neurogenesis in brain remodeling after an acute ischemic insult is well recognized (Zhang et al., 2007). Yet the prevailing concept states that the aging brain affected by a chronic neurodegenerative process has relatively modest regenerative capability. Whether brain repair can be enhanced by new therapeutic approaches remains to be addressed by future studies.

Neurovascular Uncoupling in the Aging Brain

Brain varies its blood flow according to local tissue metabolic demands. An adequate blood supply is ensured by a tight coupling between neural activity and blood flow. The link between regional synaptic activity and regional CBF, termed functional hyperemia, is the basis for functional MRI, which has revolutionized our understanding of human brain in health and disease (Drake and Iadecola, 2007). Reductions in resting CBF or altered responses to brain activation may occur in different CNS regions in AD, PD, MS, and other CNS disorders (Lo et al., 2003; Iadecola, 2004; Drake and Iadecola, 2007; Lok et al., 2007).

Modest, 20% reductions in CBF, as seen in the aging brain, are associated with diminished cerebral protein synthesis (Hossmann, 1994). More severe regional reductions in CBF, as seen in chronic neurodegenerative disorders, lead to shifts in intracellular pH and water, and accumulation of glutamate and lactate in brain ISF (Drake and Iadecola, 2007). CBF reductions greater than 50% impair ATP synthesis and decrease the ability of neurons to fire action potentials. Finally, severe reductions in CBF (>80%), similar to those found in ischemic stroke, lead to electrolyte dysbalance and ischemic neuronal death. Changes in the brain capillary unit, degeneration of brain capillaries, reductions in resting CBF, or a combination thereof may be the first signs of the disease process prior to neuronal changes and neurodegeneration.

Alzheimer's Disease

AD is characterized by a progressive cognitive decline associated with neurovascular dysfunction (Iadecola, 2004; Zlokovic, 2005), accumulation of neurotoxic Aβ on blood vessels and in the brain parenchyma (Rovelet-Lecrux et al., 2006; Hardy, 2006; Deane and Zlokovic, 2007), and intraneuronal lesions, or neurofibrillar tangles (Lee et al., 1991; Santacruz et al., 2005; Tanzi, 2005). Aβ plays a central role in the development of AD pathology (Snyder et al., 2005; Tanzi, 2005; Hardy, 2006; Rovelet-Lecrux et al., 2006; Selkoe, 2001; Deane and Zlokovic, 2007; Haass and Selkoe, 2007). Brain Aβ is elevated in patients with sporadic AD and inherited FAD. Increased Aβ42 levels in the brain ISF result in the formation of neurotoxic Aβ oligomers (Haass and Selkoe, 2007). Neurovascular accumulation of Aβ and vascular deposition of amyloid result in the development of cerebral amyloid angiopathy (CAA) (Ghiso and Frangione, 2002; Greenberg et al., 2004).

Most AD cases (~99%) present with the late onset, i.e., in individuals over 65 years of age, without evidence of Mendelian genetic transmission (Tanzi and Bertram, 2005). Late-onset AD individuals typically do not have increased production of Aβ. According to current concepts, Aβ accumulates in the brain in AD likely due to its faulty clearance from the brain (Zlokovic et al., 2000b; Selkoe, 2001; Tanzi et al., 2004; Holtzman and Zlokovic, 2007). In mouse models of AD, including APP-overexpressing *APPsw*$^{+/-}$ mice and transgenic *APP* mice harboring vasculotropic Dutch and Iowa mutations, dense plaques develop initially on blood vessels or as a classical CAA (Deane et al., 2004a; Kumar-Singh et al., 2005). It is believed that plaques are generated on blood vessels due to deficient Aβ clearance across the BBB or along Virhoff-Robin arterial spaces in the brain.

Microvascular Pathology

Reduced microvascular density, an increased number of fragmented vessels with fewer intact branches, atrophic string vessels, increased irregularity of capillary surfaces, marked changes in the vessel diameter, capillary basement membrane thickening, and collagen accumulation in the basement membrane have been described in AD (Farkas and Luiten, 2001; Bailey et al., 2004).

BBB Influx of Aβ

RAGE is a major influx transporter for Aβ across the BBB (Deane et al., 2003). In AD and transgenic models of β-amyloidosis, RAGE expression increases in the affected cerebral vessels, microglia, and neurons (Yan et al., 1996; Deane et al., 2003; Donahue et al., 2006). RAGE binds to different forms of Aβ and mediates its pathophysiologic cellular responses. Under physiological conditions, RAGE is expressed at relatively low levels at the BBB, except at the endothelium of larger brain microvessels. However, the accumulation of RAGE ligands (e.g., AGE proteins and Aβ) in the aging brain increases cerebrovascular RAGE expression. It has been shown that Aβ/RAGE interaction at the luminal membrane of the BBB (Figure 5A) results in (1) transcytosis of circulating Aβ across the BBB into the brain parenchyma and its binding to neurons; (2) NF-κB-mediated endothelial activation resulting in secretion of proinflammatory cytokines (e.g., tumor necrosis factor-α and interleukin-6), the expression of adhesion molecules (e.g., ICAM-1 and VCAM); and (3) generation of endothelin-1, which suppresses CBF. These cellular events may be implicated in disease onset and progression in AD models, and possibly in AD.

Aβ/RAGE interaction contributes to the neuronal killing directly by producing oxidative damage to RAGE-expressing neurons, and indirectly, by activating microglia (Yan et al., 1996). Inhibition of Aβ/RAGE interaction in the affected vasculature inhibits cytokine production, oxidant stress, and Aβ BBB transport (Deane et al., 2003). Thus, RAGE is an important therapeutic target in AD. The inhibitors of Aβ/RAGE interaction have been shown to stabilize the BBB functions, reduce neuroinflammation, and improve the resting CBF and the CBF responses to brain activation. Some RAGE/Aβ blockers are currently being tested in AD patients.

Recent work has confirmed that blood is a major, chronic source of soluble Aβ peptides in the brain (Clifford et al., 2007). In rats, Aβ peptides cross a defective BBB by passive diffusion followed by selective binding to certain subtypes of neurons.

