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Wiring the Vascular Network with Neural Cues: A CNS Perspective

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The vascular and the nervous system are responsible for oxygen, nutrient, and information transfer and thereby constitute highly important communication systems in higher organisms. These functional similarities are reflected at the anatomical, cellular, and molecular levels, where common developmental principles and mutual crosstalks have evolved to coordinate their action. This resemblance of the two systems at different levels of complexity has been termed the "neurovascular link." Most of the evidence demonstrating neurovascular interactions derives from studies outside the CNS and from the CNS tissue of the retina. However, little is known about the specific properties of the neurovascular link in the brain. Here, we focus on regulatory effects of molecules involved in the neurovascular link on angiogenesis in the periphery and in the brain and distinguish between general and CNS-specific cues for angiogenesis. Moreover, we discuss the emerging molecular interactions of these angiogenic cues with the VEGF-VEGFR-Delta-like ligand 4 (Dll4)-Jagged-Notch pathway.

Introduction

The Neurovascular Link

In 1543, the Belgian anatomist Andreas Vesalius (1514–1564) was the first to describe the parallel organization and alignment of arteries and nerves and thereby laid the early foundation to the concept of the neurovascular link (Carmeliet and Tessier-Lavigne, 2005).

From a functional perspective, both systems are important for information transport over long distances: whereas the nervous system processes electric signals to transfer information, the vascular system establishes long-range communication via dissolved messenger molecules and by serving as pathway for leukocyte trafficking (Nourshargh et al., 2010). The functionality of both systems requires correct patterning and guidance of their cellular and subcellular elements.

In 1890, the neuroscientist Ramon y Cajal described the specialized cellular structure at the tip of the growing axon, which he termed the "axonal growth cone" (Carmeliet and Tessier-Lavigne, 2005; Lowery and Van Vactor, 2009). Nowadays, we know that axonal growth cones extend fan-like lamellipodial and long, finger-like filopodial protrusions that sense the local microenvironment for guidance cues (de Castro et al., 2007; Lowery and Van Vactor, 2009) and thereby steer the growing axon (Figures 1A and 1C). Around 100 years later, vascular biologists discovered that sprouting blood vessels are led by cells that resemble these axonal growth cones in cellular appearance and function, exhibiting similar lamellipodia and filopodia structures. These cells have been named "endothelial tip cells" and are key structures in the pathfinding of developing, newly forming blood vessels (Carmeliet and Tessier-Lavigne, 2005; Carmeliet and Jain, 2011; Gerhardt et al., 2003; Marin-Padilla, 1985; Potente et al., 2011) (Figures 1B and 1D).

At the subcellular level, both systems sense guidance cues using structures based on the actin cytoskeleton (lamellipodia and filopodia) resulting in extension and retraction of these structures and in directed movements of growing nerves and blood vessels (Figures 1A–1D).

Axonal growth cones that steer growing axons consist of a central and a peripheral domain. Whereas the peripheral domain is composed of the lamellipodia consisting of an actin meshwork and filopodia consiting of F-actin bundles, the central domain of a growth cone contains mainly microtubules with only few

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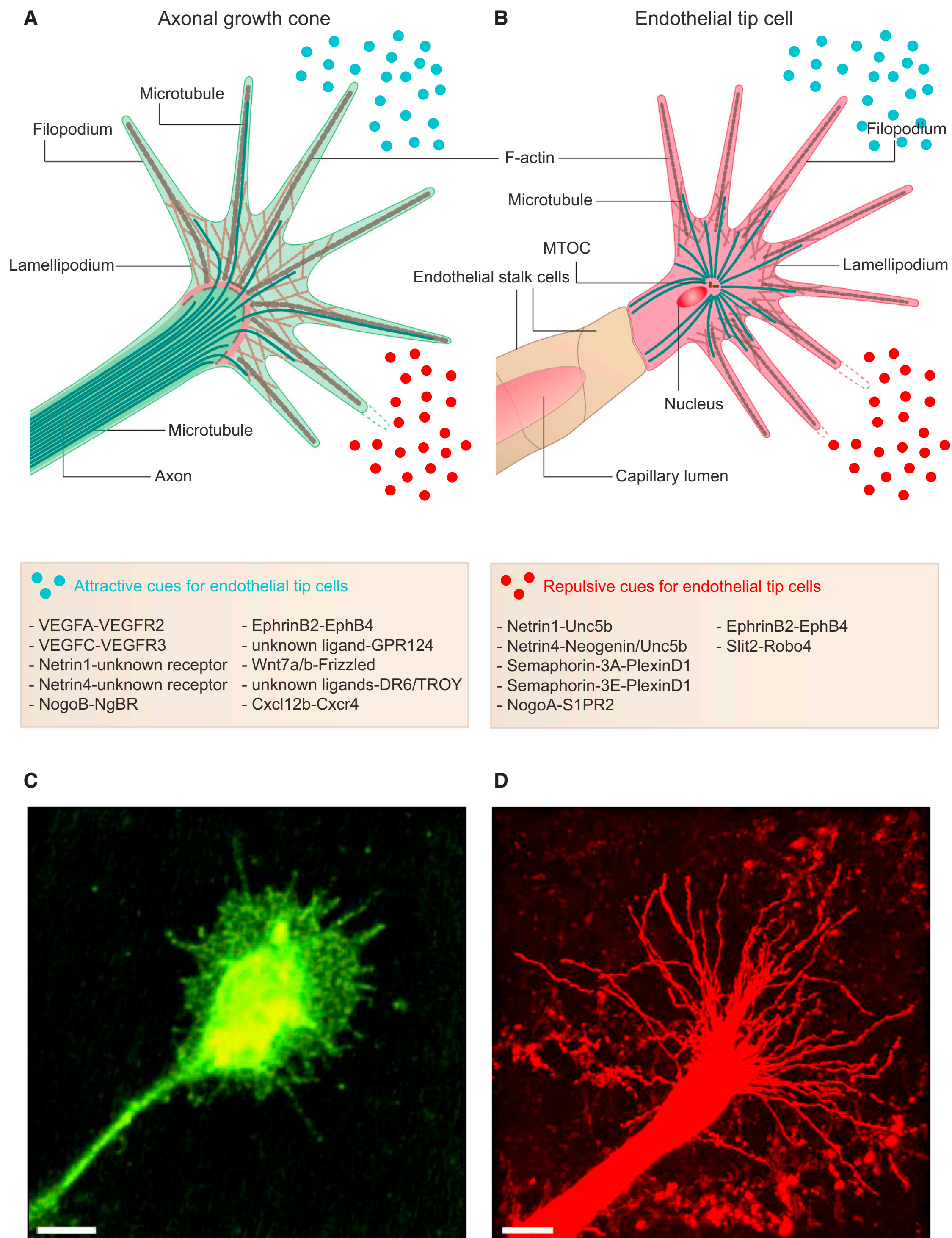

Figure 1. Cellular Similarities between the Neuronal Growth Cone and the Vascular Endothelial Tip Cell

At the forefront of growing axons and growing blood vessels, the axonal growth cone and the endothelial tip cell are specialized, "hand-like" structures that sense environmental cues using lamellipodia and "finger-like" filopodia. Thereby, the growing axons and growing blood vessels are guided to their respective targets. The tip cell is an own cellular entity of a multicellular sprouting blood vessel (consisting of other specialized endothelial cells, see below), whereas the axonal growth cone is a specialized, subcellular structure of the extending neuron. Nevertheless, they are functional analogs, as common attractive and repulsive guidance cues have been adopted by the nervous and the vascular system during evolution to guide these structures.

(A) The axonal growth cone at the leading edge of a growing axon is a specialized structure at the tip of an extending neuron, usually far away from its cell body. Actin-based structures (brown) such as lamellipodia and filopodia are used to sense and integrate attractive (blue) and repulsive (red) guidance cues in the local

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microtubule filaments reaching into the peripheral domain and sometimes even into filopodia (Figure 1A; Lowery and Van Vactor, 2009). In comparison to axonal growth cones, the cytoskeletal composition and organization of endothelial tip cells is less well described. Beside the fact that endothelial tip cells have been discovered 110 years later than axonal growth cones, this is mainly due to technical limitations. For instance, the lack of specific endothelial tip cell markers and the inability to observe endothelial tip cell behavior on flat surfaces (in contrast to axonal growth cones) limit in vitro studies on endothelial tip cells and their cytoskeletal organization. However, F-actin structures have been observed at the leading edge and in filopodia of endothelial tip cells in vivo (Figure 1B; Fraccaroli et al., 2012; Phng et al., 2013).

Interestingly, recent years have seen the discovery of common molecular cues that guide both endothelial tip cells and axonal growth cones. First, the four axonal guidance molecule families, Netrins, Semaphorins, Ephrins, and Slits, and their receptors have been shown to not only steer growing axons but also guide growing blood vessels via these specialized structures (Carmeliet and Jain, 2011; Carmeliet and Tessier-Lavigne, 2005; Quaegebeur et al., 2011) (Figures 1A and 1B).

Subsequently, a number of axonal guidance molecules like the morphogens wingless-type proteins (Wnts), Sonic Hedgehog (Shh), and Bone Morphogenetic Protein (BMP) have been shown to exert similar repulsive and attractive functions on neuronal growth cones (Charron and Tessier-Lavigne, 2007) and blood vessel endothelial tip cells (Zacchigna et al., 2008), although a direct function on tip cell guidance has not been demonstrated (Quaegebeur et al., 2011).

In addition, classical angiogenic factors like VEGF-A, FGF-2 and vessel-derived factors like Endothelin-3 and Artemin and its receptor GFRalpha3 can also direct neuronal development (Honma et al., 2002; Quaegebeur et al., 2011; Zacchigna et al., 2008). These molecules affecting both the vascular and the nervous system(s) have in consequence been termed "angioneurins" (Segura et al., 2009; Zacchigna et al., 2008). Moreover, mutual crosstalk and co-patterning of the vascular and neuronal system are also a result of direct cellular interactions: for instance, sensory neurons and Schwann cells in the PNS provide a template for the patterning of arteries but not veins during skin development, while neuronal release of VEGF induces arterial differentiation (Li et al., 2013). On the other hand, vessel-derived cues such as Artemin and Endothelin-3 can guide growing axons (Honma et al., 2002; Makita et al., 2008).

