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Neuronal and Vascular Interactions

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Abstract

The brain, which represents 2% of body mass but consumes 20% of body energy at rest, has a limited capacity to store energy and is therefore highly dependent on oxygen and glucose supply from the blood stream. Normal functioning of neural circuits thus relies on adequate matching between metabolic needs and blood supply. Moreover, not only does the brain need to be densely vascularized, it also requires a tightly controlled environment free of toxins and pathogens to provide the proper chemical composition for synaptic transmission and neuronal function. In this review, we focus on three major factors that ensure optimal brain perfusion and function: the patterning of vascular networks to efficiently deliver blood and nutrients, the function of the blood–brain barrier to maintain brain homeostasis, and the regulation of cerebral blood flow to adequately couple energy supply to neural function.

Contents

1. VASCULAR PATTERNING IN THE NERVOUS SYSTEM

1.1. Control of Vascular Patterning by Genetic Programs During Development

Normal brain function relies heavily on adequate matching between metabolic needs of neural cells and blood supply (Attwell & Laughlin 2001, Peters et al. 2004). Nerves, in turn, control blood vessel tone as well as heart rate. The functional interdependence between the nervous and vascular systems is reflected in their close anatomical apposition throughout the organism.

1.1.1. Vascular patterning and neural wiring by common guidance cues and receptors. In the periphery, nerves and vessels often run in parallel, a phenomenon called neurovascular congruency (Bates et al. 2003, Lewis 1902, Martin & Lewis 1989). In the central nervous system (CNS), neural and vascular cells form a functionally integrated network, whereby neural activity and vascular dynamics are tightly coupled (Iadecola 2004), as discussed in Section 3 of this review. Moreover, both the nervous and vascular systems comprise highly branched and complex networks. The patterning of these networks is initiated during development in a highly stereotyped fashion that is controlled by genetic programs (Carmeliet & Tessier-Lavigne 2005). However, both networks exhibit a certain degree of plasticity and undergo dynamic remodeling postnatally.

Compared to the relatively well-understood genetic programs and principles governing axon guidance and pathfinding (Huber et al. 2003, O'Donnell et al. 2009, Tessier-Lavigne & Goodman 1996), mechanisms underlying the elaboration of vascular networks remained mysterious until recent years. Hypoxia and hypoxia-induced vascular endothelial growth factor (VEGF) signaling are widely accepted as the main driving forces for vascular patterning during embryonic development (James et al. 2009, Stone et al. 1995). Whether intrinsic genetic programs are also needed and exist to control vascular patterning was not clear until a decade ago. Indeed, work from several studies showed that genetically engineered animals lacking traditional axon guidance cues and receptors display vascular patterning defects (Gitler et al. 2004, Gu et al. 2005, Lu et al. 2004).

Vascular-specific ablation of these guidance molecules recapitulates these defects, indicating that common cues are shared for wiring both the nervous and vascular systems (Adams & Eichmann 2010, Carmeliet & Tessier-Lavigne 2005). This molecular understanding of neural and vascular network patterning correlates with the structural and functional similarities between neuronal and vascular sprouts (growth cones and vascular tip cells, respectively), structures that allow neurons and vessels to sense and respond to their environments. Guidance receptors, typically expressed by neuronal growth cones and endothelial tip cells, initiate signaling upon binding to their correspondent environmental cues and control axon guidance and endothelial cell migration via regulation of cytoskeleton dynamics. Although the specific molecules used within neurons and endothelial cells are often different, recent evidence suggests that similar intracellular signaling principles underlying cytoskeletal regulation are used to control both neural and vascular guidance (Gelfand et al. 2009). The identification of traditional axon guidance cues and receptors as a new class of molecules controlling vascular patterning provides a new understanding of vascular network formation. Additionally, the realization that common guidance molecules are used to sculpt both neuronal and vascular networks provides conceptual insight into the coordinated development of both systems, a topic that has been widely reviewed previously (Adams & Eichmann 2010, Carmeliet & Tessier-Lavigne 2005, Melani & Weinstein 2010).

1.1.2. What are the basic principles underlying the establishment of neurovascular congruency? The existence of neurovascular congruency is widespread, but so far the most studied example is within the vertebrate forelimb. During development in mice with genetic mutations resulting in misguided axons, arterial branches follow misrouted axons in forelimb skin, demonstrating that peripheral sensory nerves determine the pattern of arterial differentiation and blood vessel branching (Mukouyama et al. 2002, 2005). These studies suggest that neurovascular congruency can be established by a one-patterns-the-other model, in which either the nervous or vascular system precedes in development and then instructs the second system to form, using an already established architecture as a template. Consistent with this model, evidence also suggests that vessels can express signals that attract axons. For example, artemin is expressed in smooth muscle cells surrounding vessels and attracts sympathetic axon fibers (Honma et al. 2002). Similarly, vascular endothelins direct the extension of sympathetic axons from the superior cervical ganglion toward the external carotid artery (Makita et al. 2008).

Whether a one-patterns-the-other model serves as a general mechanism to govern the establishment of neurovascular congruency in all tissues had been questionable until a recent study pointed to a different mechanism. Oh & Gu (2013) found that in the mouse whisker pad, at the root of whiskers, nerves and vessels form a stereotypic double-ring structure around each follicle, with an inner nerve ring forming first, followed by an exterior vessel ring. A one-patterns-the-other model would predict that the nerve rings attract surrounding blood vessels to establish the double-ring structure. However, in mutant mice lacking trigeminal neurons and therefore lacking nerve rings, vessel rings form normally. Likewise, in mice with deformed vessel rings, nerve rings form normally, demonstrating that the neurovascular congruency in the whisker pad occurs via an independent patterning mechanism. In this particular case, nerves and vessels respond to a common guidance cue emanating from the center of each whisker follicle, with a differential response determining their inner versus outer final position. This conclusion highlights previous findings that common guidance cues are used to forge networks of both the nervous and vascular systems and contribute to their congruent patterning.