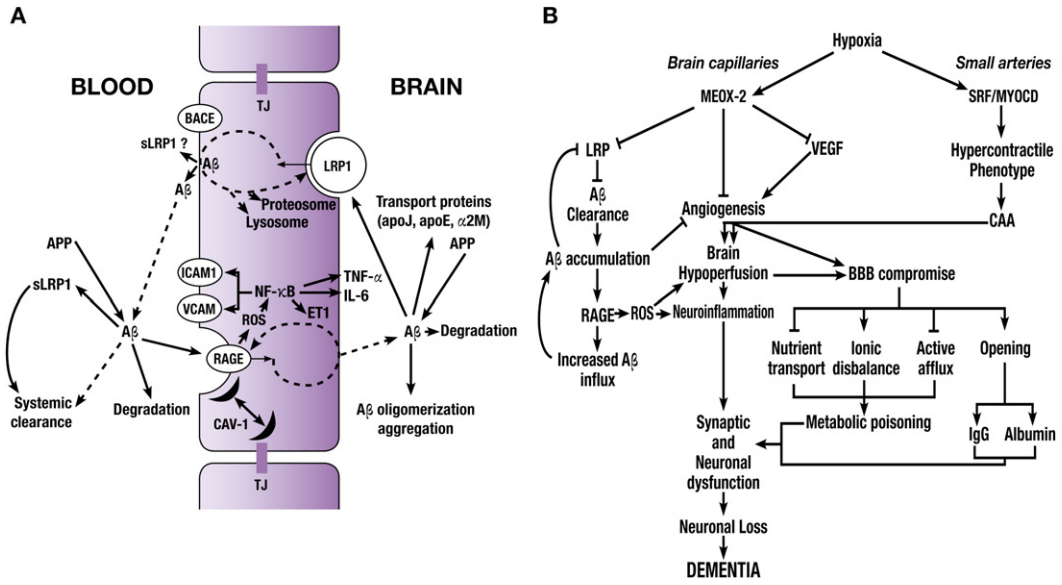

Figure 5. The Role of the BBB in the Pathogenesis of Alzheimer's Disease, or AD

(A) *Transport equilibrium for amyloid β-peptide, or Aβ.* The cell-surface LRP1 on the abluminal membrane binds different forms of Aβ (e.g., monomers, oligomers, and aggregates) and initiates Aβ transcytosis across the BBB followed by its export into the circulation. In the case of Aβ overload, LRP1 loses its normal protein conformation and undergoes the accelerated proteosomal degradation. Aβ efflux is influenced by its transport binding proteins in the brain, e.g., apoE and apoJ, or α2-macroglobulin. β-secretase (BACE) cleaves the N terminus extracellular domain of LRP1, which generates the soluble form of LRP1 (sLRP1). In human plasma, >70% of Aβ is normally bound to sLRP1. Native plasma sLRP1 is a major endogenenous peripheral "sink" agent for Aβ. The remaining Aβ in the plasma is bound to other Aβ transporting proteins (e.g., apoJ). A small fraction of plasma Aβ is free. On the luminal membrane, free Aβ that escapes the sLRP1 surveillance in the blood interacts with the receptor for advanced glycation end products (RAGE). Aβ/RAGE interaction mediates transport of Aβ from blood to brain, and activates the endothelium through reactive oxygen species (ROS)-induced nuclear translocation of NF-κB. This triggers secretion of proinflammatory cytokines (e.g., interleukin-6 [IL-6] and tumor necrosis factor-α [TNF-α]), the expression of adhesion molecules at the BBB (e.g., ICAM1 and VCAM), and secretion of endothelin-1, a suppressor of the blood flow.

(B) *The role of vascular genes.* In AD, low levels of expression of the mesenchyme homeobox gene 2 (*MEOX-2*) in the brain endothelium inhibit vascular endothelial growth factor (VEGF)-mediated angiogenesis, resulting in a premature apoptotic cell death, brain capillary regression, and reduced LRP1 expression. These events lead to both Aβ accumulation in the brain and brain hypoperfison. RAGE amplifies this pathogenic cascade. In small cerebral arteries, overexpression of serum response factor (SRF) and myocardin (MYOCD), the two transcription factors that orchestrate the vascular smooth muscle cell (VSMC) phenotype, leads to a hypercontractile VSMC phenotype overexpressing several SRF/MYOCD-regulated contractile proteins and the SRF-dependent genes that regulate Ca^{2+} homeostasis. These events reduce resting blood flow and suppress brain-activation-controlled blood flow responses. The BBB compromise and a neuroinflammatory response both aggravate synaptic and neuronal dysfunction, resulting in neuronal loss and dementia.

However, the increased CSF-to-plasma albumin ratios in AD patients with MRI-evidenced brain atrophy do not correlate with CSF-to-plasma Aβ40 and Aβ42 ratios (Matsumoto et al., 2007), suggesting that diffusion is not a key factor regulating Aβ in the CNS. Earlier studies in guinea pigs have demonstrated that plasma-derived Aβ40 and Aβ42 cross the intact BBB at slow rates by sharing a common transport system (Martel et al., 1996a). Subsequent work in AD mice has identified that RAGE is a shared influx transporter for Aβ peptides at the BBB (Deane et al., 2003). Therefore, specific influx and efflux transport mechanisms for Aβ play a key role in regulating brain Aβ (Deane and Zlokovic, 2007).

In addition to RAGE, apolipoprotein J (apoJ) can facilitate transport of plasma-derived Aβ across the BBB (Zlokovic, 1996). In contrast, circulating apoE2 and apoE3, but not apoE4, block transport of plasma Aβ into the brain (Martel et al., 1997). Transport of Aβ-apoJ complexes across the BBB is mediated via gp330/megalin or low-density lipoprotein receptor related protein 2 (LRP2) (Zlokovic et al., 1996). The role of LRP2 in transport of Aβ is still not completely understood because LRP2 is saturated by apoJ at physiological plasma levels (Shayo et al., 1997). In addition, apoJ is not a major transport protein for Aβ in human plasma (Sagare et al., 2007). Whether apoJ/LRP2-mediated transport of Aβ in the CNS has a role in disease progression and development of Aβ pathology in AD has not been explored.