The behavior of endothelial tip cells and axonal growth cones is also regulated by the interaction of guidance cues with the extracellular matrix (ECM) or by direct interactions with the ECM. For example, vascular morphogenesis is guided by the tissue distribution of VEGF-A, which depends on its ability to bind to the ECM (Gerhardt et al., 2003; Ruhrberg et al., 2002). Although the heparin-binding domain of VEGF-A (responsible for ECM binding) is not essential for vascular development, deletion of this domain leads to changes in vascular patterning and endothelial tip cell morphology (Ruhrberg et al., 2002). Interestingly, the ECM can also mediate direct signaling responses on growing vessels. For instance, the ECM regulates endothelial tip cell selection—a process mediated by Dll4-Notch signaling (see below)—via Laminin-Integrin signaling-induced expression of Dll4 in endothelial cells (Estrach et al., 2011; Stenzel et al., 2011). Moreover, recent findings describe the influence of ECM stiffness on angiogenesis and VEGF-signaling (Mammoto et al., 2009). Briefly, ECM elasticity regulates the activity of the Rho inhibitor p190RhoGAP, which, in turn, modulates the balance between the two antagonistic transcription factors TFII-I and GATA2. On soft and rigid ECM gels, p190RhoGAP activates TFII-I and inhibits GATA2, which suppresses VEGFR2 transcription and inhibits angiogenesis. On gels of intermediate ECM stiffness, however, p190RhoGAP activates GATA2 and inhibits TFII-I, thereby increasing VEGFR2 transcription and stimulating angiogenesis (Figure 4B) (Mammoto et al., 2009). It remains to be determined whether this mechanosensitive signaling pathway also regulates the tip versus stalk cell discrimination. Given that growth factors such as VEGF also regulate p190RhoGAP activity (Mammoto et al., 2009), another intriguing question is whether molecules of the neurovascular link such as Netrins or Nogo-A also regulate this mechanosensitive pathway.

Finally, the mode of action of axon guidance cues similarly depends on interactions with the ECM (Barros et al., 2011; Moore et al., 2009).

Angiogenesis, the Tip Cell Concept and the VEGF-VEGFR-Dll4-Jagged-Notch Pathway

During development, growing tissues and organs require adequate vascularization and this can occur via different mechanisms of blood vessel formation, namely vasculogenesis, sprouting angiogenesis, and intussusception (Carmeliet and Jain, 2011; Herbert and Stainier, 2011; Potente et al., 2011; Quaegebeur et al., 2011; Weis and Cheresh, 2011).

The process of sprouting angiogenesis is an important mechanism of new vessel formation in most organs, during development, but also in different pathological settings (Carmeliet and Jain, 2011; Jain and Carmeliet, 2012; Potente et al., 2011). When a new sprout forms from a pre-existing vessel, the sprout

tissue microenvironment in order to guide the extending axon to its appropriate target, where it forms a synapse. Microtubuli (green) in filopodia have been described but are rare. The central domain of an axonal growth cone is rich in microtubuli whereas its peripheral domain comprises actin-based lamellipodia and filopodia.

(B) The endothelial tip cell is a specialized vascular endothelial cell type at the tip of the newly forming blood vessel, followed by stalk cells, another specialized cell type. While tip cells migrate and sense the environment, the main function of stalk cells is to proliferate and form the vascular lumen. Phalanx cells constitute a third vascular endothelial cell type, lining the border of functional, established blood vessels (not shown). The endothelial tip cell uses actin-based (brown) lamellipodia and filopodia sense attractive (pro-angiogenic; blue) and repulsive (anti-angiogenic; red) guidance cues in the local tissue microenvironment. Thereby, the extending blood vessel reaches its target, for example another developing blood vessel constituting a fusion partner (anastomosis). Microtubuli (green) in filopodia have not been detected so far.

(C) Visualization of an axonin-1$^+$ axonal growth cone of a dissociated spinal cord commissural neuron from an embryonic day 5 (E5) chick. Note the numerous filopodial extensions emerging from the lamellipodia. The growth cone image is taken from Joset et al. (2011), with permission. The scale bar represents 10 μm.

(D) Visualization of an Isolectin B4 (IB4)$^+$ endothelial tip cell (red) in the mouse forebrain cortex at postnatal day 8 (P8). Note the numerous IB4$^+$ filopodial extensions emerging from the tip cell body. The scale bar represents 10 μm.

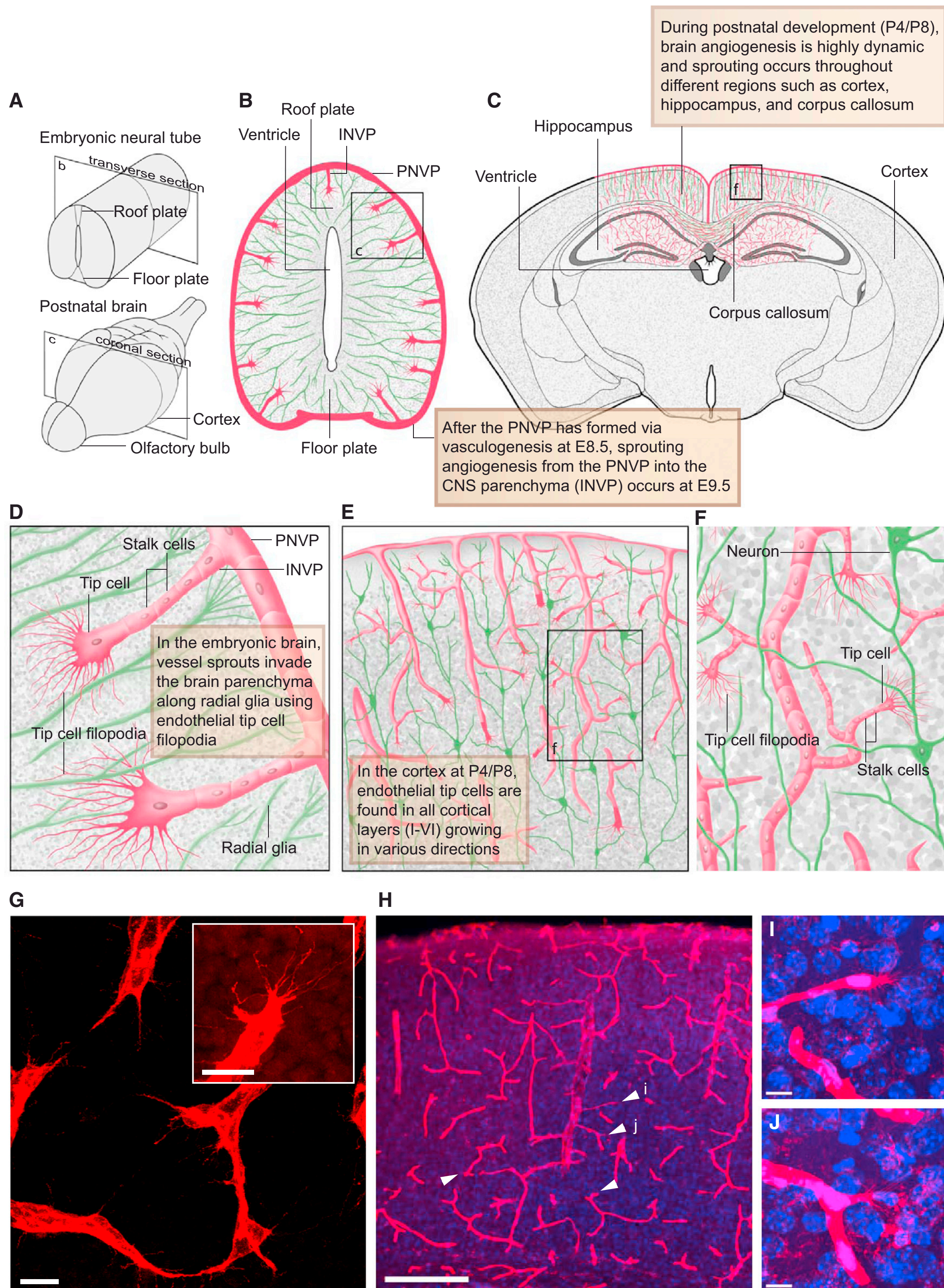

Figure 2. Embryonic and Postnatal CNS Vascularization

(A) Scheme of an embryonic mouse neural tube (top) and of a postnatal mouse brain (bottom). The transverse (embryonic neural tube) and coronal (postnatal brain) cutting planes are indicated.

(B) Schematic representation of sprouting angiogenesis into the neural tube during mouse embryogenesis. The perineural vascular plexus (PNVP, red) is formed by vasculogenesis from mesodermally derived angioblasts at around E8.5. Subsequently, at around E9.5 angiogenic sprouts invade the CNS parenchyma and

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is guided by endothelial tip cells (Figures 1B and 1D), later fuses with another vessel sprout in a process called anastomosis, and subsequently establishes an extended network of perfused vasculature (De Smet et al., 2009; Potente et al., 2011; Wacker and Gerhardt, 2011; Wälchli et al., 2015). Behind the tip cell, stalk cells proliferate, supporting the elongation of the growing blood vessel and form a lumen (Carmeliet and Jain, 2011; Geudens and Gerhardt, 2011; Potente et al., 2011; Quaegebeur et al., 2011; Wacker and Gerhardt, 2011; Wälchli et al., 2015) (Figure 1B). Recent experimental data and computational modeling suggest that endothelial tip and stalk cell specification is dynamically regulated by a feedback loop between VEGF-VEGFR signaling and the Dll4-Jagged-Notch pathway (Jakobsson et al., 2010). Upon stimulation with VEGF-A, activated endothelial cells expressing VEGFR1, 2, and 3 as well as Neuropilin-1 (Nrp-1) dynamically compete for the tip cell position by upregulating Dll4 (Jakobsson et al., 2010). Dll4 activates Notch signaling in adjacent stalk cells, which through transcriptional downregulation of VEGFR2,3 and Nrp-1, and upregulation of VEGFR1, restricts their ability to acquire the tip cell position (Blanco and Gerhardt, 2013). Dll4-Notch signaling limits the number of endothelial tip cells and tip cell filopodia, and blocking this pathway leads to increased tip cell and filopodia numbers. In addition to this feedback between tissue-induced activation and cell-cell contact-dependent lateral inhibition, also blood-born signals influence endothelial tip cell formation. BMP9 and 10, presumably provided by blood flow to the luminal endothelial surface, activate Alk1 and downstream Smad1/5/8 signaling, which converge on common transcriptional targets together with Notch signaling to limit tip cell formation (Larrivée et al., 2012; Moya et al., 2012). Interference with this central pattern generator of endothelial tip versus stalk cell specification fundamentally disturbs the angiogenic balance and vessel function in health and disease by causing excessive numbers of tip cells and tip cell filopodia (Blanco and Gerhardt, 2013; Geudens and Gerhardt, 2011; Potente et al., 2011). This VEGF-VEGFR-Dll4-Jagged-Notch pathway can additionally be modulated by extracellular matrix interactions (Germain et al., 2010; Stenzel et al., 2011) and other signaling cascades, e.g., involving molecules of the neurovascular link (Mancuso et al., 2008; Potente et al., 2011).

CNS Angiogenesis, the Neurovascular Unit and the Blood-Brain Barrier

These cellular and molecular processes involved in sprouting angiogenesis are also crucial for the vascularization of the brain tissue (Mancuso et al., 2008; Quaegebeur et al., 2011).