What is the logic for having two different principles establishing neurovascular congruency (**Figure 1**)? During pathfinding toward a target, or when a target tissue has a planar structure, a one-patterns-the-other model allows for parallel nerve and vessel trajectories, independent of

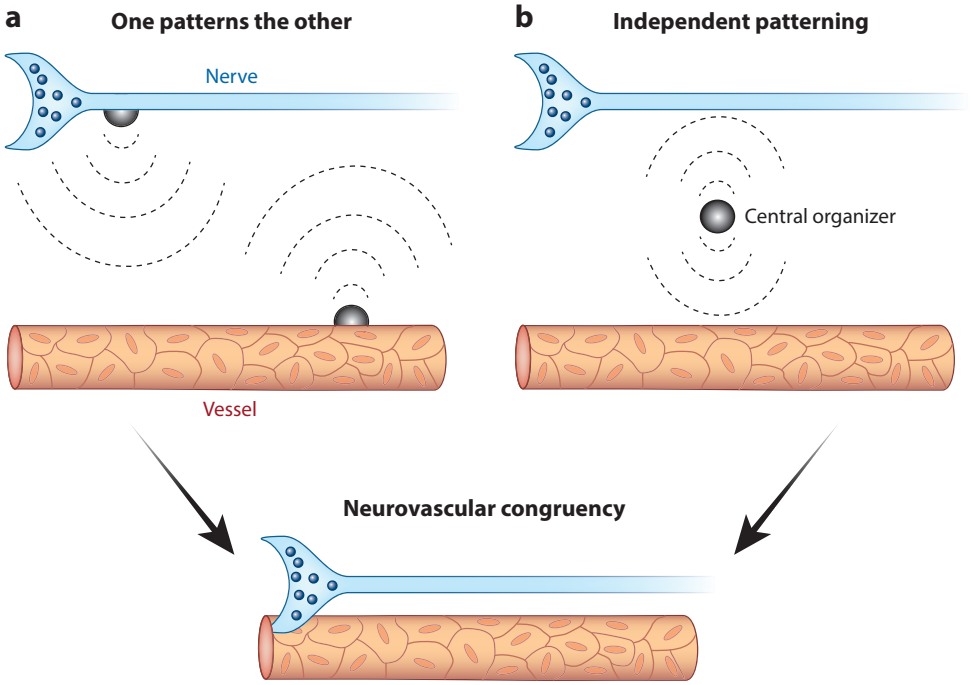


Figure 1

Two models of neurovascular congruency. (*a*) One-patterns-the-other model, in which either the nervous or vascular system precedes developmentally and then instructs the other system to form, using its established architecture as a template. This model allows parallel trajectories of nerves and vessels to develop, independent of their position relative to their surroundings. (*b*) Independent patterning model, in which balanced attractive and repulsive signals originate from a central organizer within a target tissue. This central organizer acts to pattern neurovascular congruency. When nerves and vessels reach a target tissue with a complex, three-dimensional structure, the precise architecture of nerves, vessels, and the target tissue becomes functionally relevant, as dictated by the unique requirements of the target tissue environment.

their position relative to their surroundings. However, in complex, three-dimensional target tissue structures, the relative orientation of nerves, vessels, and target tissues becomes functionally relevant. The independent or coordinate patterning model enables the target tissue to act as a central organizer to coordinate the development of multiple tissue subcomponents. Given the diversity of neurovascular structures in different tissues, local signals provided by a central organizer may be a critical mechanism used to establish neurovascular congruency. As was the case in studies of the mouse whisker pad, genetic approaches used to selectively manipulate one system at a time could be a powerful approach to probe the neurovascular interactions in the CNS.

1.1.3. Cerebrovascular patterning during development. The physical relationships between neuronal and vascular networks in the brain are much more complex than the often congruent (parallel) structure observed in the periphery. The developing brain is vascularized via ingression of blood vessels from the outside. At embryonic day 10 in mice, new capillaries sprout from perineural vessels and invade the neuroectoderm. Relative hypoxia in the growing brain is a major driving force for the ingression and refinement of the complex vascular bed that serves it. Other angiogenic signaling pathways have also been shown to play important roles in shaping cerebrovascular networks, including VEGF, Notch, Wnt/β-catenin, semaphorins/neuropilins/plexins, bone

morphogenetic proteins, orphan G protein–coupled adhesion receptor GPCR124, transforming growth factor-β, and Nogo-A (Ruhrberg & Bautch 2013, Wittko-Schneider et al. 2014). The neural environment plays a role in the initial ingression, elaboration, pruning, and stabilization of blood vessels. Multiple cell types including microglia, pericytes, neuroepithelial radial glia, neuroblasts, and astrocytes are associated with blood vessels and influence their density and branching patterns (Arnold & Betsholtz 2013; Lee & McCarty 2014; Ma et al. 2012, 2013). Although the patterning of large brain arteries (external and surface arteries) is stereotyped, the question still remains whether smaller arteries, penetrating arterioles, and capillaries also exhibit stereotyped patterns.

1.2. Control of Cerebrovascular Patterning by Neural Activity

Although the vascular network is initiated early during embryogenesis, expansion of vascular networks continues postnatally, and vascular remodeling occurs in both physiological and pathological conditions. Whether neural activity influences the formation of cerebrovascular networks remains controversial. Greenough and colleagues first postulated that during postnatal development, the brain adapts to increased metabolic demands by creating new vessels (Black et al. 1987, 1990, 1991). These milestone studies introduced the concept of vascular remodeling during maturation of the brain, but the neuronal contribution to vascular patterning after birth remains elusive.

1.2.1. The role of neural activity in cerebrovascular patterning during postnatal development and in pathological conditions. From studies in the rat cerebral cortex, the once prevailing view held that requirements of neural tissues (e.g., synaptogenesis, neuropil expansion) influence the maturation of underlying capillary networks (Black et al. 1987, Sirevaag et al. 1988) and that high metabolic activity correlates with higher vascular density (Riddle et al. 1993). Moreover, several studies proposed the existence of anatomical matching relationships between neuronal and vascular modules within cortical columns in the rat somatosensory cortex (Cox et al. 1993, Patel 1983). Such anatomical matching suggests that neuronal and vascular modules may instruct each other to build a precisely wired network for optimized local interactions, similar to the neurovascular congruency observed in the periphery. However, researchers later demonstrated that cortical microvascular domains do not display any obvious topological matching relationship with underlying neuronal columns (Woolsey et al. 1996). Consistent with this observation, recent studies using novel imaging and computational techniques, with three-dimensional reconstructions of cerebrovascular networks, further demonstrated that microvascular topology does not match neuroarchitecture in the mouse cerebral cortex (Blinder et al. 2013, Lacoste et al. 2014, Tsai et al. 2009). Therefore, neuronal structure is not necessarily involved in vascular patterning, but rather, neural activity may play a role.