BBB Clearance of Aβ

Low-density lipoprotein receptor related protein 1 (LRP1) is a major efflux transporter for Aβ across the BBB (Shibata et al., 2000). LRP1 is a member of the LDL receptor family and acts as a multifunctional scavenger and signaling receptor. Binding of Aβ to LRP1 at the abluminal side of the BBB initiates Aβ clearance from brain to blood via transcytosis across the BBB (Figure 5A) (Shibata et al., 2000; Deane et al., 2004a; Cirrito et al., 2005; Bell et al., 2007). In the liver, LRP1 mediates Aβ systemic clearance (Tamaki et al., 2006).

β-secretase cleaves the N terminus extracellular domain of LRP1 (von Arnim et al., 2005), which releases soluble LRP1 (sLRP1) in plasma. In humans, sLRP1 normally binds 70%–90% of Aβ in plasma (Sagare et al., 2007). It has been shown that binding of Aβ to sLRP1 is compromised in AD, which in turn may contribute to elevated Aβ levels in the brain. Recombinant LRP1 clusters, such as cluster IV (LRP-IV), can effectively sequester Aβ in AD plasma and *APPsw*$^{+/-}$ mice, resulting in Aβ efflux from the mouse brain (Sagare et al., 2007). Thus, LRP-IV

and other LRP1 fragments have a therapeutic potential as novel Aβ clearance agents or sLRP replacement therapy for AD with an enhanced peripheral "sink" action for Aβ.

Reduced expression of LRP1 has been reported during normal aging in rodents and nonhuman primates, and in AD individuals associated with positive staining of cerebral vessels for Aβ40 and Aβ42 (Shibata et al., 2000; Deane et al., 2004a; Donahue et al., 2006). Mice with severe functional deficiency in LRP1 at the BBB develop accumulations of Aβ when crossed with APP-overexpressing mice (Van Uden et al., 2002). However, the overexpression of LRP-IV minigene on neurons promotes Aβ retention in the CNS, suggesting that LRP1 on neurons mediates retention of Aβ in the brain (Zerbinatti et al., 2004). In contrast, LRP1 or another lipoprotein receptor on astrocytes mediates degradation of amyloid deposits via apoE (Koistinaho et al., 2004). Therefore, different cells of the vasculo-glial unit may act together to eliminate Aβ. Binding of Aβ to apoJ, apoE, and α2-macroglobulin critically alters Aβ clearance rates from the brain (Figure 5A) and can influence its vascular and parenchymal accumulation (Holtzman and Zlokovic, 2007; Bell et al., 2007).

Mice that lack P-gp at the BBB (knockouts for *mdr1a* and *mdr1b* genes) have reduced clearance of Aβ from the CNS and lower levels of LRP1 in brain capillaries (Cirrito et al., 2005). Crossing *mdr1a*/*mdr1b* null mice with APP-overexpressing mice accelerates accumulation of Aβ and amyloid deposition, raising a possibility that *mdr1a* and *mdr1b* genes may influence Aβ clearance either directly through P-gp or indirectly through LRP1.

In addition to receptor-mediated transport, free diffusion of Aβ via the ISF-CSF bulk flow contributes to Aβ removal from the CNS (Silverberg et al., 2003). The exact contribution of this pathway to overall Aβ clearance is not known. It has been estimated that the diffusional component of Aβ clearance might eliminate up to 10%–15% of Aβ in mice (Shibata et al., 2000).

Aberrant Angiogenesis

Recent findings suggest that degeneration of the BEC in AD and AD models may reflect an aberrant angiogenesis. AD BEC express extremely low levels of the mesenchyme homebox gene 2 (*MEOX-2*), a transcription factor which normally regulates vascular cell differentiation and remodeling, and whose expression in the adult brain is restricted to the vascular system. Low levels of MEOX-2 expression mediate abnormal angiogenic responses of AD BEC to VEGF and other angiogenic factors (Wu et al., 2005), resulting in premature vessel regression, reduced resting CBF, and improper formation of the BBB (Figure 5B). Low levels of *MEOX-2* promote proteasomal degradation of LRP1, which lowers the Aβ clearing capability at the BBB, which in turn potentially leads to Aβ accumulation on the blood vessels. It has been shown that Aβ accumulations on the outer membrane of the blood vessels are anti-angiogenic, and therefore might contribute to the observed reductions in the brain capillary density in AD models and AD (Paris et al., 2004a, 2004b). Aberrant angiogenesis may have an amyloidogenic effect in the brain due to compromised BBB clearance of Aβ (Deane et al., 2004b).

Arterial Component

Aβ is a potent vasoconstrictor in cerebral circulation (Thomas et al., 1996). In APP-expressing mice, impaired endothelium-dependent regulation of neocortical microcirculation (Iadecola et al., 1999) and reductions in functional hyperemia (Niwa et al., 2000) have been observed at an early stage. A mismatch between CBF, metabolism, and brain activity has been shown in sporadic AD (Smith et al., 1999; Bookheimer et al., 2000; Ruitenberg et al., 2005; Drake and Iadecola, 2007).

Pial and intracerebral arteries in AD are affected by CAA. The VSMC layer is often reduced, resulting in the rupture of the vessel wall and intracerebral bleeding (Ghiso and Frangione, 2002; Greenberg et al., 2004). Pathogenic levels of vasculotropic mutant forms of Aβ (e.g., Dutch, Iowa, Arctic, Flemish, and Italian) accelerate VSMC degeneration, contributing to hemorrhagic strokes, as in familial forms of AD.

Recent studies have demonstrated that the expression of two transcription factors that control VSMC cell differentiation, namely serum response factor (*SRF*) and myocradin (*MYOCD*), is increased in AD, resulting in a hypercontractile arterial phenotype, brain hypoperfusion, and diminished functional hyperemia (Chow et al., 2007) (Figure 5B). These events may contribute to hypoperfusion observed in AD brains.