During mouse embryogenesis, the perineural vascular plexus (PNVP) around the neural tube forms via vasculogenesis at embryonic 8.5 (E8.5) and later gives rise to the arteries and veins of the leptomeninges (pia mater and arachnoidea) (Figure 2B). Experimental work with avian embryos showed that the formation of the PNVP depends on neural tube-derived VEGF-A signaling through VEGFR2 expressed on angioblasts forming the PNVP (Mancuso et al., 2008). Subsequently, at E9.5, endothelial sprouts emanate from the PNVP and invade into the CNS parenchyma, thereby forming the intraneural vascular plexus (INVP) via sprouting angiogenesis (Daneman et al., 2010) (Figures 2B–2D). The migration of endothelial cells into the CNS parenchyma and toward the subventricular zone (SVZ) is regulated by different signaling pathways. VEGF-A-VEGFR-Neuropilin-1 signaling has a critical role for appropriate vessel ingression and patterning and thus for the formation of the INVP (Mackenzie and Ruhrberg, 2012). Whereas during developmental stages neurons are the predominant source of VEGF-A, glial cells become the predominant producers of VEGF-A in the CNS once vascular remodeling is completed around postnatal day (P) 24 (Mancuso et al., 2008). Accordingly, ectopic overexpression of VEGF-A isoforms or the soluble VEGF-A decoy receptor sFlt-1 resulted in aberrant vessel ingression and vascular patterning of the avian neural tube (Bautch and James, 2009). Recent evidence shows that the Wnt ligands Wnt7a and Wnt7b as well as the G protein-coupled receptor GPR124 are also crucial for proper vessel ingression into the CNS parenchyma and the formation of CNS-specific properties of the INVP (Anderson et al., 2011; Cullen et al., 2011; Daneman et al., 2009; Kuhnert et al., 2010; Stenman et al., 2008). Angiogenic sprouting and vascular remodeling is further regulated by VEGF-VEGFRs-Nrp-1, Dll4-Notch signaling, Angiopoietins-Tie receptors, Integrin receptors, Wnts-Frizzled receptors TGFβ signaling, the axonal guidance ligand-receptor pair Slit2-Robo4, as well as DR6/TROY receptors (Jeansson et al., 2011; Mancuso et al., 2008; Stenzel et al., 2011; Tam et al., 2012).

migrate towards the ventricle, where pro-angiogenic factors such as VEGF-A and Wnts are produced. At the forefront of these angiogenic sprouts, endothelial tip cells guide the CNS-invading blood vessels using endothelial tip cell filopodia. The boxed area is enlarged in (C).

(C) Schematic representation of a coronal section of a mouse brain during postnatal development. Blood vessels (red) and nerves (green) are indicated in the cortex, corpus callosum, and the hippocampus (blood vessels only).

(D) Sprouting angiogenesis into the CNS parenchyma is regulated via a number of attractive and repulsive molecular cues presumably acting on endothelial tip cell filopodia: endothelial sprouts invading the CNS parenchyma from E9.5 onward grow along radial glia fibers towards the ventricle. Molecularly, sprouting angiogenesis into the CNS is regulated by non-CNS-specific cues for angiogenesis such as VEGF-A-VEGFR2/Neuropilin-1, Semaphorin-3A/Semaphorin-3E-Plexin-D1, Slit2-Robo4, as well as by CNS-specific cues such as GPR124, Wnt7a/b-Frizzled6, and DR6/TROY.

(E and F) Postnatally (e.g., at P4/P8), CNS angiogenesis is highly dynamic and the complex vessel network is mainly established via sprouting angiogenesis (E). At the forefront of vascular sprouts, endothelial tip cells guide the growing vessel, thereby further expanding the vascular network (F). At, e.g., P4 and P8, endothelial tip cells can be found in all cortical layers (I–VI) (E). Only few molecular cues are known that regulate postnatal brain angiogenesis (in contrast to the well-described postnatal retinal angiogenesis), for instance, Nogo-A. Angiogenic endothelial tip cells of growing blood vessels are also abundant in the hippocampus and the corpus callosum at P8 (not shown). The boxed area is enlarged in (F).

(G) Immunofluorescent staining of IB4^{+} endothelial sprouts invading the CNS tissue of the embryonic neural tube (hindbrain) at E8.5. The inset shows an endothelial tip cell with its filopodia. The scale bars represent 10 μm.

(H) Immunofluorescent staining of IB4^{+} blood vessels (red) and endothelial sprouts (arrowheads) in the postnatal cortex at P8. Cell nuclei (DAPI, blue). Labeled arrowheads (I and J) indicate tip cells that are highlighted on the right side (I and J). The scale bar represents 200 μm.

(I and J) Two IB4^{+} endothelial tip cells (red) in the mouse forebrain cortex at (P8). Note the numerous filopodial extensions exploring the local microenvironment for guidance cues. Cell nuclei (DAPI, blue). The scale bars represent 10 μm.

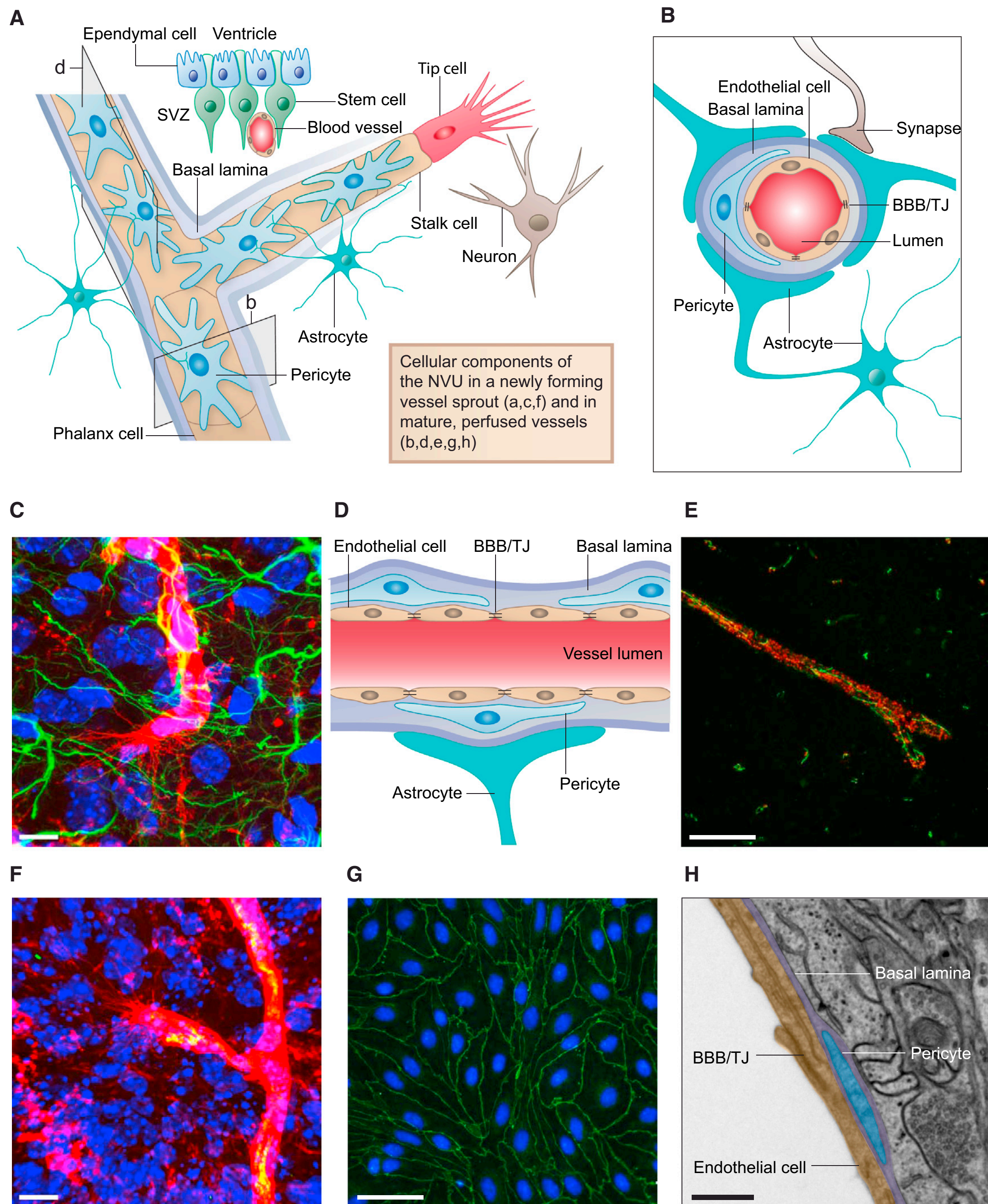

Figure 3. The Neurovascular Unit and the Blood-Brain Barrier

(A) Scheme of the neurovascular unit (NVU) for a newly forming vascular sprout showing involved perivascular cell types such as astrocytes, pericytes, and neurons. An endothelial tip cell guides the sprout throughout the tissue, followed by endothelial stalk cells. Phalanx cells are quiescent endothelial cells still capable of sensing angiogenic stimuli. At the level of the endothelial tip cell, the basal lamina is not (fully) established. The perivascular stem cell niche in the subventricular zone (SVZ) including ependymal cells, neuronal stem cells, and endothelial cells is shown as well. Cutting planes for (D) and (E) are indicated.

(B and D) Scheme of the NVU for established blood vessels that is composed of a variety of cell types including endothelial cells, pericytes, astrocytes, and neurons. Transverse (B) and longitudinal (D) sections of the scheme in (A) are shown. Endothelial cells and pericytes are ensheathed by a common basal lamina, the endothelial basement membrane (composed of the endothelial and the parenchymal basement membrane). The blood-brain barrier (BBB) is formed by microvascular endothelial cells that are connected via complex tight junctions (TJ), thereby inhibiting paracellular diffusion of watersoluble molecules. The endothelial cells regulating the transport of molecules between the blood and the brain parenchyma via the expression of influx and efflux transporters.

(C) An IB4$^+$ endothelial tip cell (red) in the mouse forebrain cortex at P8. The vascular endothelial tip cell extends numerous, finger-like filopodial extensions that explore the local microenvironment for guidance cues. GFAP$^+$ astrocytes and GFAP$^+$ radial glia (green), cell nuclei (DAPI, blue). The scale bar represents 10 μm.

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Postnatally, the CNS vasculature is further remodeled and expanded via sprouting angiogenesis (Figures 2E–2I) (Wälchli et al., 2015). Only few molecular cues besides VEGF-A (Ogunshola et al., 2000) are currently known to regulate postnatal vascular patterning. One recently identified example is, for instance, the axonal growth inhibitor Nogo-A (Wälchli et al., 2013). How these signaling pathways interact among each other and with the VEGF-VEGFR-Dll4-Jagged-Notch pathway to regulate brain angiogenesis is only poorly understood (Eichmann and Thomas, 2013; Mancuso et al., 2008).