Studies postulating that sensory stimulation positively influenced brain angiogenesis first introduced the concept of activity-induced vascular plasticity during postnatal development (Argandona & Lafuente 1996, 2000; Black et al. 1987; Sirevaag et al. 1988). Thus, after birth, sensory-related neural activity may refine cerebrovascular networks into their mature form, much like it does for neuronal circuits (Katz & Shatz 1996, Zhang & Poo 2001). By simultaneously visualizing neuronal and vascular modules, investigators recently examined the direct effect of sensory neural activity on cerebrovascular development during a critical postnatal period under physiological conditions. Lacoste et al. (2014) found that vascular density and branching, as well as endothelial cell proliferation, were decreased in layer IV of the primary somatosensory cortex when sensory input was reduced by either a complete deafferentation, a genetic impairment of neurotransmitter release at thalamocortical synapses, or a selective reduction of sensory-related neural activity. In contrast, increased sensory stimulation resulted in a vascular network with greater vessel density

and branching. These findings suggest that, in addition to angiogenic programs that regulate the initial vascular patterning, sensory-related neural activity appears necessary for cerebrovascular refinement during early postnatal development, with changes in neural activity being sufficient to trigger changes in vascular networks.

Brain vascular structure may be regulated differently under pathological conditions in which neural activity is affected. Investigators recently showed that excessive neural activity following hyperactivation of sensorimotor systems impairs cerebrovascular network formation during a critical postnatal window. Whiteus et al. (2014) found a severe reduction of angiogenesis in the cerebral cortex following either vigorous locomotor exercise, persistent auditory stimulation, or chemically induced seizures. In the adult rat brain, however, previous studies using similar hyperactivation paradigms evidenced increased angiogenesis in the cerebellum following intense locomotor exercise (Isaacs et al. 1992) or in the hippocampus after electroconvulsive seizures (Newton et al. 2006), emphasizing the difference between the immature and mature brain in terms of vascular plasticity. Importantly, this angiogenic capability of the adult brain might be relevant in ischemic conditions such as stroke. Researchers have shown that angiogenesis is increased in the penumbra of the ischemic adult mouse barrel cortex following enhancement of sensory-related neural activity by whisker stimulation (Whitaker et al. 2007), an effect that involves VEGF/VEGFR2 signaling (Li et al. 2011) and that can be amplified by inhibition of de novo cholesterol synthesis by statins (Zhang et al. 2012).

1.2.2. How does neural activity control cerebrovascular patterning? The question remains whether neural activity affects angiogenesis directly via neurotransmitter release, growth factor release, or both—for instance, by thalamocortical axons—or indirectly via pathways that are activated following neural activation, which involve cortical interneurons and glial cells. Pyramidal (excitatory) neurons, inhibitory interneurons, and astrocytes are recruited by somatosensory inputs (Lecrux et al. 2011) and in turn release vasoactive mediators that control vascular tone and cerebral blood flow (CBF) (Cauli & Hamel 2010, Drake & Iadecola 2007). Researchers have yet to resolve whether these neural modules also release angiogenesis regulators upon neural activity changes. Among many possibilities, astrocytes might be involved. Astrocytes are in close contact with both neuronal synapses and cerebral microvessels and are thus well positioned to couple neural activity to vascular growth. Indeed, in addition to their role in the control of CBF (Attwell et al. 2010, Iadecola & Nedergaard 2007, Lind et al. 2013), astrocytes respond to glutamate by releasing proangiogenic lipids (epoxyeicosatrienoic acids) as potent as VEGF (Munzenmaier & Harder 2000, Potente et al. 2003, Pozzi et al. 2005, Zhang & Harder 2002). Moreover, Ma et al. (2012) recently demonstrated that astrocytes are essential for the normal postnatal development of cortical vasculature. Future in vivo studies should investigate the precise mechanisms through which neural activity controls the release of astroglial angiogenesis modulators and their effects on cerebrovascular patterning. Finally, as we discuss in Section 3, local hypoxia may trigger activity-induced angiogenesis.

2. THE BLOOD–BRAIN BARRIER

2.1. Historical Perspectives: Location and Development of the Blood–Brain Barrier

The blood–brain barrier (BBB) provides one of the best examples of how the neuronal-vascular interface functions to ensure a homeostatic environment for proper brain function. As opposed to the periphery, in which a fenestrated endothelium allows for the rapid transport of solutes and fluids to and from the blood (Aird 2007), the CNS requires a tightly controlled environment

free of various toxins and pathogens to provide the proper chemical composition for synaptic transmission. This environment is maintained by the BBB, which is characterized by a thin layer of continuous, nonfenestrated endothelial cells that line the walls of CNS blood vessels. This endothelial cell layer serves as the physiological barrier that seals the CNS and controls substance influx and efflux (Armulik et al. 2010, Bell et al. 2010, Daneman et al. 2010b). Additionally, astrocytes and pericytes provide functional support for the BBB and, together with endothelial cells, are referred to as the neurovascular unit (**Figure 2**) (Siegenthaler et al. 2013).

Historically, three seminal lines of investigation established the existence of a barrier between the blood and brain. First, scientists observed over a century ago that systemic injection of water-soluble trypan blue dye resulted in the staining of several tissues, notably excluding the brain (Ehrlich 1885). Second, the advent of electron microscopy (EM) allowed Reese & Karnovsky (1967) to identify CNS endothelial cells as the cell type that possesses barrier properties. When they injected horseradish peroxidase (HRP) into the circulation as a subcellular tracer, they observed no extravasation from vessel lumen to brain parenchyma (Reese & Karnovsky 1967). Furthermore, pinocytotic vesicles were not present to transport the tracer across cerebral endothelial cells, and the tracer movement between cells was blocked by tight junctions. When HRP was injected directly into the brain, it diffused past astrocytic endfeet and the basement membrane but was again blocked at tight junctions between endothelial cells, indicating that these cells are the site of the BBB (Brightman & Reese 1969). Third, the uniqueness of the BBB to the CNS was investigated years later through the generation of quail-chick transplantation chimeras (Stewart & Wiley 1981). In these experiments, nonvascularized embryonic mesoderm engrafted into the brain, but not vice versa, developed vasculature with barrier properties, demonstrating an inductive role for the CNS microenvironment in the development of the BBB.