Large cerebral arteries do not develop CAA. But in AD, they are frequently affected by atherosclerosis (Casserly and Topol, 2004; Beach et al., 2007). Atherosclerosis reduces brain perfusion and may precipitate a chronic ischemic condition in AD. The Rotterdam Scan Study demonstrated that silent brain infarcts detected with MRI are associated with dementia in elderly people (Vermeer et al., 2003). The nun study found that demented AD individuals with amyloid and tau pathology have numerous brain microinfarctions (Snowdon et al., 1997). It has been suggested that cerebrovascular disease and brain infarction may contribute to the severity of cognitive decline in AD (Song et al., 2007b; Sheng et al., 2007). However, the exact pathways by which atherosclerosis and arteriolosclerosis contribute to cognitive decline, and the relationship between vascular brain damage and white matter hyperintensities on MRI and cognitive decline, are still not completely understood (Chui et al., 2006).

The link between ischemia and increased Aβ production (Iadecola, 2004), on one hand, and the accumulation of hyperphosphorylated tau in cortical neurons and filament formation similar to that present in human neurodegenerative tauopathies and AD (Gordon-Krajcer et al., 2007; Wen et al., 2007), on the other hand, have been reported in rodent models of stroke. Thus, brain hypoperfusion may create, at least in animal models, AD-like pathological changes in the brain.

Vascular Factors

Vascular risk factors might be responsible for cognitive decline in the elderly according to several epidemiologic studies, including the largest population-based Rotterdam study (Hofman et al., 1997; Ruitenberg et al., 2005). A number of risk factors for AD and vascular dementia overlap, including old age, atherosclerosis, stroke, homocysteine, hypertension, hyperlipidemia, head injury, transient ischemic attacks, high serum viscosity, thrombogenic factors, cardiac disease, apoE4 (Iadecola, 2004; Zlokovic, 2005; de la Torre, 2006), and diabetes (Luchsinger et al., 2007). Brains of AD patients are typically hypoperfused and hypoxic compared with those of normal subjects. Notably, hypoxia downregulates MEOX-2 in the brain endothelium (Wu et al., 2005) and stabilizes the expression of MYOCD and SRF in the VSMC in small cerebral arteries (Chow et al., 2007). It is possible that hypoxia might be upstream of the gene expression changes

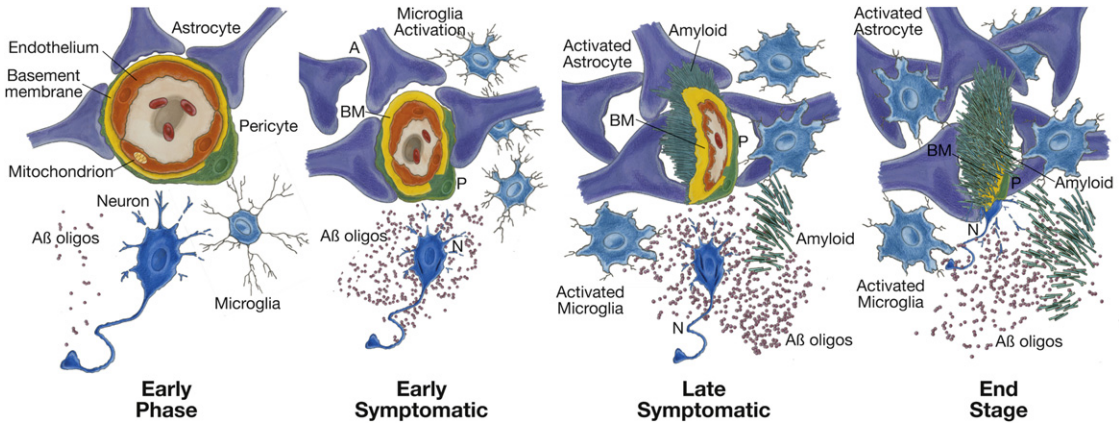

Figure 6. Schematic of the Involvement of the Neurovascular Unit in the Pathogenesis of AD

Early phase. Brain hypoperfusion and impaired BBB clearance of Aβ lead to accumulation of neurotoxic Aβ oligomers in the brain. Reduced blood flow and accumulated Aβ oligomers can both initiate the neuronal injury. *Early symptomatic.* More pronounced reductions in the blood flow, activation of the endothelium and pericytes, a loss of BBB Aβ clearance properties, an increasing accumulation of Aβ in the brain, and activation of microglia and astrocytes create a chronic problem for normal synaptic transmission and neuronal function. Neurofibrillary tangles may accumulate in neurons in response to both ischemic injury and Aβ. *Late symptomatic*. Degeneration of the endothelial cell wall and pericytes precludes clearance of Aβ and eliminates the blood flow from the capillary unit, resulting in accumulation of metabolic waste products, changes in the pH, and electrolyte unbalance. These chemical changes in the brain microenvironment present an insurmountable challenge for synaptic and neuronal function. Amyloid accumulates on the vessel wall. There is also a more prominent accumulation of the intraneuronal tangles. *End stage*. This stage is characterized by a profound neuroinflammatory response and a collapse of the capillary unit, accompanied with a loss of axonal-dendritic synapses and neurons, which disappear under the amyloid deposits.

in the vascular system that set in motion a disruption of the neurovascular unit in AD. More work is needed to clarify the exact relationship between hypoxia and the vascularly restricted genes in the pathogenesis of AD.

Neurovascular Disease Pathway

A number of models have been proposed to explain the disease pathway or pathways in AD. The limited scope of this review precludes me from comparing and discussing all existing models and theories of AD and including several important clinical observations linking cerebrovascular disease and AD, and cerebrovascular disease and cognitive decline. Instead, I will briefly summarize major findings that have been discussed in greater detail in earlier sections, and focus on the neurovascular cascade in AD.