At the cellular level, endothelial cells invading the CNS interact with cells of the surrounding CNS-parenchyma, including neurons, astrocytes, pericytes, postnatally also oligodendrocytes, as well as neural stem cells (Eichmann and Thomas, 2013; Mancuso et al., 2008; Quaegebeur et al., 2011) (Figures 3A–3F). Neurons, astrocytes, pericytes, and endothelial cells form the neurovascular unit (NVU) (Figures 3A–3E) are functionally coupled to regulate cerebrovascular interactions and contribute to the regulation of CNS angiogenesis (Eichmann and Thomas, 2013; Mancuso et al., 2008; Quaegebeur et al., 2011).

The importance of the interactions between the nervous and the vascular system was first illustrated by the early observation that the CNS parenchyme provides instructive signals that regulate endothelial cell sprouting into the CNS and simultaneously induce CNS-specific properties in endothelial cells (Stewart and Wiley, 1981; Tam and Watts, 2010). These specific properties of CNS blood vessels are, for instance, represented by the formation of the blood-brain barrier (BBB), the best-studied feature distinguishing CNS capillaries from vessels outside the CNS (Zlokovic, 2008) (Figures 3D–3G). The particular barrier properties of the endothelial cells forming the BBB are established during development, mainly by extrinsic cues provided by the CNS microenvironment (Stewart and Wiley, 1981; Tam and Watts, 2010). Properties intrinsic to the CNS endothelium have been proposed (Vasudevan et al., 2008) but a functional role during barriergenesis is unknown. The BBB comprises of complex tight junctions (Zlokovic, 2008) (Figures 3D–3G) and associated selective transport mechanisms to form a regulated physical permeability barrier that can become leaky in CNS pathologies (Storkebaum et al., 2011; Zlokovic, 2008, 2011). Interestingly, at certain sites in the CNS such as the SVZ, these barrier properties can locally be modified (Tavazoie et al., 2008).

Similar to the blood-brain barrier, the retina forms a highly sophisticated interface between the retinal tissue and the blood vascular system, called the blood-retina barrier (Runkle and Antonetti, 2011). The retina constitutes a part of the CNS that is vascularized postnatally, therefore allowing easy access. The relatively simple and flat geometry of the retinal tissue and its vascularization via the initial radial growth of blood vessels facilitates visualization of sprouting angiogenesis. Therefore, the retina is a commonly used model to study effects on sprouting angiogenesis and endothelial tip cell behavior (Pitulescu et al., 2010; Sawamiphak et al., 2010a). Other regions of the CNS such as the cortex, hindbrain, and even the spinal cord have a more complex three-dimensional structure that complicates a comprehensive description of vascular patterning. However, the emerging concept of organ- and region-specific mechanisms of angiogenesis (see below) highlights the importance of studying these brain tissues in more detail. Table 1 summarizes the methods currently available to study angiogenesis and barriergenesis in the CNS (brain, spinal cord, and retina) (Table 1).

In light of the importance of CNS tissue-derived signals for the differentiation of the brain endothelium and given that the role of common guidance cues on angiogenesis has been well described outside the CNS and in the CNS tissue of the retina (Carmeliet and Tessier-Lavigne, 2005; Eichmann and Thomas, 2013) but not in the brain, we aimed to review the effects of molecules involved in the neurovascular link on brain angiogenesis as compared to peripheral tissues.

Recent reviews have focused on the roles of all four of the classical families of axon guidance cues in angiogenesis (Eichmann and Thomas, 2013; Quaegebeur et al., 2011). Here, we first discuss two typical examples, namely Netrins and Semaphorins, and the Nogo family of proteins, before highlighting recent evidence on CNS-specific angiogenic cues. We further describe possible effects of these cues on blood-brain barrier formation and try to understand how these molecules can be integrated into the current concept of the neurovascular link. A special focus will be on molecular crosstalks of these angiogenic cues with VEGF-related pathways. We emphasize developmental processes but provide also occasional examples on neurovascular crosstalks in pathological conditions such as tumors and ischemic conditions.

General Mechanisms of Angiogenesis

Netrins and Their Receptors in Angiogenesis

Netrin-1. Netrin-1 signaling in angiogenesis and vascular guidance is facilitated by its interaction with the receptor Unc5b expressed on endothelial cells (Castets and Mehlen, 2010; Lu et al., 2004), which regulates angiogenesis in peripheral tissues as well as the CNS (Figure 4A; Table S1). In the zebrafish embryo, Netrin-1a is highly expressed in the ventral neural tube and the muscle pioneer cells at the horizontal myoseptum (HMS), revealing no major expression differences between CNS and non-CNS tissues (Lim et al., 2011; Wilson et al., 2006). At the functional level, morpholino-mediated knockdown of Unc5b or Netrin1a caused increased vessel branching of intersomitic (and thus non-CNS) vessels (ISVs) and caused guidance defects leading to aberrant ISV pathfinding (Lu et al., 2004), whereas knockdown effects on CNS vessels were not investigated.

(E) Established vessel in the adult mouse cortex displaying blood-brain barrier characteristics: Claudin5$^+$ (yellow) tight junctions connecting neighboring CD31$^+$ endothelial cells (red). VE-cadherin$^+$ cell-cell junctions (green). The scale bar represents 10 μm.
(F) IB4$^+$ endothelial tip cell (red) and PDGFRβ^+ pericytes (white) in the mouse forebrain cortex at P8. Cell nuclei (DAPI, blue). The scale bar represents 10 μm.
(G) Cultured mouse brain microvascular endothelial cells stained for the tight junction-marker occludin (green). Cell nuclei (DAPI, blue). The scale bar represents 10 μm.
(H) Electronic microscopy picture of the blood-brain barrier in the adult mouse brain cortex. Note the tight junctions between neighboring endothelial cells. The scale bar represents 10 μm.

Table 1. Methods to Address/Investigate Angiogenesis in the CNS

System	Method	Angiogenesis	Barriergenesis	References
Developmental Brain and Spinal Cord Angiogenesis				
Developmental brain and spinal cord angiogenesis	Embryonic mouse hindbrain angiogenesis	Yes	Yes	Fantin et al. (2013a, 2013b); Wälchli et al. (2015)
Developmental brain angiogenesis	Postnatal mouse brain angiogenesis	Yes	Yes	Harb et al. (2013); Wälchli et al. (2013); Whiteus et al. (2014)
Developmental CNS (brain) and non-CNS angiogenesis	Embryonic zebrafish angiogenesis	Yes	Yes	Bussmann et al. (2011); Ellertsdóttir et al. (2010); Lenard et al. (2013); Tam et al. (2012)
Developmental brain and spinal cord angiogenesis	Postnatal mouse brain angiogenesis (tracer injections, e.g., Evans blue, sulfo-NHS-biotin)	No?	Yes	e.g., Wang et al. (2012)
Developmental Retinal Angiogenesis CNS: RETINA				
Developmental retinal angiogenesis	Postnatal retina angiogenesis	Yes	Yes	Pitulescu et al. (2010); Sawamiphak et al. (2010a)
Developmental retinal angiogenesis	Postnatal retina angiogenesis—Miles assay	No?	Yes	e.g., Koch et al. (2011)
Developmental retinal angiogenesis	Postnatal retina angiogenesis	Yes	Yes	Gerhardt et al. (2003)

In the mouse, Unc5b, but not Unc5a and DCC, is expressed on blood vessel endothelial cells at different developmental time points (E10.5–E12.5) of both CNS and non-CNS tissues (Lu et al., 2004), including endothelial cells of the intersomitic arteries (Larrivée et al., 2007), as well as CNS endothelial cells of the INVP and intra-ocular vasculature (Lu et al., 2004). Interestingly, Unc5b is preferentially expressed on arteries (not veins) and also found on endothelial tip cells of the retina at P4 (Lu et al., 2004) (Figure 4A). In developing embryos of chick and quail, Unc5b is highly expressed on INVP endothelial cells and endothelial cells of vessels invading the limb bud, thereby displaying expression in CNS and peripheral blood vessels (Bouvrée et al., 2008). Similar to the situation in the mouse (Larrivée et al., 2007; Lu et al., 2004), Unc5b is predominantly expressed on arterial endothelial cells (Bouvrée et al., 2008) and downregulated in CNS and non-CNS vessels at later embryonic stages (E10–E13 CAM), when vessels acquire a quiescent state (Larrivée et al., 2007), further supporting Unc5b's role in restricting sprouting angiogenesis.

Genetic deletion of Unc5b leads to an increase in vessel branching and endothelial tip cell and filopodia number in the embryonic (E10.5/E12.5) mouse CNS (hindbrain, neural tube) as well as in non-CNS blood vessels like the internal carotid artery and the ISVs (at E10.5) (Lu et al., 2004). Moreover, intraocular injections of recombinant Netrin-1 induces retraction of endothelial tip cell filopodia and reduces the number of endothelial tip cells as well as the number of filopodia per tip cell in the mouse retina at P5 and in the mouse hindbrain at E10.5 (Larrivée et al., 2007; Lu et al., 2004). Netrin-1-Unc5b signaling mainly acts on sprouting angiogenesis and vessel branching in- and outside the CNS (Larrivée et al., 2007; Lu et al., 2004), as Netrin-1 does not regulate vasculogenesis (Bouvrée et al., 2008) and Unc5B mutants (Lu et al., 2004) show no effects on arterio-venous specification. Whether Netrin-1-Unc5b signaling interacts with VEGF-A-VEGFR2 signaling to regulate PNVP and INVP formation in the developing neural tube and brain remains unanswered. The relative normal appearance of the INVP in Unc5b mutant mice (Lu et al., 2004) suggests that Unc5b might modulate vascular patterning in the CNS without affecting the initial vessel ingression into the nervous tissue.

Molecularly, recent evidence suggests an interaction of Unc5b signaling with the VEGF-VEGFR2 signaling pathway (Koch et al., 2011) (Figure 4B; Table 2; Table S1). Koch and colleagues identified an interaction between Unc5b and Robo4, a vascular-specific receptor for Slit2 (Carmeliet and Tessier-Lavigne, 2005; Jones et al., 2008, 2009; Koch et al., 2011). Robo4-Unc5b binding and subsequent Unc5b signaling counteracts VEGF-VEGFR2 signaling via competition for Src protein recruitment—a downstream target of the VEGF-VEGFR2 signaling pathway (Koch et al., 2011) (Figure 4B). Accordingly, Unc5b could negatively regulate angiogenesis via direct regulation of VEGF signaling in vitro, but the relevance of these findings for angiogenesis in vivo is currently unclear. Whether Netrin-1-Unc5b signaling regulates angiogenesis via modulation of the VEGF pathway in different tissues in- and outside the CNS remains an open question.

Interestingly, the embryonic expression pattern (Lu et al., 2004) of Unc5b (on arteries and sprouting capillaries but not on veins) is recapitulated in pathological angiogenesis models such as oxygen-induced retinopathy (OIR) at P17 and subcutaneous tumor angiogenesis, exerting anti-angiogenic functions (Larrivée et al., 2007).