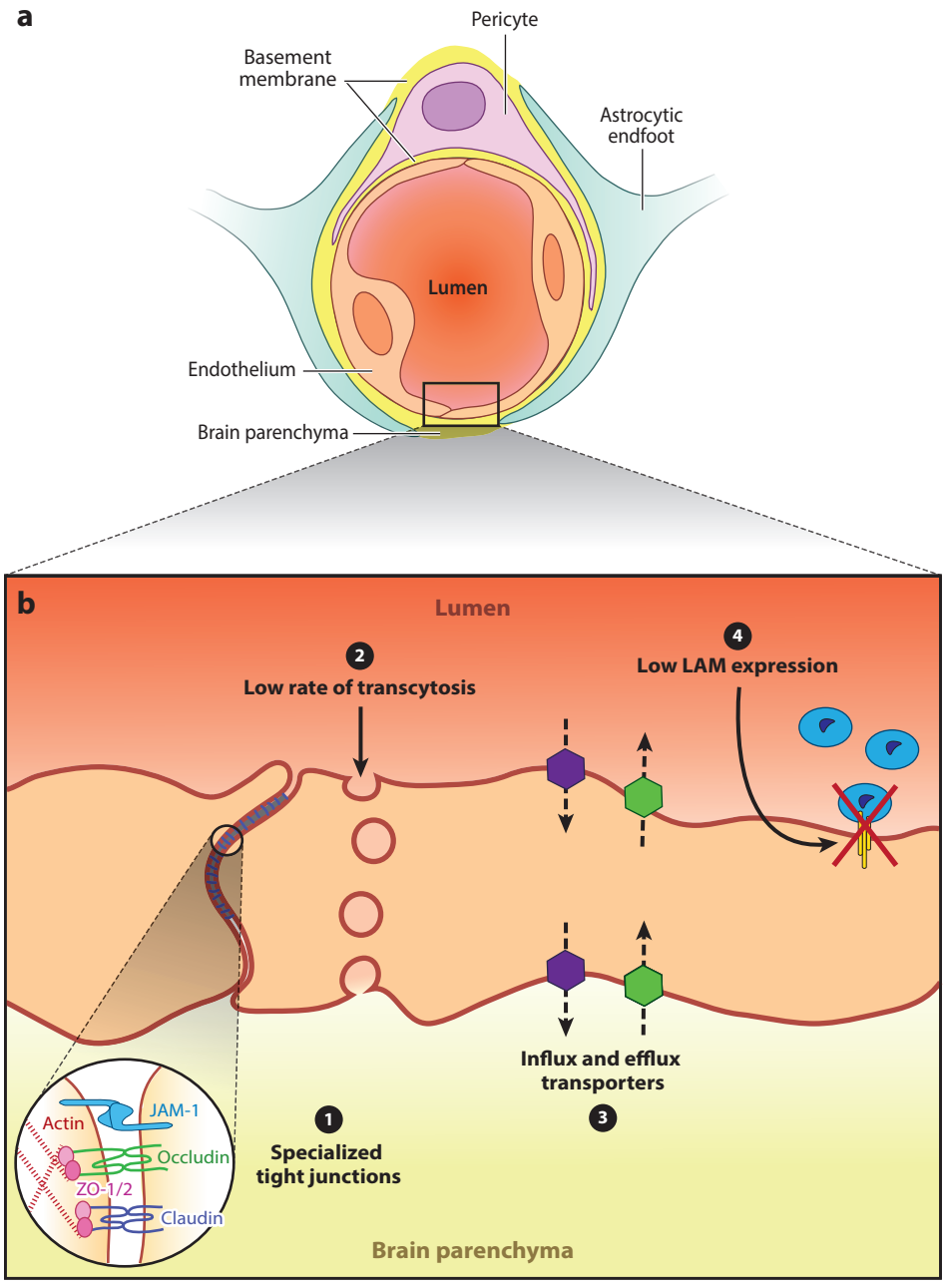
Several groups have investigated the question of BBB development in diverse species with different experimental paradigms, the history of which has been reviewed extensively (Saunders et al. 2012). Once a point of contention, the developmental time point at which the BBB becomes functional has been resolved in recent years. Several studies have used high-resolution imaging techniques to show that circulating tracers are completely excluded from the brain parenchyma at embryonic time points in rodent models, demonstrating that the BBB becomes nonleaky and thus functional before birth (Bauer et al. 1995, Ben-Zvi et al. 2014, Daneman et al. 2010b).

2.2. What Are the Cellular and Molecular Underpinnings of Blood–Brain Barrier Functionality?

Once an outstanding question in the neurovascular field, studies over the past several years have begun to shed light on the mechanisms whereby the neurovascular unit confers BBB properties upon CNS endothelium. Here, we review the current understanding of the cellular and molecular pathways by which each neurovascular unit cell type contributes to BBB development and functionality.

2.2.1. Endothelial cells. As the cells forming the BBB, endothelial cells of the CNS have four specific properties that contribute to BBB integrity (**Figure 2**). First, the tight junctions between endothelial cells prevent the passage of ions and nutrients within the paracellular space (Hawkins & Davis 2005). Although tight junctions are present in peripheral endothelial cells, they are tighter in CNS endothelium, as evidenced by both their ultrastructural characteristics under EM (Reese & Karnovsky 1967) and the observation that no water-soluble molecules can pass freely through tight junctions in the CNS. Second, compared to peripheral endothelium, which readily uses cargo-filled vesicles to transport macromolecules from the blood to underlying tissues, CNS endothelial

cells display remarkably low rates of vesicular trafficking between the luminal and abluminal cell membranes, a process termed transcytosis (Reese & Karnovsky 1967, Siegenthaler et al. 2013). Third, instead of using vesicle trafficking, brain endothelial cells express numerous transporters to deliver nutrients such as glucose, amino acids, and metabolically relevant ions to the brain, as well as to remove potentially neurotoxic substances and drugs (Saunders et al. 2013). Finally, CNS endothelial cells have low expression levels of leukocyte adhesion molecules (LAMs) and thus play



a role in preventing the movement of immune cells into the immunoprivileged brain environment (Rössler et al. 1992). In general, these four endothelial cell properties can be categorized into those that confer tightness to (tight junctions and transporter expression) and prevent leakiness of (low rates of transcytosis and LAM expression) the BBB.

Emerging evidence suggests that endothelial cells express molecules that are essential for the establishment of these CNS-specific properties. Much of this work has been guided by endothelial cell transcriptome studies that have produced lists of candidate genes that may be important for BBB function, some of which have been investigated in depth (Armulik et al. 2010, Ben-Zvi et al. 2014, Daneman et al. 2010a, Tam et al. 2012). Recently, major facilitator domain containing protein 2A (MFSD2A) was identified as a molecule expressed specifically in CNS endothelial cells that promotes BBB formation and function primarily by maintaining low rates of transcytosis (Ben-Zvi et al. 2014), suggesting that CNS endothelial cells may promote BBB integrity by endogenously expressing machinery to suppress this process that readily occurs in the periphery. Additionally, this observation highlights the importance of the regulation of transcytosis, an often overlooked feature of CNS endothelial cells. Interestingly, a separate group identified MFSD2A as a transporter for fatty acids in brain endothelial cells (Nguyen et al. 2014). Future studies will address whether MFSD2A serves a dual role in CNS endothelial cells or if both functions are required to promote BBB integrity (Betsholtz 2014, Zhao & Zlokovic 2014).