According to the proposed model (Figure 5B), changes in the expression of key vascular genes and receptors in brain capillaries and small cerebral arteries may compromise (directly or indirectly) several BBB functions. This in turn leads to reductions in the resting CBF and attenuated CBF responses to brain activation, accumulation of Aβ, and a neuroinflammatory response, resulting in BBB breakdown. In an early phase, faulty clearance of Aβ at the BBB may favor accumulation of neurotoxic Aβ oligomers in the brain ISF. Aβ oligomers and focal reductions in the capillary blood flow can affect synaptic transmission, cause the neuronal injury, and initiate recruitment of microglia from the blood or within the brain (Figure 6A). At an early symptomatic stage, the BBB starts losing properties of an Aβ clearing membrane, and the activated endothelium secretes proinflammatory cytokines and CBF suppressors. This results in more pronounced synaptic dysfunction, accumulation of intraneuronal tangles, and activation of microglia. At a late symptomatic stage, the capillary unit is distorted with the degenerated endothelial barrier. There is a severe loss of Aβ clearing capability, resulting in amyloid formation on the outer side of the capillary membrane, an increased number of neurofibrillary tangles, and an increased number of activated microglia and astrocytes. At the final stage, the capillary unit disappears under the amyloid deposits contemporaneous with synaptic and neuronal loss.

Parkinson's Disease

PD is a chronic, progressive neurodegenerative movement disorder. Tremors, rigidity, slow movement (bradykinesia), poor balance, and difficulty walking (parkinsonian gait) are primary symptoms. PD results from the degeneration of dopamine-producing nerve cells in the brain, specifically in the substantia nigra and the locus coeruleus, although an initial locus in the dorsal motor nucleus of the vagus nerve (in the medulla) has also been suggested (Braak et al., 2006). When dopamine production is depleted, the motor system nerves are unable to control movement and coordination. PD individuals have typically lost over 80% of their dopamine-producing cells by the time symptoms appear.

BBB Transport

Dopamine restorative therapy with its precursor L-DOPA has provided symptomatic benefit to PD patients. The effectivenes of L-DOPA is a perfect example of how the BBB transport systems can be utilized to deliver neurotherapeutics. Namely, L-DOPA, but not dopamine, is transported across the BBB in humans via the L1 facilitative transporter (Hawkins et al., 2006). After transport across the BBB, L-DOPA is converted to dopamine, likely at the surviving dopaminergic terminals and at serotonergic and adrenergic nerve terminals that contain decarboxylase.

It has been suggested that absorption or metabolism of putative PD toxins, and their faulty elimination across the BBB, may play a role in the pathogenesis of PD. Low activity of P-gp efflux transporter at the BBB in the midbrain of individuals at risk for PD has been proposed as a mechanism mediating the retention of

putative PD toxins. The involvement of the BBB P-gp transporter in PD pathogenesis has been demonstrated by verapamil (a P-gp substrate) retention on PET scans of the midbrain, but not of other brain regions, in PD individuals (Kortekaas et al., 2005).

The importance of P-gp in the pathogenesis of PD has been also suggested by the *MDR1* gene polymorphism studies in Chinese populations (Lee et al., 2004). These studies have demonstrated that some polymorphisms in the *MDR1* gene may be protective and reduce the risk of PD. Other studies have suggested that mutation in the *MDR1* gene may predispose carriers to damaging effects of pesticides, leading to a PD phenotype (Drożdzik et al., 2003).

Studies with paraquat, an insecticide frequently used to produce parkinsonism in rodents, have demonstrated that its transport across the BBB can be blocked by competitive inhibition of the BBB L1 AA transporter (McCormack and Di Monte, 2003). This suggests that the L1 AA transporter may be involved in mediating the entry of parquat in the brain. Interestingly, some species, like guinea pigs, may exhibit selective regional transport of dopamine across the BBB in the caudate-putamen, but not in other brain regions (Martel et al., 1996b). This, however, has not been found in other species, including humans.

In a hemiparkinsonian rat model, L-DOPA therapy increases endothelial proliferation and its own transport into the basal ganglia, subventricular zone, and hippocampal dentate gyrus (Westin et al., 2006). It has been suggested that these changes correlate with the development of dyskinesia, a side effect of L-DOPA.

Circumventing the BBB

Glial-derived neurotrophic factor (GDNF) can regenerate damaged dopaminergic nerve terminals in animal models of PD, but does not cross the BBB. In a recent clinical trial, GDNF was administered directly into the putamen, which enhanced dopaminergic function in the immediate vicinity of the catheter tip, but generated no clinical improvement (Lang et al., 2006). In general, clinical trials with neurotrophic factors, gene therapy, or peptide neurotheraputics face a major challenge in how to circumvent the BBB (Pardridge, 2007).

Microvascular Changes

Early studies have demonstrated that melanin-containing neurons of the zona compacta have a very close spatial relationship with the blood supply (Issidorides, 1971). The neuronal vascular contact is so intimate that the capillary appears to have an almost intracellular position. In PD individuals, normal contacts between nigral neurons and capillaries are lost at an early disease stage, and capillaries do not maintain their normal shape. Capillary basement membrane thickening and collagen accumulation have also been shown in PD (Farkas et al., 2000).

Neuroinflammation

Neuroinflammation appears to be a ubiquitous finding in PD patients and experimental models of PD. Phagocyte activation, increased synthesis and release of proinflammatory cytokines, complement activation, activation of microglia, and release of reactive oxygen species (ROS) have been described (Whitton, 2007).

CBF Dysregulation

Orthostatic hypotension is one of the many autonomic disturbances observed in PD. Normally brain perfusion does not depend on systemic blood pressure, a mechanism known as cerebral autoregulation. Impaired autoregulation of brain perfusion, independent of dopaminergic treatment, has been demonstrated in PD patients subjected to a drop in blood pressure compared with controls (Vokatch et al., 2007).

Amyotrophic Lateral Sclerosis

ALS is a chronic neurodegenerative disorder of motor neurons in the brain, brainstem, and spinal cord that results in a progressive paralysis that kills individuals within 3 to 5 years of onset (Boillée et al., 2006a). About 10% of patients have a familial history, whereas 90% of cases are sporadic. Mutations in superoxide dismutase-1 (SOD1) are the most common form of inherited ALS, accounting for almost 25% of familial cases.

Neurovascular Unit

The current model of ALS disease suggests that toxicity derived from microglia and astrocytes contributes to disease progression and motor neuron degeneration (Boillée et al., 2006a, 2006b; Beers et al., 2006; Di Giorgio et al., 2007; Nagai et al., 2007).