In addition to the anti-angiogenic roles of Netrin-1-Unc5b signaling (Bouvrée et al., 2008; Larrivée et al., 2007; Lu et al., 2004), several reports propose a contrasting pro-angiogenic role for Netrin-1 (Castets and Mehlen, 2010; Park et al., 2004; Wilson et al., 2006). For instance, Park and colleagues described that Netrin-1 induces angiogenesis in the chick chorioallantoic membrane (CAM) as well as in a mouse corneal micropocket assay in vivo, where it acts synergistically with

VEGF-A (Park et al., 2004). In contrast to previous reports (Lu et al., 2004), Li and colleagues found that Unc5b$^{-/-}$ mice show no vascular patterning- and angiogenesis defects in-and outside the CNS at E10.5–E11.5 (Wilson et al., 2006). Interestingly, however, Netrin-1-Unc5b signaling exerts vascular bed-specifc effects on angiogenesis in mouse and zebrafish, as placental anigogenesis in Unc5b mutant mice and parachordal vessels (PAVs) development in Netrin1a- and Unc5b morphant zebrafish is impaired (Navankasattusas et al., 2008; Wilson et al., 2006). Moreover, Netrin1a is involved in co-patterning of vessels and nerves as it regulates patterning of motoneuron axons which is important for normal PAV sprouting (Lim et al., 2011).

Neuro-vascular interactions at the functional level were observed in diabetic mice where Netrin-1 and Netrin-4 increased capillary density of the vasa nervosa, leading to increased motor and sensory nerve conduction velocities (Wilson et al., 2006).

In vitro, Netrin-1 and Netrin-4 promote tube formation, proliferation, and migration of peripheral endothelial cells such as HUVECs and HUAECs but since none of the known Netrin-1 receptors (DCC, Unc5a-d, Neogenin, Adenosin2b [A2b]) could be detected in these endothelial cells, Wilson et al. suggested that Netrin's proangiogenic effects are mediated via yet unidentified Netrin receptors (Wilson et al., 2006).

Taken together, Netrin-1 has the capacity to act—similar to its function in axonal guidance—as a bifunctional guidance cue in the vascular system: repulsion via endothelial Unc5b (Larrivée et al., 2007; Lu et al., 2004), attraction via yet unknown receptors on endothelial cells (Castets and Mehlen, 2010; Wilson et al., 2006). Unc5b is preferentially expressed on arteries and capillary endothelial tip cells and Netrin-1-Unc5b signaling mostly restricts sprouting angiogenesis, tip cell filopodia extension, and vessel branching during development in-and outside the CNS (Larrivée et al., 2007; Lu et al., 2004).

The debate about these contradicting reports on the negative and positive regulation of angiogenesis by Netrin-1 is still ongoing (Castets and Mehlen, 2010; Larrieu-Lahargue et al., 2012), but neither the anti- nor the pro-angiogenic roles of Netrin-1 or Netrin-4 have shown CNS specificity (Navankasattusas et al., 2008; Wilson et al., 2006) (Table 3; Table S1).

Netrin-4. Netrin-4 can either act as an anti-angiogenic molecule through binding to Neogenin-Unc5B and negative regulation of VEGF signaling (Lejmi et al., 2008) or as a pro-angiogenic molecule in the lymphatic system via regulation of integrin function (Larrieu-Lahargue et al., 2010) (Figure 4A; Table S1). In the developing zebrafish, Netrin-4 is expressed in blood vessel endothelial cells of the trunk, e.g., ISVs, and in CNS-blood vessel endothelial cells of the brain, retina, and eye, as well as in neurons of certain fiber tracts (Lambert et al., 2012). Functionally, morpholino-mediated knockdown of Netrin-4 leads to severe defects in non-CNS vessels (lack of ISV outgrowth), as well as of the cranial vasculature, suggesting that the pro-angiogenic effect of Netrin-4 is not tissue specific regarding the CNS versus non-CNS domains (Lambert et al., 2012). In the mouse, Netrin-4 is broadly expressed at embryonic (E11.5–E18.5), postnatal (P20), and adult stages, including various regions of the CNS (brain, spinal cord) and the periphery such as pancreas, kidney, intestine, and thymus (Yin et al., 2000).

In vitro, Netrin-4 acts as pro-angiogenic stimulus to regulate survival, proliferation, migration, tube formation, and sprouting angiogenesis of non-CNS endothelial cells (HUVECs and HUAECs) (Lambert et al., 2012). Interestingly, these effects are mediated via phosphorylation of the protein kinases FAK, Akt, JNK1/2, and ERK1/2 in HUVECs (Lambert et al., 2012), which are common downstream targets of VEGF-VEGFR2 signaling, therefore suggesting a crosstalk between the Netrin-4 and these VEGF-A related pathways (Figure 4B; Table 2).

Netrin-4 has described pro-angiogenic roles also in angiogenesis of CNS pathologies, for instance, in a mouse model of cerebral ischemia (Hoang et al., 2009).

In contrast to these pro-angiogenic effects, others have found anti-angiogenic roles for Netrin-4. Netrin-4 inhibits VEGF-induced HUAEC (but not HUVEC) migration, tube formation, and branching in vitro (Lejmi et al., 2008), likely mediated via binding of Netrin-4 to Neogenin and recruitment of Unc5b (Figures 4A and 4B). Mechanistically, Netrin-4 increases the interaction between Unc5b and Neogenin in VEGF-stimulated (or FGF-2-stimulated) HUAECs and Netrin-4 signaling exerts its anti-angiogenic effects via inhibition of VEGF-induced FAK-phosphorylation in HUAECs (Lejmi et al., 2008) (Figure 4B; Table 2). This mechanism might also be at work in pathological angiogenesis, as Netrin-4 overexpression reduced VEGF- and FGF2-induced tumor angiogenesis in a subcutaneous xenograft tumor model in vivo (Lejmi et al., 2008). Therefore, it will be interesting to investigate whether Netrin-4 and VEGF signaling crosstalk to exert pro-angiogenic effects on neo-angiogenesis in CNS pathologies, e.g., in brain tumors.

In summary, Netrin-1 and Netrin-4 and their receptors act as repulsive or attractive cues—partially via regulation of VEGF signaling (Koch et al., 2011; Lejmi et al., 2008)—in developmental angiogenesis in- and outside the CNS.

Semaphorins and Their Receptors in Angiogenesis

Semaphorins signal via Plexin receptors to regulate angiogenesis (Figure 4A), while a functional interaction with the Neuropilin-1 (Nrp-1) receptor in angiogenesis has not been convincingly established (see discussion below). In accordance with their inhibitory roles in axonal guidance (Dickson, 2002), Semaphorins usually inhibit angiogenesis (Figure 4B), although some family members can be stimulatory (Capparuccia and Tamagnone, 2009). Here, we will mainly focus on class 3 Semaphorins as they are the best-described Semaphorins in developmental angiogenesis.

Semaphorin-3A. Semaphorin-3A and its receptor Plexin-D1 negatively regulate angiogenesis in zebrafish through modulation of VEGF signaling (Figures 4A and 4B; Table S2). The role of Semaphorin-3A in mouse angiogenesis is controversial (Figure 4A; Table S2).

In the developing zebrafish, Semaphorin-3A1 and -3A2 are expressed in the somites but absent from intersomitic boundaries containing ISVs, whereas the Semaphorin receptor Plexin-D1 is specifically expressed throughout the zebrafish vasculature including ISVs (Torres-Vázquez et al., 2004). Semaphorin-3A1/Semaphorin-3A2 and Plexin-D1 morphants, as well as the Plexin-D1 mutant *out of bounds* (*obd*) display vascular patterning defects of ISVs: while ISVs usually grow between somite blocks, ISVs of morphants and *obd* mutants do not follow

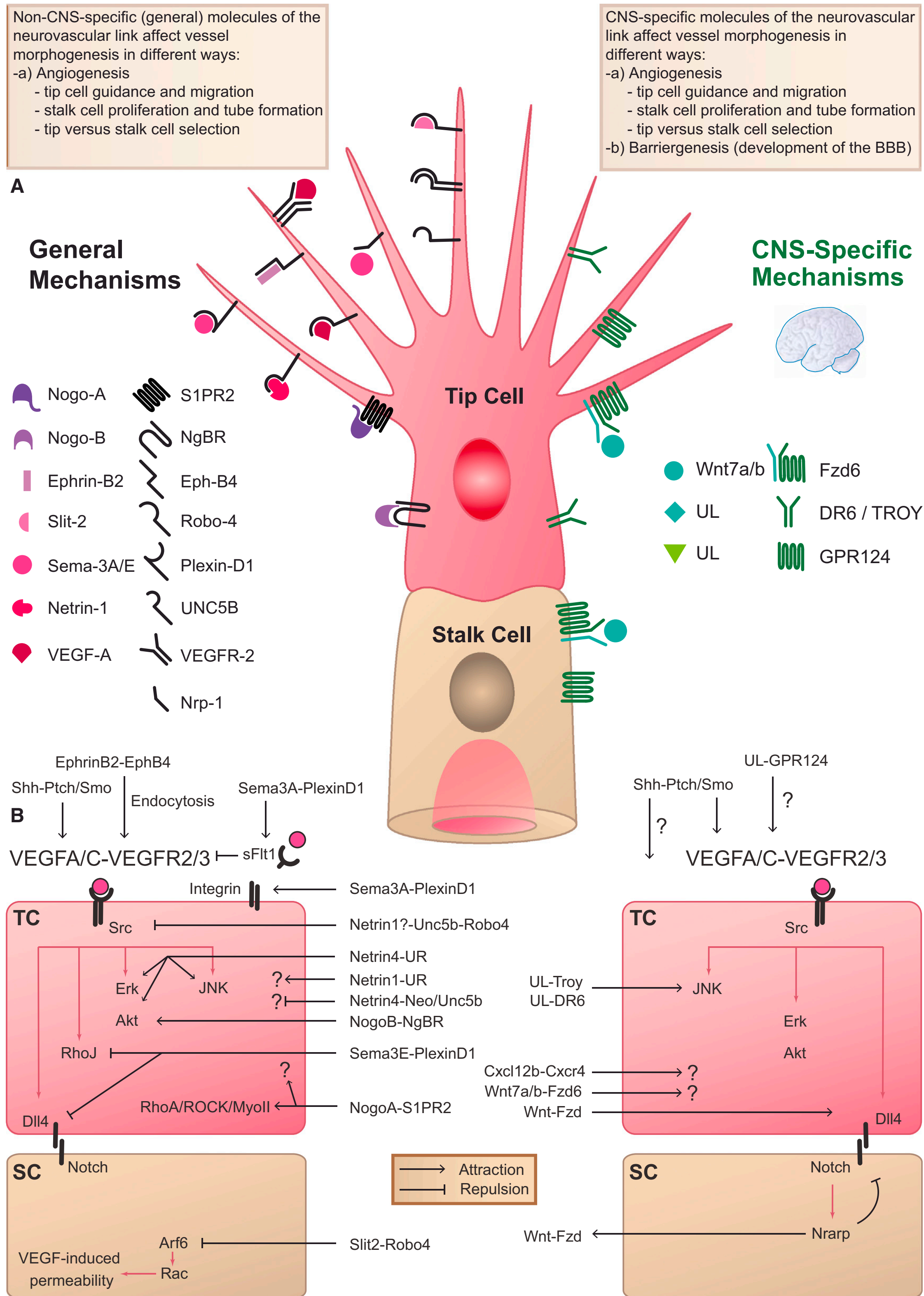

Figure 4. General- and CNS-Specific Mechanisms of Angiogenesis and Endothelial Tip Cell Guidance