2.2.2. Neural progenitors. During embryonic development, neural progenitors secrete factors that regulate angiogenesis and the first steps of BBB formation. The most well-characterized examples are the Wnt family of morphogens, including Wnt7a and 7b. Wnt7a and 7b are expressed by neural progenitors in ventral regions of the brain at the same time that nascent endothelial cells expressing β-catenin begin to ingress (Daneman et al. 2009, Stenman et al. 2008). If Wnt/β-catenin signaling is genetically abolished, severe defects in angiogenesis are observed specifically in the CNS, including loss of capillary beds and vascular malformations adjacent to the meninges. In addition, loss of the Wnt receptor Frizzled4 leads to BBB breakdown, specifically in the cerebellum (Wang et al. 2012). With regard to the specific BBB properties that Wnt cues regulate, neuronally derived Wnt7a induces the expression of BBB-specific transporters, most notably glucose transporter-1. Temporal ablation of β-catenin in endothelial cells postnatally also results in decreased expression of the tight junction component claudin-3 and a loss of BBB integrity (Liebner et al. 2008), suggesting that neuron-endothelium interactions may facilitate the

Figure 2

CNS endothelial cell properties contributing to BBB functionality. (*a*) Cross section of the neurovascular unit at the level of a CNS capillary. Vessels are lined by a single layer of nonfenestrated endothelial cells that exhibit barrier properties. Astrocytes and pericytes surround the abluminal surface of CNS endothelial cells and provide additional functional support for the establishment and maintenance of the BBB. (*b*) A magnified view of the CNS endothelium, highlighting the four cellular properties that contribute to BBB integrity by stringently controlling the exchange of ions and nutrients between the blood and brain. ❶ Specialized tight junctions prevent paracellular flux between endothelial cells. Cell-cell interactions are mediated by tight junction proteins, including JAM-1, occludin, and members of the claudin family. The cytoplasmic adaptor proteins ZO-1 and ZO-2 link these transmembrane proteins to the cytoskeleton. ❷ Endothelial cells exhibit extremely low rates of transcytosis, as vesicular trafficking of ions and nutrients across cells is kept to a minimum. ❸ Endothelial cells express influx (*purple*) and efflux (*green*) transporters that shuttle specific nutrients into the brain and remove potentially harmful toxins and other small molecules from the brain, respectively. ❹ The low expression of LAMs aids in maintaining low levels of immune surveillance in the CNS. Abbreviations: BBB, blood–brain barrier; CNS, central nervous system; JAM-1, junctional adhesion molecule-1; LAM, leukocyte adhesion molecule; ZO, zonula occludens.

maintenance of BBB tight junctions. Together, these studies suggest that neuronally derived Wnt cues contribute to the tightness properties (tight junctions and transporters) of the BBB in CNS endothelial cells. The canonical Wnt/β-catenin signaling pathway likely results in transcriptional regulation of effector proteins in endothelial cells with BBB function. For example, expression of downstream Wnt/β-catenin signaling targets DR6 and TROY, which are upregulated in endothelial cells with increased β-catenin levels, is necessary for BBB formation and vascular pattering (Tam et al. 2012). Canonical β-catenin signaling, however, is not the only Wnt pathway relevant to BBB function. A recent study has shown that Wnt5a activates the planar cell polarity pathway in vitro to promote tight junction integrity in endothelial cells (Artus et al. 2014), opening up the field for further study of how nonclassical Wnt signaling regulates BBB integrity in vivo. One conceptual caveat to studying Wnt signaling in BBB development, however, is that disruption of this pathway leads to defects in vascular patterning, making it difficult to differentiate the processes of angiogenesis and barrier genesis in these conditions (Daneman et al. 2009).

2.2.3. Astrocytes. Researchers have long known that astrocytes, a major glial subtype in the brain, ensheathe CNS vessels and confer BBB properties to endothelial cells. For example, early experiments showed that transplantation of astrocytes can induce peripheral endothelial cells to form nonleaky vessels (Janzer & Raff 1987). Unlike the early role played by neural progenitors in BBB development, however, astrocytes do not appear at the neurovascular unit until after birth and are generally thought to aid in the maintenance of BBB functionality (Obermeier et al. 2013, Siegenthaler et al. 2013). For example, astrocytes express molecules that regulate properties of BBB integrity in endothelial cells. Most notably, astrocyte-secreted Sonic hedgehog (Shh) improves barrier function both in vitro and in vivo (Alvarez et al. 2011). Shh signaling in CNS endothelial cells increases the expression of the tight junction components occludin and claudin-5 and decreases the expression of chemokine and cell adhesion molecule expression, suggesting it has a role in both tight junction integrity and immune surveillance at the BBB. In addition to Shh, astrocyte-secreted angiopoietin-1 and angiotensinogen both signal to endothelial cells to promote tight junction integrity (Lee et al. 2003, Wosik et al. 2007). Together, these studies show that cross talk between astrocytes and CNS endothelial cells functions to maintain the integrity of the BBB, primarily through the expression and regulation of interendothelial tight junctions. Recently, researchers showed that retinoic acid, produced by fetal astrocytes, plays an important role in the development of the BBB, calling into question the view that astrocytes are only required for BBB maintenance after birth (Mizee et al. 2013). As retinoic acid regulates both Shh and Wnt signaling in the CNS (Halilagic et al. 2007), this study also illustrates the complex interactions between different cell types and signaling pathways in the establishment and maintenance of the BBB.

2.2.4. Pericytes. The relatively recent notion in the field of neuroscience that pericytes are key regulators of BBB integrity during both developmental and adult stages is now well established. Pericytes, contractile cells of the neurovascular unit at the capillary level, are recruited by release of platelet-derived growth factor-b (Pdgf-b) from nascent endothelial cells during embryonic development, which binds to Pdgf receptor-β (Pdgfrβ) expressed on the pericyte cell surface (Hellstrom et al. 1999, Lindahl et al. 1997). The identification of Pdgfrβ as a marker for brain pericytes offered the first entry point into understanding the role of this cell type in BBB function, as mice lacking Pdgfrβ completely lack brain pericytes (Lindahl et al. 1997). Although they are embryonically lethal, pericyte-deficient mice are characterized by high vascular permeability at embryonic stages, indicating a defect in BBB development (Daneman et al. 2010b). In these animals, endothelial cells display numerous membrane protrusions into the vessel lumen and an increased density of cytoplasmic vesicles, as visualized by EM, indicating increased rates of

transcytosis. Additionally, loss of pericytes leads to the expression of several LAMs, whereas the expression of occludin, claudin-5, and several transporters remains unaltered. In a separate study, Armulik et al. (2010) showed that adult mice with decreased pericyte coverage display BBB leakiness that correlates to the extent of pericyte loss. Moreover, leakiness in these animals occurs through a transcytotic route. Interestingly, MFSD2A expression is reduced in pericyte-deficient mice (Ben-Zvi et al. 2014), pointing to a possible link between pericyte loss and BBB permeability, a potential topic of future investigation (Betsholtz 2014).