Blood Vessels

The role of blood vessels in the pathogenesis of ALS is not clear. Human data suggest that two angiogenic factors, i.e., VEGF (Lambrechts et al., 2003) and angiogenin (Greenway et al., 2006), might have a role in ALS. Mice with a mutation that eliminates hypoxia-responsive induction of the *Vegf* gene ($Vegfa^{\delta/\delta}$) develop late-onset motor neuron degeneration (Oosthuyse et al., 2001). It has been reported that treatment of $SOD1^{G93A}$ rats with intracerebroventricular VEGF (Storkebaum et al., 2005), or of $SOD1^{G93A}$ mice with a VEGF-expressing lentiviral vector that is transported retrogradely to motor neurons (Azzouz et al., 2004), reduces pathology and extends survival.

BBB Breakdown

Earlier studies in ALS patients have suggested a possible blood-CSF barrier and BBB breakdown by demonstrating increased levels of albumin, IgG, and complement components in the CSF or in the spinal cord (Engelhardt and Appel, 1990; Meucci et al., 1993). More recent studies that focused on the axonal damage markers in the CSF (e.g., tau and neurofilaments) reported a mild increase in the CSF-to-serum albumin ratio in 28% of ALS patients (Brettschneider et al., 2006). Leakages into the spinal cord of serum plasma proteins (i.e., Ig) and deposition of red-blood-cell-derived neurotoxic products such as hemoglobin have been shown in $SOD1^{G93A}$ mutant mice at the onset of the disease (Z. Zhong and B.V. Zlokovic, unpublished data). Figure 7 illustrates the Zlokovic-Cleveland model for the possible role of BBB breakdown in the pathogenesis of ALS. Based on this model, BBB breakdown resulting in a leakage of serum proteins may generate edema with focal hypoxic conditions in the spinal cord tissue. In addition, leakage of Igs that interact with motor neuron antigens can produce ROS (Wentworth et al., 2000) and initiate an autoimmune response, which in turn can cause demyelination, disruption of neuronal transmission, and cell death. Hemoglobin released from extravasated red blood cells confers direct toxicity to neurons (Regan and Guo, 1998), which is associated with ROS production, lipid peroxidation, and neuronal cell death.

More studies are needed, however, to clarify when and how the BBB breakdown contributes to motor neuron injury in experimental models of ALS. For example, it is not clear whether BBB

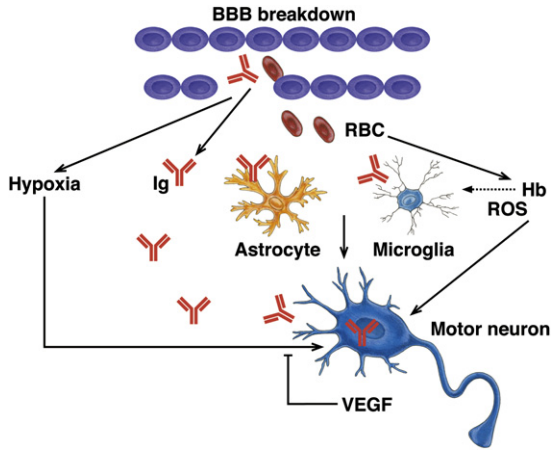

Figure 7. Schematic of the BBB Involvement in the Pathogenesis of Amyotrophic Lateral Sclerosis, or ALS: Zlokovic-Cleveland Model
Focal BBB breakdown with edema and serum protein leakage (e.g., albumin and Igs) results in focal tissue hypoxia. Red blood cell (RBC) extravasation results in release of neurotoxic hemoglobin (Hb)-derived products focally in the spinal cord tissue. Free Hb is directly toxic to motor neurons through generation of ROS. Focal Ig leakage may promote activation of microglia and astrocytes, contributing to nonautonomous cell death. Leakage of Ig that interacts with motor neuron antigens may exert direct toxic effects on motor neurons. VEGF promotes angiogenesis and protects neurons from hypoxic injury and toxicity resulting from Hb and Ig leakage.

breakdown precedes motor neuron degeneration and the inflammatory response. The future studies should also elucidate whether BBB breakdown is a common ramification of all SOD1 mutations with different biochemical characteristics.

Blood Flow

It has been shown that the spinal cord ischemia worsens motor neuron degeneration and functional outcome in *Vegfa*$^{\delta/\delta}$ mice, while similarly, the absence of hypoxic induction of VEGF in mice that develop motor neuron disease from expression of ALS-linked mutant SOD1^{G93A} yields substantially reduced survival (Lambrechts et al., 2003). Although these studies have suggested that hypoxia may play a role in inducing motor neuron disorder, the contribution of the spinal cord hypoperfusion to the development of ALS pathology still remains unclear.

Multiple Sclerosis

MS damages the myelin sheaths that surround and protect nerve cells in the brain and spinal cord. The symptoms of the disease may vary and can include visual disturbances, muscle weakness, trouble with coordination and balance, sensations such as numbness or prickling, and thinking and memory problems. MS begins between the ages of 20 and 40 and affects women more than men. Currently, there is no cure for MS, although some therapeutics may slow down the disease progression and help control symptoms.

The cause of MS is unknown. The current view is that MS is an autoimmune disease. Early studies have considered the CNS as an immunologically privileged site. However, today a body of evidence suggests that immune reactions take place in the CNS with distinctive features that are dictated in part by specific CNS anatomy; this includes the lack of endogenous antigen-presenting cells, lack of the lymphatic system, and the presence of the immunological BBB. As discussed below, the immunological BBB controls exchanges of immune cells and their mediators between blood and brain.