(A) Molecules of the neurovascular link and their receptors implicated in general- and CNS-specific mechanisms of developmental angiogenesis. Receptors in both categories are expressed on endothelial tip cell (filopodia) as well as on endothelial stalk cells. Most of these receptors are expressed on both endothelial tip

(legend continued on next page)

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Table 2. Molecular Interactions with the VEGF-VEGFR2 Signaling Pathway

Ligand-Receptor Pair	Ligand-Receptor Level	Signaling (Protein) Level	Transcriptional (mRNA) Level
Ligand-Receptor Pairs with Global Functions in Angiogenesis and Vascular Patterning			
VEGF-A-VEGFR2			Plexin-D1↑ (Kim et al., 2011), Netrin-4, Neogenin, Unc5b↑ (Lejmi et al., 2008)
Unc5b-Robo4	?	Src (recruitment) (Koch et al., 2011)	
Netrin-1-Unc5b	?	?	?
Netrin-4-UR	?	Erk, Akt, JNK (Lambert et al., 2012)	
Netrin-4-Neo/Unc5b	?	FAK (Lejmi et al., 2008)	?
Sema3A-Plexin-D1		Integrin (Serini et al., 2003)	sFlt expression↑ (Zygmunt et al., 2011)
Sema3E-Plexin-D1	?	RhoJ (Fukushima et al., 2011)	Dll4 expression↓ (Kim et al., 2011)
Sema3F-unknown receptor Sema7a-unknown receptor	?	?	?
EphrinB2-EphB4	VEGFR2/3 endocytosis (Sawamiphak et al., 2010b; Wang et al., 2010b)	?	?
Slit2-Robo4	?	Arf6/Rac (Jones et al., 2009)	?
NogoA-S1PR2?		RhoA? (Wälchli et al., 2013)	?
NogoB-NgBR	?	Akt (Zhao et al., 2010)	
Sonic hedgehog-Ptch/Smo			VEGF-A isoforms↑ (Pola et al., 2001)
CNS-Specific Regulators of Angiogenesis			
Wnt7a/b-(Fzd6)	?	?	?
Norrin-Fzd4	?	?	VEGF-A, VEGFR1, VEGFR2, Nrp1, Nrp2↑ (Wang et al., 2012)
Unknown ligand-GPR124	?	?	VEGF-A↑ (Cullen et al., 2011)
Unknown ligand-DR6/TROY	?	JNK (Tam et al., 2012)	?

the intersegmental boundaries but form ectopic branches along the trunk (Torres-Vázquez et al., 2004). This mispatterning was described for ISVs, but it is unknown whether CNS vessel patterning is also affected (Torres-Vázquez et al., 2004).

Addition of Semaphorin-3A to Plexin-D1-expressing HUVECs leads to collapse of actin stress fibers and migration inhibition in vitro suggesting a direct guidance mechanism via effects on the actin cytoskeleton (Torres-Vázquez et al., 2004). However, a recent study proposed an alternative mechanism, namely interaction of Semaphorin-3A1/A2-Plexin-D1 with the VEGF signaling pathway (Zygmunt et al., 2011) (Figure 4B; Table 2). Plexin-D1 was shown to be necessary in endothelial cells for the regulation of soluble Flt1 (sFlt1) expression, a splice variant of VEGFR1 acting as a decoy receptor (blocking VEGFR signaling) (Figure 4B). Accordingly, the loss of sFlt1 in Plexin-D1 mutants leads to an increased number of endothelial tip cells and subsequent hyperbranching (Zygmunt et al., 2011). Although Semaphorins-3A1/3A2 and Plexin-D1 seem to be expressed in the CNS (Torres-Vázquez et al., 2004; Zygmunt et al., 2011), no functional data with regard to its effects on brain angiogenesis is available in zebrafish.

During embryonic mouse development, Semaphorin-3A is expressed at E10 in vascular endothelial cells in the spinal cord and in the dorsal aorta (Serini et al., 2003). Interestingly, at E12.5, endothelial cells of perineural blood vessels that sprout into the brain parenchyma express Semaphorin-3A, indicating its expression on active, sprouting endothelium (Serini et al., 2003). Thus, endothelial Semaphorin-3A expression does not

and stalk cells but are—for simplicity—only displayed on one endothelial cell type. The different (CNS-specific or generally/globally acting) ligand-receptor pairs regulate multiple aspects of angiogenesis: vessel guidance (either as attractive or repulsive cues), endothelial proliferation and tube formation as well as tip/stalk cell selection. CNS-specific cues for angiogenesis additionally regulate blood-brain barrier-formation and differentiation. See also Tables S1–S4.
(B) Molecules of the neurovascular link (either CNS-specific or general/non-CNS specific) interact with the canonical angiogenic VEGF-A/C-VEGFR2/3 pathway at multiple levels to affect different angiogenic functions: (1) to affect tip cell guidance via cytoskeleton regulation, e.g., Netrin-1-Unc5b, Semaphorin-3A-Plexin-D1, Semaphorin-3E-Plexin-D1 (RhoJ), or EphrinB2-EphB4, (2) to determine the tip-to-stalk cell ratio via Dll4/Notch, e.g., Semaphorin-3E-Plexin-D1 or Wnt/β-catenin, (3) to regulate sprouting angiogenesis via interactions with the VEGF-pathway, e.g., Nogo-B-NgBR (Akt), DR6/TROY (JNK), or Unc5b (Src), (4) to modulate VEGF receptor endocytosis, e.g., EphrinB2-EphB4, and (5) to modulate vascular permeability and tube formation, e.g., Slit2-Robo4 (Arf6, Rac). However, some evidence also suggests that not all molecular players converge toward the VEGF-VEGFR-Dll4-Jagged-Notch pathway. For example, the CNS-specific cue GPR124 as well as Nogo-A seem to regulate angiogenesis without massive effects on VEGF-A-VEGFR2-Dll4-Jagged-Notch signaling. Other examples of molecules that regulate vascular morphogenesis independently of the VEGF pathway are FGF and BMP. See also Tables S1–S4. TC, tip cell; SC, stalk cell; UL, unknown ligand; UR, unknown receptor.

Table 3. Developmental Brain/SC versus Retina versus Non-CNS Angiogenesis

Developmental Brain and Spinal Cord Angiogenesis		Developmental Retinal Angiogenesis	
Embryonic Angiogenesis			
Developmental process	Ligand-receptor pair	Developmental process	Ligand-receptor pair
E8.5: PNVP formation (vasculogenesis)	VEGF-A-VEGFR2	The neuronal retina remains avascular during embryonic stages	
E9.5: INVP formation (sprouting from PNVP into CNS parenchyma):	VEGF-A-VEGFR2 Wnt7a/7b-unknown receptor Unknown ligand-GPR124		
E9.5–P25: INVP formation (angiogenic sprouting and migration toward the SVZ)	VEGF-A-VEGFR2 Unknown ligand-Neuropilin Semaphorin-3E-Plexin D1 Ephrin B2-Eph B4 Slit 2-Robo 4		
Postnatal Angiogenesis			
E9.5–P25: INVP formation (sprouting angiogenesis and remodeling?)	VEGF-A-VEGFR2 Nogo-A-(S1PR2?) Thyroid hormone-unknown receptor?	P0–P7: formation of superficial retinal vascular plexus (sprouting angiogenesis [radial, 2D])	VEGF-A/B/C-VEGFR1/2/3 Netrin-1-Unc5b Semaphorin-3E-Plexin D1 Ephrin B2-Eph B4 Slit 2-Robo 4 Nogo-A-(S1PR2?)
		P7–P14: formation of deeper retinal vascular plexi (sprouting angiogenesis into deeper plexi [vertical, 3D] and sprouting angiogenesis within deeper plexi [radial, 2D])	Wnt-VEGFR1 Norrin-Frizzled4 Nogo-A-(S1PR2?)
Ligand-Receptor Pairs with Global Functions in Angiogenesis and Vascular Patterning			
Ligand-receptor pair	Retinal angiogenesis	Brain/s.c. angiogenesis	Non-CNS angiogenesis
VEGFA/B/C-VEGFR1/2/3	Yes (Gerhardt et al., 2003; Stone et al., 1995)	Yes (Carmeliet et al., 1996; Ferrara et al., 1996)	Yes (Carmeliet et al., 1996; Ferrara et al., 1996)
Netrin-1-Unc5b	Yes (Lu et al., 2004)	Yes (Lu et al., 2004)	Yes (Lu et al., 2004; Park et al., 2004)
Netrin-4-Unc5b	Yes (Lejmi et al., 2008), pathological angiogenesis only	?	Yes (Wilson et al., 2006)
Semaphorin-3A-Plexin-D1	?	?	Yes (Serini et al., 2003; Torres-Vázquez et al., 2004)
Semaphorin-3E-Plexin-D1	Yes (Kim et al., 2011)	?	Yes (Gu et al., 2005)
Semaphorin-3F-unknown receptor Semaphorin-7A-unknown receptor	?	?	?
EphrinB2-EphB4	Yes (Sawamiphak et al., 2010b; Wang et al., 2010b)	Yes (Sawamiphak et al., 2010b)	Yes (Sawamiphak et al., 2010b; Wang et al., 2010b)
Slit2-Robo4	Yes (Jones et al., 2008; Wang et al., 2003)	?	Yes (Bedell et al., 2005)
NogoA-S1PR2?	Yes (Wälchli et al., 2013)	Yes (Wälchli et al., 2013)	?
NogoB-NgBR	?	?	Yes (Miao et al., 2006; Zhao et al., 2010)
Sonic hedgehog-Ptch/Smo	?	?	Yes (Nagase et al., 2008; Pola et al., 2001)
CNS-Specific Regulators of Angiogenesis			
Ligand-receptor pair	Retinal angiogenesis	Brain/s.c. angiogenesis	Non-CNS angiogenesis
Wnt7a/b-(Fzd6)	?	Yes (Daneman et al., 2009; Stenman et al., 2008)	No (Daneman et al., 2009; Stenman et al., 2008)
Norrin-Fzd4	Yes (Xu et al., 2004), regulation of BBB integrity in the brain	Yes (Wang et al., 2012)	Yes (Luhmann et al., 2005; Rehm et al., 2002)
Unknown ligand-GPR124	?(Kuhnert et al., 2010) - GPR124 is also expressed in retinal ECs	Yes (Anderson et al., 2011; Cullen et al., 2011; Kuhnert et al., 2010)	No (Anderson et al., 2011; Cullen et al., 2011; Kuhnert et al., 2010)
Unknown ligand-DR6/TROY	?	Yes (Tam et al., 2012)	No (Tam et al., 2012)

reveal any specificity between the CNS and non-CNS organs but seems to be selectively expressed on angiogenic but not on quiescent endothelial cells.