2.3. The Blood–Brain Barrier in Disease and Neurodegeneration

Neuronal-vascular abnormalities, including BBB breakdown, can have deleterious effects on neuronal function. For example, BBB leakiness has been shown to precede age-dependent neuronal loss, decreased dendritic spine density and length, memory impairment, and neuroinflammation (Bell et al. 2010). BBB breakdown has also been implicated in numerous neurodegenerative diseases, such as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, and multiple sclerosis. The relationship between loss of BBB integrity and disease pathology has been reviewed extensively (Obermeier et al. 2013, Zlokovic 2008).

2.4. Emerging Therapeutic Strategies for Blood–Brain Barrier Manipulation for Central Nervous System Drug Delivery

The control of BBB integrity in health and disease is a double-edged sword: Although an intact BBB is clearly essential for normal brain function, a sealed BBB is the major obstacle for CNS drug delivery. In fact, a large percentage of neurotherapeutic agents, including recombinant proteins and antibodies, do not cross the BBB under normal circumstances (Zlokovic 2008). Therefore, a major research focus has unsurprisingly been devoted to designing therapeutic methods of BBB manipulation for drug delivery to the CNS. Current approaches focus on altering the properties of CNS endothelial cells, allowing for the controlled penetration of therapeutic agents (Pardridge 2007, 2012). As opposed to modifying intercellular tight junctions (which may damage the structural integrity of the neurovascular unit) or using transporters (which requires designing drugs that mimic the molecular structure of endogenous substrates), hijacking transcytotic pathways in endothelial cells provides an appealing strategy for drug delivery to the CNS.

A subset of nutrients and macromolecules, such as insulin and iron, are known to cross the BBB by binding to surface receptors at the luminal endothelial cell plasma membrane with subsequent delivery to the brain parenchyma, a process termed receptor-mediated transcytosis (Tuma & Hubbard 2003). Manipulation of receptor-mediated transcytosis pathways through the use of molecular Trojan horse (MTH) technology is a favorable strategy for CNS drug delivery. To design these agents, neurotherapeutic agents are conjugated to an antibody immunoglobulin G (IgG) domain that targets an endogenous BBB receptor (Pardridge 2012, Xia et al. 2009). Specifically, several groups have used the transferrin receptor (TfR), which facilitates the delivery of transferrin-bound iron to the brain, as a target for MTH design to treat pathological conditions such as Alzheimer's disease and brain tumors (Jones & Shusta 2007, Yu & Watts 2013). Although these strategies have had some success, an incomplete understanding of how TfR trafficking occurs in CNS endothelial cells confounds their interpretation. Indeed, studies have suggested that MTHs targeting the TfR remain trapped in the CNS endothelium and that this may relate to the affinity of the IgG to the TfR (Couch et al. 2013, Manich et al. 2013, Yu et al. 2011), calling into question the ability of these tools to readily deliver neurotherapeutic agents to the brain.

To attempt to resolve these discrepancies, two recently published studies further investigated how MTHs targeting the TfR are trafficked in CNS endothelium. First, Niewoehner et al. (2014)

generated an MTH that engages the TfR monovalently, which they showed significantly increases the brain delivery of an antiamyloid-β antibody. In contrast, a traditional bivalent version of the same MTH is sorted to the endothelial lysosomes, supporting a model in which the binding mode to the TfR is the critical factor that determines the efficiency of drug delivery. Bien-Ly et al. (2014) propose that anti-TfR affinity is the key factor affecting MTH efficiency. The authors demonstrated that high-affinity TfR binding both reduces brain TfR levels and drives MTH lysosomal degradation, compared to a low-affinity MTH that allows more effective drug delivery to the brain.

Although these studies provide novel insights guiding the future design of more effective MTH agents, the question remains whether targeting receptor-mediated transcytosis pathways is the most effective strategy of BBB manipulation for drug delivery. One major caveat to this method is that the targeted receptors, such as TfR, are expressed throughout the body, which leads to MTH clearance into peripheral tissues and reduces the amount of the agent available for brain delivery (Yu & Watts 2013). Targeting molecules that are expressed specifically in endothelial cells, such as MFSD2A, may provide an alternative strategy for CNS drug delivery. Also, the transcytotic pathway that is upregulated in the absence of MFSD2A has not been shown to place limits on the size or chemical properties of transported cargo (Armulik et al. 2010, Ben-Zvi et al. 2014), making this pathway particularly appealing from a drug delivery perspective (Betsholtz 2014).

2.5. What Are the Future Directions in Blood–Brain Barrier Research?

As the critical role of the BBB in both normal and pathological conditions has become increasingly apparent, the effort to develop new technologies to study its function is moving to the forefront of the field. Although EM studies of endothelial cells and imaging of fixable injected tracers remain landmark techniques, they provide only a static snapshot and do not allow investigators to visualize the consequences of manipulating the properties of CNS endothelium in real time. Emerging techniques using two-photon microscopy to visualize the molecular components of CNS endothelial cells are beginning to address these outstanding issues. For example, Knowland et al. (2014) have used a transgenic *eGFP-Claudin5* mouse (Evans et al. 2000), which fluorescently labels tight junctions, to show for the first time how tight junction dynamics are altered in vivo in response to stroke. Similar studies will become increasingly useful in the future to assess how endothelial cells respond to a variety of genetic and pharmacological assaults. In particular, it will be important to develop tools to allow the visualization of different transcytotic pathways, as the regulation of transcytosis is emerging as a critical mechanism of maintaining BBB integrity (Armulik et al. 2010, Ben-Zvi et al. 2014, Daneman et al. 2010b).

The field also needs an in vitro system for high-throughput screening of drugs that can alter BBB permeability—one that is simple, is widely acceptable, and reliably recapitulates the in vivo barrier. Ideally, such a cellular model would express the molecular constituents of CNS endothelial cells (transporters and tight junction molecules), recapitulate endothelial cell architecture and polarity, and, most importantly, possess highly restrictive paracellular and transcellular barriers. As multiple neurovascular unit cell types maintain BBB functionality in vivo, however, CNS endothelial cells readily lose barrier properties in culture. For example, transendothelial electrical resistance across CNS endothelial monolayers, a commonly used metric of tight junction integrity, is severely reduced in vitro (Grant et al. 1998), indicating a nonrestrictive barrier (Wilhelm et al. 2011).