Leukocyte Entry into the CNS

Few leukocytes are normally present in the CNS. Most work on the trafficking of leukocytes in the CNS came from studies using disease or injury models. These studies revealed at least three distinctive routes for leukocyte entry into the CNS: (1) from blood to CSF across the choroid plexus; (2) from blood to subarachnoid space; and (3) from blood to parenchymal perivascular space (Ransohoff et al., 2003; Engelhardt and Ransohoff, 2005; Man et al., 2007). The blood-CSF route has physiological significance since the CSF of healthy individuals contains ~3,000 leukocytes per ml. In the CSF, T cells represent ~80% of leukocytes. In the second route, leukocytes extravasate across postcapillary venules at the pial surface of the brain into the subarachnoid space, and from there enter the Virchow-Robin perivascular spaces. The perivascular spaces are considered probable sites of lymphocytic interaction with antigen-presenting cells, and are important for immune surveillance. The activated T cell blasts can also extravasate across the postcapillary venules into the brain parenchyma (third route).

BBB Transport

The BBB mechanisms critically regulate immune responses of the CNS in conditions such as MS and experimental models of MS, such as EAE. During the course of EAE, autoaggressive CD4+ T lymphocytes are activated outside the CNS. They accumulate in the brain and CSF by crossing the BBB and the blood-CSF barrier (Ransohoff et al., 2003; Engelhardt and Ransohoff, 2005; Man et al., 2007). It has been proposed that CSF central-memory CD4+ T lymphocytes carry out routine immunosurveillance of the CNS by searching within the CSF-filled subarachnoid spaces for recall antigens presented by either subarachnoid space macrophages or pericytes.

In the EAE models, transport of different subsets of cytotoxic T lymphocytes from blood to brain is critical for lymphocytic infiltration of the CNS, which in turn results in neuronal killing. It is believed that Th17 lymphocytes, which secrete interleukin-17, may play an important role in neuronal killing (Stockinger and Veldhoen, 2007). But whether Th17 lymphocytes are activated from pre-Th17 subsets of T cells in peripheral organs and transported across the BBB, or whether Th1, Th0, or other pre-Th17 lymphocytes initially penetrate the BBB followed by maturation into Th17 cells in the CNS locally, is still a matter of debate. Recent studies suggested that Th17 cells can penetrate the BBB (Kebir et al., 2007). The BBB mechanisms that mediate transport of different subsets of T cells into the CNS represent potentially important therapeutic targets.

Figure 8 shows factors determining the transport of leukocytes across the cerebral endothelium in inflamed postcapillary venules on the pial surface. Leukocyte extravasation takes place in different steps that include rolling, activation, adhesion, and transmigration (Engelhardt and Ransohoff, 2005; Man et al., 2007). In a simplified scenario, the interaction of selectins and their ligands during the tethering/rolling phase, chemokines and G-protein coupled receptors during the activation phase, integrins and endothelial cell adhesion molecules (CAMs) during the adhesion phase, and chemokines, chemokine receptors,

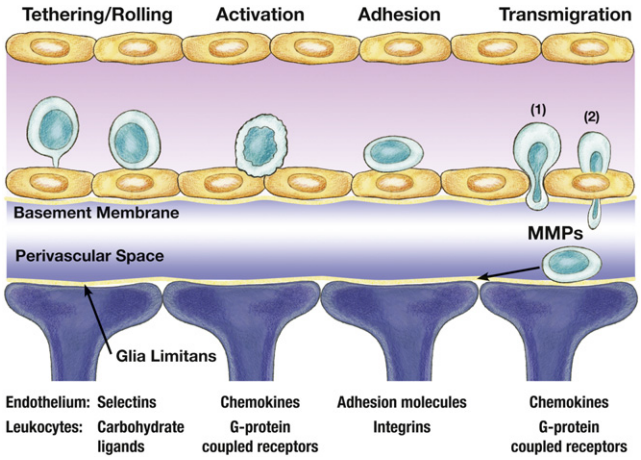

Figure 8. Schematic of the BBB Involvement in the Pathogenesis of Multiple Sclerosis, or MS

Leukocyte extravasation across the activated endothelium of the pericapillary venules on the pial surface of the brain involves four stages: tethering and rolling, activation, adhesion, and transmigration (or diapedesis). Leukocytes *tether* to endothelial cells through binding of selectins to their carbohydrate ligands. A simplified schematic on the bottom illustrates selectins only on endothelium, although selectins can also be expressed by leukocytes. *Rolling* requires interaction between chemokines expressed at the luminal membrane of the BBB with G protein-coupled chemokine receptors on leukocytes. Interaction between chemokine receptors on leukocytes with endothelial luminal chemokines initiates signals that lead to clustering and conformational changes of the cell surface integrins on leukocytes. *Adhesion* requires transformation of integrins on leukocytes into a form that can bind to their ligands, typically CAMs on the luminal side of the BBB, with high affinity. This high-affinity/high-avidity reaction between integrins and adhesion molecules mediates leukocyte arrest and adhesion. *Transmigration* happens after the arrest phase by paracellular transport across the endothelial junctions, transcellularly across the endothelial cells, or both. Leukocytes locomote on the endothelial surface until they identify the interendothelial junctions. Next, they extend their protrusions through the interendothelial junction and search for chemokines expressed on the abluminal side of the BBB. Chemokine-chemokine receptor interactions guide the extravasation of leukocytes. These interactions mediate the cytoskeletal changes in leukocytes, resulting in a change of their shape to allow transmigration. Transmigration follows chemotactic gradients. Extravasating cells cluster in the perivascular space, between the endothelial basement membrane and the basement membrane of the glia limitans, awaiting transfer into the brain extracellular space. This requires an additional transport step across the glia limitans mediated by matrix metalloproteinases (MMPs).

and MMPs during the transmigration phase (Figure 8) direct the entry of recently activated or chronic memory leukocytes into different CNS sites. The later stages of extravasation require interactions between G protein-coupled receptor on leukocytes (e.g., chemokine receptors) and an appropriate ligand (e.g., chemokines). Chemokines mediate the activation of integrins on leukocytes to achieve adhesion, i.e., a state of high-affinity binding with CAMs on the endothelium. A second set of signals through chemokines on the luminal and abluminal side of the BBB and G protein-coupled receptors on endothelium leads to cytoskeletal reorganization, which permits transmigration of leukocytes across the BBB. A number of TJ molecules participate, including JAM-A and ESAM. Leukocytes can extravasate by paracellular or transcellular route across the BBB.