Functionally, endothelial Semaphorin-3A is a regulator of vascular branching in mouse and chick CNS and non-CNS organs (Acevedo et al., 2008; Serini et al., 2003). Semaphorin-3A$^{-/-}$ mice showed decreased vascular branching in cranial blood vessels (CNS) and in trunk ISVs (non-CNS) at E9.5 (Serini et al., 2003). Remodeling of the developing chick PNVP into small capillaries and large-caliber vessels is severely affected by overexpression of Semaphorin-3A/F, of Nrp-1, or of Plexin-A1. This PNVP-remodeling is integrin dependent (Serini et al., 2003). Accordingly, in vitro, Semaphorin-3A inhibited integrin-mediated adhesion of endothelial cells on the ECM-ligands vitronectin and fibronectin and also inhibited directed migration of HUVECs toward fibronectin and vitronectin gradients in a Plexin-A1- and Nrp-1-dependent manner (Serini et al., 2003).

According to Serini et al., Semaphorin-3A negatively regulates integrin activity to modulate endothelial cell adhesion and migration therefore allowing proper vascular branching and remodeling during sprouting angiogenesis in vivo (Serini et al., 2003). Semaphorin-3A also inhibits developmental angiogenesis in the chick CAM (Acevedo et al., 2008) and the quail limb buds (Bates et al., 2003). However, despite the above-mentioned evidence, the role of Semaphorin-3A on developmental angiogenesis has been questioned as other studies showed that Semaphorin-3A$^{-/-}$ mice do not display any vascular phenotype in vivo (Vieira et al., 2007) and no effects on endothelial cell migration and adhesion in vitro (Pan et al., 2007).

In the postnatal mouse retina (P5 to P8), Semaphorin-3A-Nrp-1 signaling regulates sprouting angiogenesis and vessel remodeling (Pan et al., 2007). Intraocular injection of anti-Nrp-1 antibody blocking Semaphorin-3A-Nrp-1 binding shows that Semaphorin-3A-Nrp-1 signaling induces vascular remodeling in the mature parts of the superficial retinal plexus (Pan et al., 2007). In contrast, the anti-Nrp-1 antibody as well as an anti-VEGF antibody inhibited sprouting angiogenesis into the deeper layers of the retina, suggesting pro-angiogenic roles for Semaphorin-3A. Surprisingly, in vitro, addition of anti-Nrp-1 antibody had only minor effect on VEGF-VEGFR2 signaling (Figure 4B).

Taken together, although these data suggest that Semaphorin-3A regulates angiogenesis in the CNS and the periphery, the molecular mechanisms including the interactions with the VEGF-VEGFR pathway remain elusive (Figures 4A and 4B; Tables 2 and 3; Table S2).

Semaphorin-3E. Semaphorin-3E is a special case among the Class 3 Semaphorins because it is the only family member that does not bind to Nrp-1 but directly to the signal-transducing unit Plexin-D1 to initiate downstream signaling (Gu et al., 2005) (Figure 4A). Semaphorin-3E-Plexin-D1 signaling negatively regulates angiogenesis in- and outside the CNS via interaction with the VEGF-VEGFR-DLL4-Jagged-Notch pathway (Figures 4A and 4B; Tables 2 and 3; Table S2).

In zebrafish, Semaphorin-3E and Semaphorin receptors Plexin-D1 and -B2 are expressed in endothelial cells of the dorsal aorta (DA), from which ISVs form (Lamont et al., 2009). Semaphorin-3E and Plexin-B2 morphants show a delayed outgrowth of ISVs but no apparent guidance defects, which is distinct from the Plexin-D1 mutant *obd* and Plexin-D1 morphants described above (Torres-Vázquez et al., 2004). Transplantation experiments in zebrafsh indicate that endothelial Semaphorin-3E signals to endothelial Plexin-B2 in paracrine and autocrine manners to regulate the precise timing of ISV formation, by a yet unknown molecular mechanism (Lamont et al., 2009). These data suggest that different Semaphorin-Plexin ligand-receptor pairs display different functions in zebrafish ISV angiogenesis, i.e., Semaphorin-3A-Plexin-D1 acting on vascular guidance/patterning (Torres-Vázquez et al., 2004; Zygmunt et al., 2011), whereas Semaphorin-3E-Plexin-B2 controls the initiation and timing of ISV sprouting. The expression of Semaphorin-3E and Plexin-B2 on CNS vessels and possible functions on brain angiogenesis in these mutants were not investigated.

In the mouse, Semaphorin-3E is a negative regulator of ISV angiogenesis (Gu et al., 2005). In contrast to the situation in zebrafish (Lamont et al., 2009), Semaphorin-3E is expressed on somites, whereas similar to zebrafish, mouse Plexin-D1 is expressed on ISV endothelial cells (Gu et al., 2005), suggesting different cellular mechanism between these species.

Binding studies showed that Semaphorin-3E specifically binds Plexin-D1 but not Nrp-1 in vivo and in vitro (Gu et al., 2005). Accordingly, Plexin-D1$^{-/-}$ mice display excessive branching of ISVs into Semaphorin-3E expressing somites, while in vivo overexpression of Semaphorin-3E in chicken somites creates regions devoid of blood vessels, indicating that Semaphorin-3E-Plexin-D1 negatively regulates angiogenesis and functions as repulsive cue in vascular guidance (Gu et al., 2005). Interestingly, Semaphorin-3E-Plexin-D1 function is not restricted to non-CNS vessels: in the mouse retina at P2–P6, endothelial Plexin-D1 expression is highly enriched at the sprouting front including endothelial tip and stalk cells as well as veins but is absent from mature vessels and arteries, indicating its role in active sprouting angiogenesis (Fukushima et al., 2011; Kim et al., 2011). At the same developmental time points, Semaphorin-3E is expressed in retinal ganglion cells over the entire retina and therefore not restricted to sites of active angiogenesis (Fukushima et al., 2011; Kim et al., 2011), indicating that the receptor rather than the ligand expression spatially specifies angiogenic sites.

Functionally, Semaphorin-3E$^{-/-}$ and Plexin-D1$^{-/-}$ mice display vascular patterning defects characterized by an unevenly growing retinal vascular front (Kim et al., 2011), whereas injection of function-blocking Plexin-D1-Fc increased vascular branching and neuronal Semaphorin-3E restricts endothelial migration into the deeper retinal plexus (Fukushima et al., 2011), thereby suggesting negative regulatory effects of Semaphorin-3E-Plexin-D1 on postnatal angiogenesis via neuro-vascular interactions in the CNS. Whether Semaphorin-3E-Plexin-D1 signaling interacts with VEGF-A-VEGFR2 signaling to regulate PNVP and INVP formation in the developing neural tube and brain remains to be explored.

Mechanistically, Semaphorin-3E-Plexin-D1 signaling leads to downstream activation of the small GTPase RhoJ expressed in endothelial cells and subsequent retraction of retinal endothelial filopodia (Fukushima et al., 2011) (Figure 4B). In line with this idea, intraocular injection of Plexin-D1-Fc induced the formation of more filopodia in tip cells and increased vascular density in the

plexus, likely via RhoJ (Fukushima et al., 2011). This molecular interaction is effective during retinal development, in cultured non-CNS endothelial cells (HUVEC) in vitro as well as in ischemic retinopathy in vivo, where Plexin-D1-RhoJ selectively inhibits pathological extraretinal neovessel ingrowth without affecting normal (non-pathological) retinal vessels (Fukushima et al., 2011).

Furthermore, Semaphorin-3E-Plexin-D1 activation inhibits VEGF-induced Dll4 expression in retinal endothelial cells in vivo and in HUVECs in vitro, thereby regulating the tip/stalk cell selection in CNS and non-CNS endothelial cells (Fukushima et al., 2011) (Figure 4B; Table 2). Semaphorin-3E$^{-/-}$ mice display increased Dll4 levels in retinal endothelial cells leading to increased Notch signaling and a subsequent decreased number of endothelial tip cells and this phenotype could be rescued by the Dll4/Notch signaling inhibitor DAPT (Kim et al., 2011). Accordingly, intraocular injection of Semaphorin-3E caused a downregulation of endothelial Dll4, whereas injection of function blocking Plexin-D1-Fc upregulated endothelial Dll4 expression (Kim et al., 2011).

Interestingly, VEGF-A-VEGFR2 and Semaphorin-3E-Plexin-D1 are involved in a negative feedback mechanism involving Dll4 (Figure 4B): VEGF-A-VEGFR2 signaling leads to upregulation of endothelial Dll4 and Plexin-D1 in tip cells, while Semaphorin-3E-Plexin-D1 signaling downregulates endothelial Dll4 expression in tip cells (Kim et al., 2011). Therefore, VEGF-A directly activates Dll4-Notch signaling and indirectly inhibits Dll4-Notch signaling via Plexin-D1 upregulation (Kim et al., 2011). Accordingly, intraocular injection of VEGF-A leads to an expanded Plexin-D1 expression toward the more mature vascular plexus to inhibit active sprouting.

The observed phenotypes suggest two distinct underlying molecular mechanisms at work in the developing retina: repulsive effects on migration to regulate sprouting into the deeper retina and modulation of tip/stalk cell selection to ensure normal sprouting at the retinal vascular front, presumably via the modulation of the VEGF-VEGFR pathway (Figure 4B; Table 2). Whether these mechanisms hold true in tissues outside the CNS remains to be determined. Although Nrp-1 is a receptor for Semaphorin ligands in neural development, the precise role for the ligand receptor pair Semaphorin-3 s-Nrps on angiogenesis is debated: whereas Semaphorin-3E interacts with Plexin-D1 independently of Nrp-1 to inhibit angiogenesis (Gu et al., 2005), the precise role for the ligand receptor pair Semaphorin-3A-Nrps on developmental angiogenesis in vivo is less clear. As Nrp-1 binds Semaphorin-3 s but also VEGF-A (Soker et al., 1998), it is difficult to precisely distinguish between effects of Semaphorin-3A-Nrp-1 versus VEGF-A-Nrp-1 signaling (Gu et al., 2005; Serini et al., 2003).

While the in vivo function of Semaphorin-3A in mouse is controversial (see above), Nrp-1 has a well-described function in tip cells during angiogenic sprouting in the CNS (Fantin et al., 2013a; Gerhardt et al., 2004). In the embryonic mouse, Nrp-1 is expressed in endothelial tip and stalk cells of vascular sprouts invading the brain parenchyma at E10.5–E11.5 as well as on neural progenitors and on macrophages (Fantin et al., 2013a; Gerhardt et al., 2004). In Nrp-1$^{-/-}$ mice, endothelial tip cell filopodia fail to reorient from their initial direction along radial glia processes to a perpendicular direction along the ventricles, suggesting a guidance function for Nrp-1 (Gerhardt et al., 2004). Interestingly, endothelial cells with higher Nrp-1 levels have a higher chance to become a tip cell rather than a stalk cell during CNS sprouting angiogenesis in vivo, suggesting an important role for Nrp-1 in tip cell selection and function (Fantin et al., 2013a).