Several groups have recently made strides toward improving in vitro BBB models. In an attempt to recapitulate the neurovascular unit in vitro, endothelial cells are often co- and tricultured with astrocytes and pericytes, resulting in increased transendothelial electrical resistance measurements (Hatherell et al. 2011). Paolinelli et al. (2013) demonstrated that activating the Wnt/β-catenin

pathway in vitro increases the restrictiveness of endothelial cell monolayers without the need to coculture with additional cell types, raising the interesting notion that stimulating the cellular signaling pathways necessary for BBB function in culture may be sufficient to form a restrictive barrier. One recurring discrepancy among the various in vitro BBB models relates to cell type, as isolated primary endothelial cells and immortalized lines from various species have been used to varying degrees of success (Gumbleton & Audus 2001). Recent evidence has demonstrated that endothelial cells with barrier properties can be generated from human pluripotent stem cells (Lippmann et al. 2014, Lippmann et al. 2012). In vitro BBB models from stem cell sources may provide an ideal platform for future work, as they circumvent the variability generated by the use of isolated primary cells and can be propagated in vitro from all-human cell sources.

Despite all the progress since the discovery of the BBB, the field is in many ways still in its infancy, with many fundamental questions remaining to be answered: What are the key molecular regulators and pathways essential for the development of the BBB? How do these core components work together to ensure BBB functionality? Are endothelial cell tight junctions regulated by unique mechanisms in the CNS? How is transcytosis, the most promising pathway to manipulate for therapeutic purposes, regulated in CNS endothelial cells? These topics deserve to be a major focus of future research in neuroscience.

3. NEUROVASCULAR COUPLING

The first evidence of a spatiotemporal coupling between brain activity and CBF (or neurovascular coupling) was provided in 1890 by Roy & Sherrington, who challenged the doctrine that active changes in brain vessel diameter were impossible (Friedland & Iadecola 1991, Roy & Sherrington 1890). A key conclusion of that work remains valid today: "[T]he brain possesses an intrinsic mechanism by which its vascular supply can be varied locally in correspondence with local variations of functional activity" (Roy & Sherrington 1890, p. 105). Since then, researchers have made a tremendous effort investigate mechanisms governing the regulation of CBF by neural activity at various levels along the vascular tree, from arterioles to capillaries. Hence, studies pertaining to the vasomotor function of nerves, proposed in the late 1920s by Talbott and colleagues (1929) and involving various neurotransmitters and signaling molecules (Cauli & Hamel 2010), gave birth to a fast-evolving field in neurobiology with far-reaching implications in both health and disease. Indeed, thorough investigation of the relationships between neural activity and CBF is essential for adequate analysis of blood-oxygenation-level-dependent (BOLD) functional brain imaging (Hillman 2014) and for fundamental understanding of vascular dysfunctions (Iadecola 2004, Zlokovic 2010).

The neurovascular unit is the anatomical substrate of neurovascular interactions in the brain (Lecrux & Hamel 2011, Lo & Rosenberg 2009). Along the vascular tree, cellular components of the neurovascular unit integrate messages conveyed by neural activity to modify the diameter of brain vessels and regulate CBF. In the control of vessel diameter, the main difference between intracerebral arterioles and capillaries is the nature, position, and abundance of contractile cells surrounding the external (abluminal) vessel surface (**Figure 3**). Arterioles are fully covered by a single layer of vascular smooth muscle cells (VSMCs), whereas capillaries, which by definition lack VSMCs, are partly covered by contractile pericytes with the highest pericyte density in the CNS (Armulik et al. 2005, Cipolla 2009). In the neocortex, the regulation of CBF by neural activity is thought to happen mainly at the arteriole level (Fergus & Lee 1997, Hillman et al. 2007), although the involvement of pericytes at the capillary level was recently established; this has opened a new debate about the contribution of each vascular compartment to the regulation of CBF as well as to the generation of the BOLD signal used as a readout for neural activity in functional brain imaging.

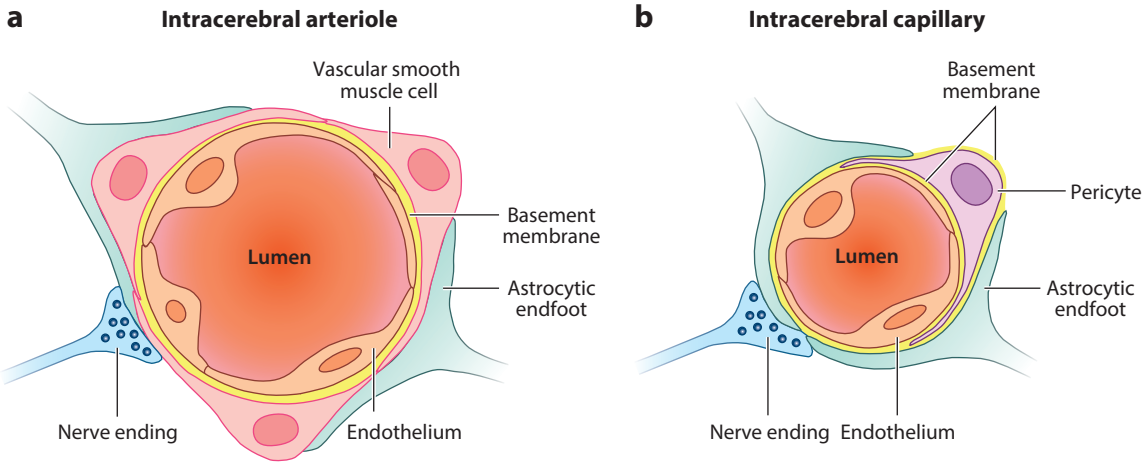


Figure 3

Schematic representation of the neurovascular unit. Inside the brain, endothelial cells are organized into a multicellular complex together with contractile and glial cells, an assembly called the neurovascular unit. The main difference between intracerebral arterioles and capillaries is the nature, position, and abundance of contractile cells that surround the external vessel surface. (*a*) At the level of intracerebral arterioles, the endothelium is fully covered by a single layer of vascular smooth muscle cells, which provide contractile properties to the arteriole. Astrocytes send their processes, called endfeet, around the arteriole, providing further support as well as a functional connection to surrounding neural tissues. (*b*) Intracerebral capillaries lack vascular smooth muscle cells but are partly covered by contractile pericytes.