New therapies based on blocking transport of central memory T cells, effector memory T cells, and activated monocytes with Natalizumab (therapeutic neutralizing monoclonal antibody to α4 integrin) across the BBB have been reported (Ransohoff, 2007). T cells express α4β1 integrins on the cell surface, and their transport across the BBB into the CSF and into the MS lesions was blocked by Natalizumab. Natalizumab binds and inactivates the integrin molecule on leukocytes. Its therapeutic effects include dramatic reductions of the BBB breakdown in recipients with active MS and lowering the counts of CSF leukocytes by over 60%, compared with subjects with no inflammatory diseases.

AIDS Dementia

The AIDS Dementia Complex (ADC) is the most common and clinically important CNS complication of late HIV-1 infection. It is a source of great morbidity and, when severe, is associated with limited survival. While its pathogenesis remains unclear, ADC is generally believed to be caused by HIV-1 itself, rather than by another opportunistic infection.

BBB-regulated cellular trafficking is critical for the development of CNS pathology caused by HIV-1, as well as other neuroinflammatory conditions including meningitis and encephalitis. HIV-1 infiltrates the CNS largely via infected monocytes and macrophages. According to the current concept, HIV-1 infiltration is mediated by its envelope glycoprotein, gp120, which activates protein kinase C isoforms in BEC to alter the BBB permeability and allow monocyte migration (Kanmogne et al., 2007). The involvement of MMPs during HIV-1 infiltration, as well as in other disorders of the CNS such as MS and cerebral ischemia, as discussed above, and migraine and brain tumors, is well established. Transport of peripheral monocytes carrying the HIV-1 virus across the BBB into the CNS precedes development of AIDS dementia. Better understanding of the BBB molecular mechanisms is not only important for understanding the pathogenesis of the disease, but will likely lead to the discovery of new therapeutic targets and agents to control ADC.

Future Challenges

As discussed in earlier sections, a growing body of evidence suggests that neurovascular mechanisms and disruption of the BBB may precede, accelerate, or contribute to chronic disease processes in neurodegenerative disorders of the adult and aging nervous system. The examples include, but are not limited to (1) faulty BBB clearance of potential brain toxins in AD and PD; (2) inefficient clearance of excitotoxins across the BBB after an ischemic insult or traumatic brain injury; (3) increased transport of leukocytes across the activated BBB in MS, AIDS dementia, and AD, and during neuroinflammatory CNS responses, and (4) BBB breakdown in ALS, AD, epilepsy and MS.

Numerous challenges remain. At the BBB level, a number of carrier-mediated transporters, such as those for neuropeptides, choline, thyroid hormones, vitamins, and nucleobases, remain to be cloned. Many active efflux transporters and receptor-mediated transporters have not been cloned. The transport mechanisms that regulate levels of different proteinacious aggregates inside the brain, such Aβ, need to be refined in greater detail, and for others, e.g., α-synucleins, huntingtin, SOD1, they remain to be discovered. The role of the TJ proteins in the pathogenesis of neurodegenerative disorders remains to be explored as well.

Developing new genomic and proteomic discovery platforms will enable us to identify new BBB transporters and junctional

barrier proteins. Exploring polymorphisms in these transporters and the junctional interendothelial proteins to look for genetic susceptibility to neurodegenerative disorders is an important priority. If BBB transporters, receptors, and interendothelial proteins contribute to susceptibility or progression of neurodegeneration, then manipulation of such transport systems and barrier function may offer neuroprotective and treatment strategies. New BBB transporters and junctional proteins could also be potentially utilized as portals of entry for cerebral drug targeting systems.

The transport mechanisms of the BBB have not been fully characterized in humans. Better knowledge of human BBB transport systems is essential for translating findings from animal disease models to humans. Progress in neuroimaging should allow us to measure in vivo barrier activity and function of key human BBB transporters. Construction of a human BBB molecular atlas could be a major advancement toward understanding the role of the BBB in health, disease, and drug delivery to the brain.

Challenges for the future include understanding the crosstalk between nonneuronal cell types (e.g., glia and microglia), cells of the vessel wall (e.g., endothelium and pericytes), and neurons (with each other), and possibly between neurons and peripheral hematopoetic cells and vascular niches and neurogenic loci in the brain. Identifying how these cells respond to, process, or synthesize different receptors, and identifying the ligands that mediate their interactions, is critical to understanding how these same cells regulate the neuronal milieu.

Experiments that selectively examine leukocyte trafficking into meningeal, parenchymal, and ventricular sites should further clarify immunological specialization of the CNS. Investigation of the molecular mechanisms of transmigration of different leukocyte subpopulations across the BBB is critical for developing specific strategies to control the neuroinflammatory response.

An overall goal of future research in drug targeting is to expand the CNS drug space from lipid-soluble small molecules to the much larger space of neurotherapeutics that includes molecules that do not normally cross the BBB. For example, future studies should continue to explore the translational potential of approaches that are currently in different stages of preclinical development, including (1) the molecular Trojan horses that use either monoclonal antibodies against the BBB receptors (e.g., insulin and transferrin) to ferry across the BBB an attached drug, protein, antisense agent, or nonviral plasmid DNA, or pegylated immunoliposomes to deliver short hairpin RNA to control neurotransmitter levels or growth factor activities; (2) delivery of pharmaceuticals encapsulated into nanoparticles conjugated to ligands for BBB receptors (such as LDL apoproteins) to carry drugs across the BBB via the BBB LDL receptor; and (3) delivery via the BBB receptors that are particularly expressed in the disease state, as for example RAGE in AD, diabetic vasculopathy, or stroke.

With the present exciting developments and new future directions, continued BBB research will change the face of neuromedicine in the decades to come, with hundreds of millions of people worldwide predicted to benefit from our better understanding of the role of the BBB in neurodegenerative disorders and its potential role in new diagnostic and therapeutic approaches.

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Dr. Zlokovic is the scientific founder of Socratech L.L.C., a start-up biotechnology company with a mission to develop new therapeutic approaches for stroke and Alzheimer's disease.

This review is dedicated to my teacher and friend Hugh Davson, a father of the modern BBB concept, who died in 1996.

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