3.1. Control of Cerebrovascular Function at the Level of Arterioles

The net effect of neural activity on arteriole diameter—the induction of either vasodilation or vasoconstriction—reflects the integration by the neurovascular unit of signals controlling the electric state and contractile properties of VSMCs (Hamel 2006, Hillman 2014). The major players in cortical neurovascular coupling are (*a*) projection neurons sending fibers to the neocortex from subcortical centers (Cauli et al. 2004, Elhusseiny & Hamel 2000, Zhang et al. 1995), (*b*) local excitatory and inhibitory neurons (Cauli & Hamel 2010, Lecrux et al. 2011), and (*c*) astrocytes (Attwell et al. 2010, Howarth 2014, Zonta et al. 2003), which all release vasoactive substances. More details about the regulation of CBF at the level of cerebral arterioles can be found in recent reviews (Cauli & Hamel 2010, Hillman 2014).

3.2. Control of Cerebrovascular Function at the Level of Capillaries

CNS capillaries lack VSMCs, and at least 80% of their abluminal surface is covered by pericytes (Armulik et al. 2005, Peppiatt et al. 2006). These contractile cells contribute to the capillary neurovascular unit. Pericytes were long considered as support cells for the endothelium, involved in the formation and maintenance of the BBB (Armulik et al. 2010, Daneman et al. 2010b, Mäe et al. 2011). The regulation of capillary diameter by pericytes in response to neural activity has been clarified over the past decade (Hamilton et al. 2010, Itoh & Suzuki 2012, Peppiatt et al. 2006). Most recently, investigators reevaluated the contribution of pericytes to CBF regulation in more physiological conditions. Using two-photon microscopy in mice with genetically labeled pericytes, Fernandez-Klett et al. (2010) demonstrated in real time that pericytes are effective regulators of capillary diameter and flow without contributing significantly to global CBF regulation. In contrast, Hall et al. (2014) demonstrated that pericytes increase blood flow in the somatosensory cortex following whisker pad stimulation, through relaxation (vasodilation) initiated at the capillary level.

Overall, the activity-induced control of capillary diameter (and flow) by pericytes must also be considered as an integrative process in the context of cerebrovascular anatomy. Indeed, the population of pericytes is heterogeneous along the vascular tree. Subtypes of pericytes respond differentially to neurotransmitters or vasomodulators (Fernandez-Klett et al. 2010, Hall et al. 2014, Peppiatt et al. 2006), and pericytes adjacent to small capillaries are most probably noncontractile, as they are negative for smooth muscle α–actin (Nehls & Drenckhahn 1991). This suggests that activity-induced CBF regulation by pericytes might happen locally within defined microvascular segments, with contractile signals possibly propagating between pericytes and spreading back to upstream arterioles (Hall et al. 2014, Peppiatt et al. 2006).

3.3. A New Concept for Neurovascular Interactions in the Early Postnatal Brain

Recent studies in rodents and humans have shown that the phenomenon of neurovascular coupling is not functional until a few weeks after birth (three weeks in rats, seven to eight weeks in humans). Whereas sensory stimulation in adult rodents and humans leads to a positive BOLD signal, reflecting a local increase in CBF, the identical stimulus in newborn infants or rat pups results in an inverted response with negative BOLD signals (Anderson et al. 2001, Born et al. 2002, Kozberg et al. 2013, Muramoto et al. 2002, Yamada et al. 2000). These studies suggest that negative BOLD signals in response to sensory stimulation result from either decreased perfusion or increased oxygen consumption. The absence of a neurovascular coupling response to neuronal activation implies that, during early postnatal development, the immature brain must rely on alternative mechanisms to adequately match oxygen and nutrient supply to increasing energy demands. One potential mechanism during postnatal development could be the control of cerebrovascular patterning by neural activity, which we discussed in Section 1 of this review. Indeed, Lacoste et al. (2014) recently demonstrated that during postnatal development, sensory-related neural activity promotes the formation of vascular networks in the mouse barrel cortex.

Could hypoxia be involved in the control of cerebrovascular patterning by neural activity? After birth, the maturation of neuronal networks involves energy-consuming processes such as neurogenesis, synaptogenesis, maturation of astrocytes, and changes in brain cytoarchitecture. Thus, in the early postnatal brain, in the absence of neurovascular coupling, neuronal network activation might generate a local and transient hypoxic state. Hypoxia-induced growth factors represent the main driving force of vascular development not only during embryogenesis (Haigh et al. 2003, Provis et al. 1997, Raab et al. 2004, Stone et al. 1995) but also during postnatal cerebrovascular remodeling (Rey & Semenza 2010) and particularly in pathological conditions such as ischemia and cancer (Silpanisong & Pearce 2013). Local reduction in oxygen tension leads to activation of the transcription factor hypoxia-inducible factor 1 (HIF-1). Activated HIF-1 regulates the expression of virtually all the key angiogenic factors, including VEGF, angiopoietin-2, and placental growth factor, upon binding to their hypoxia response elements (Jiang et al. 1997, Rey & Semenza 2010, Semenza et al. 1997). Future studies could investigate whether sensory stimulation, which leads to increased vascular density and branching in the cerebral cortex (Lacoste et al. 2014), is due to a local and transient hypoxic state that in turn triggers hypoxia-induced, proangiogenic pathways (Yuen et al. 2014).

4. CONCLUSION

In this review, we have highlighted the current understanding of how the nervous and vascular systems interact to ensure proper brain function. Neuronal and vascular networks are established during development by using a common set of guidance cues, providing the closely juxtaposed

anatomical framework for the delivery of oxygen and nutrients from the blood to underlying neuronal tissue. This framework can be refined during postnatal development by neural activity in response to the demands of the environment. Additionally, the control of cerebrovascular function at different levels along the vascular tree is crucial in regulating CBF and ensuring that the metabolic needs of neurons are met within the brain. Yet the regulation of CBF is not functional until several weeks after birth. This implies that the early postnatal brain relies on alternative mechanisms, such as neural activity–driven angiogenesis, for efficient delivery of oxygen and glucose. Finally, the integrity of the BBB is critical in maintaining the safe and homeostatic environment necessary for the function of neural circuits. As neuroscience research progresses, the functional relevance of a proper neuronal-vascular interface within the brain becomes increasingly clear. A fundamental understanding of neuronal and vascular interactions has far-reaching benefits in developing strategies to treat psychological and neurodegenerative diseases, brain tumors, and stroke, highlighting the importance of future research in this field of neuroscience.

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