In summary, Semaphorin-3A and Semaphorin-3E regulate angiogenesis in CNS and non-CNS tissues acting predominantly as negative angiogenic cues (Table 3; Table S2).

Other Semaphorins. Notably, some pro-angiogenic roles of Semaphorins apart for Semaphorin-3A (see above) have been described for Semaphorin-6D and Plexin-A1 (Toyofuku et al., 2004), Semaphorin-4A and Plexin-D1 (Toyofuku et al., 2007), and for Semaphorin-5A (Fiore et al., 2005), whereas all these ligand-receptor pairs do not show any specificity for CNS angiogenesis.

Nogo Proteins and Their Receptors in Angiogenesis

Nogo-B. Nogo-B and its specific receptor NgBR, which was initially characterized in the vasculature, play a crucial role as pro-angiogenic cues during developmental non-CNS angiogenesis in the embryonic zebrafish via interaction with the VEGF signaling pathway (Figures 4A and 4B; Tables 2 and 3; Table S3) (Miao et al., 2006; Zhao et al., 2010).

At 24 hpf, Nogo-B expression was found on somites and in the brain, while NgBR was expressed on endothelial cells of ISVs and of the DA, as well as in neural tissue of the brain (Zhao et al., 2010). Morpholino-mediated knockdown of Nogo-B or NgBR lead to absent or misoriented ISVs at 24 hpf. However, vasculogenesis (angioblast proliferation and migration) were not disturbed upon NgBR knockdown, suggesting that the Nogo-B-NgBR ligand receptor pair acts primarily to regulate sprouting angiogenesis in vivo (Zhao et al., 2010). Nogo-B and NgBR expression patterns on CNS endothelial cells as well as effects of Nogo-B or NgBR knockdown on CNS angiogenesis were not investigated but would be interesting to address in light of the expression of these proteins in the brain (see above).

In the adult mouse, Nogo-B is expressed in endothelial cells and vascular smooth muscle cells of different (non-CNS) blood vessels including the femoral and carotid arteries and the coronary vessels as well as on HUVECs (Acevedo et al., 2004). With regard to the CNS, Nogo-B is detectable in adult mouse brain extracts as well as in adult and postnatal mouse cortical endothelial cells (Acevedo et al., 2004; Wälchli et al., 2013), suggesting a Nogo-B expressed in endothelial and other cell types in- and outside the CNS. NgBR, the receptor of Nogo-B, is also expressed in the adult mouse, in a variety of non-CNS organs such as the heart, liver, kidney, and pancreas but was not detectable in brain extracts (Miao et al., 2006) suggesting a possible role of NgBR on angiogenesis in vascular patterning only outside the CNS. NgBR is expressed in HUVECs (Acevedo et al., 2004; Miao et al., 2006; Zhao et al., 2010) but its expression on CNS endothelial cells as well as the role of Nogo-B-NgBR in CNS angiogenesis is not known.

Current literature suggests that Nogo-B is a positive and Nogo-A a negative regulator of angiogenesis (Wälchli et al., 2013; Zhao et al., 2010). Interestingly, Nogo-A/B$^{-/-}$ mice are viable and fertile (Schwab, 2010) and show no obvious defects

in developmental (P21) ear skin angiogenesis and vascular patterning (Yu et al., 2009). Given the opposing roles of the two Nogo-isoforms (Acevedo et al., 2004; Wälchli et al., 2013), it is therefore possible that the lack of vascular phenotype in the double knockout mice is due to a compensation mechanisms between the two Nogo isoforms or due to a compensation by other molecules as shown for the Nogo-A KO mouse where several Semaphorins and Ephrins are upregulated (Kempf et al., 2013). Taken together, Nogo-B's in vivo expression and function in developmental angiogenesis in- and outside the CNS awaits further investigation.

Mechanistically, Nogo-B-NgBR signaling and VEGF-VEGFR2 signaling crosstalk at the level of Akt (Figure 4B; Table 2; Table S3), which is a common downstream target (Miao et al., 2008; Zhao et al., 2010). Small interfering RNA-mediated knockdown of NgBR in HUVECs reduces VEGF-stimulated HUVEC migration and tube formation via reduced Akt phosphorylation (Zhao et al., 2010). Thus, VEGF-A and Nogo-B induce Akt phosphorylation in a NgBR-sensitive way. Accordingly, constitutively active Akt was able to partially rescue these inhibitory effects of NgBR knockdown on VEGF-induced HUVEC migration in vitro as well as the ISV sprouting defects in Nogo-B- and NgBR zebrafish morphants in vivo (Zhao et al., 2010).

Taken together, Nogo-B promotes developmental angiogenesis of ISVs via endothelial NgBR and interacts with VEGF-related pathways at the level of Akt (Acevedo et al., 2004; Miao et al., 2006; Zhao et al., 2010). The precise expression pattern of Nogo-B/NgBR on endothelial cells (i.e., tip versus stalk cell expression), a possible functional relevance of Nogo-B-NgBR during developmental CNS angiogenesis as well as further investigation of the above-described interactions between Nogo-B/NgBR and VEGF-VEGFR2-Akt are interesting questions for future investigations.

In several models of pathological neo-angiogenesis outside the CNS, for instance, VEGF-induced ear angiogenesis and wound-healing angiogenesis, Nogo-B and NgBR are expressed on smooth muscle cells and endothelial cells of angiogenic and mature blood vessels, consistent with their role as pro-angiogenic cues (Acevedo et al., 2004; Kritz et al., 2008; Miao et al., 2006). However, Nogo-B also exerts repulsive effects on vascular cells, as it inhibits vascular smooth muscle cell (VSMC) migration in vitro (Acevedo et al., 2004; Kritz et al., 2008). In adult mice, loss of endothelial and VSMC Nogo-B from the femoral artery vessel wall after injury leads to increased neointima formation caused by VSMC proliferation and migration and subsequent vessel stenosis (Acevedo et al., 2004; Kritz et al., 2008), thereby further supporting an inhibitory effect of Nogo-B on blood vessels.

Nogo-A. Nogo-A's important function for growing neurons during development and after CNS injuries is supported by a large body of data (Schwab, 2010), yet very little is known about Nogo-A's function in angiogenesis in- and outside the CNS (Table 3; Table S3). We have recently identified Nogo-A as a negative regulator of developmental CNS angiogenesis (Wälchli et al., 2013) and showed that Nogo-A is expressed throughout the postnatal mouse brain as well as in the retina at postnatal stages P4 and P8 (Wälchli et al., 2013). At the cellular level, Nogo-A is found in postnatal cortical neurons and retinal ganglion cells, in immediate vicinity of CNS blood vessels and endothelial tip cells and their filopodia, but not on endothelial cells.

Nogo-A protein was not expressed in postnatal cortical endothelial tip cells in vivo, as well as in postnatal and adult brain MVECs (Wälchli et al., 2013). Nogo-A gene deletion (Nogo-A$^{-/-}$) or treatment with anti-Nogo-A antibody (Oertle et al., 2003) leads to a significantly increased blood vessel density in the aforementioned brain regions at P8 (Wälchli et al., 2013). Moreover, in the retina, Nogo-A$^{-/-}$ mice revealed an increased radial migration of the forming vessel plexus at P4, and an increased vessel density in the deeper retinal layers in Nogo-A$^{-/-}$ and Nogo-A Ab-treated animals (Wälchli et al., 2013). These results suggest a cellular mechanism where Nogo-A expressed by CNS neurons interacts with vascular endothelial cells, thereby negatively regulating sprouting angiogenesis in the postnatal CNS in vivo. Accordingly, the Nogo-A-specific domain Nogo-A Delta 20 inhibited the spreading, migration and sprout formation of brain MVECs (Wälchli et al., 2013). Addition of soluble Nogo-A Delta 20 to mouse brain MVEC cultures led to quick retraction of MVEC lamellipodia and filopodia (Wälchli et al., 2013). The repulsive effects of Nogo-A Delta 20 on CNS endothelial cells and its filopodial and lamellipodial protrusions were mediated via the Rho-A ROCK-Myosin II pathway, which has described roles in lamellipodial and filopodial motility in non-CNS endothelial cells (De Smet et al., 2009; Fischer et al., 2009). Notably, the second inhibitory domain of Nogo-A, Nogo-66, did not show any inhibitory effects on brain-derived MVEC cell spreading, migration, and lamellipodia and filopodia motility in vitro (Wälchli et al., 2013). This is in contrast to Nogo-A's described role on neurons, where Nogo-66 and Nogo-A Delta 20 both exert inhibition of neurite outgrowth and lead to growth cone collapse (Oertle et al., 2003).

Non-CNS angiogenesis was not tested so far but since Nogo-A expression has been described at developmental stages in different peripheral tissues such as heart and skin (Schwab, 2010), it is tempting to speculate that angiogenesis and vascular patterning in vascular beds outside the CNS could also be affected by Nogo-A. Whether Nogo-A signaling interacts with VEGF-A-VEGFR2 signaling to regulate CNS angiogenesis in the developing neural tube and brain is currently unclear.

The number of brain cortical endothelial tip cells was increased in conditions lacking functional Nogo-A (Wälchli et al., 2013). Whether this regulation of the tip cell number involves an interaction with the VEGF-VEGFR2-Dll4-Jagged-Notch signaling axis is not understood (Figure 4B; Table 2; Table S3). Although earlier studies have reported an upregulation of VEGF-A mRNA in spinal cords treated with anti-Nogo-A Ab (Bareyre et al., 2002), protein levels of phosphorylated (activated) and total VEGFR2 protein as well as mRNA levels of VEGFA, VEGFR2, Dll4, and Notch4 were unchanged in P8 Nogo-A$^{-/-}$ whole brain lysates (Wälchli et al., 2013). Moreover, MVECs treated with Nogo-A Delta 20 showed no effect on p-VEGFR2 and total VEGFR2 levels (Wälchli et al., 2013). Given the predominant role of VEGF-VEGFR-Dll4-Jagged-Notch pathway (Blanco and Gerhardt, 2013) in the regulation of the number of endothelial tip cells and in light of the observations described above, possible interactions of the Nogo-A- and the VEGF-pathways deserve further investigation.

The role of Nogo-A on neo-angiogenesis in pathological conditions is almost completely unknown. Only one recent article described an increased vascularization of hydrogels implanted into the lesioned spinal cord of rats supplemented with anti-NgR1 antibodies (Wei et al., 2010), thereby suggesting a repulsive function for the Nogo-66 receptor NgR1 in pathological angiogenesis. However, as we and others found neither NgR1 expression in CNS endothelial cells during development and in the adult (Acevedo et al., 2004; Wälchli et al., 2013) nor effects of Nogo-66 on in vitro CNS endothelial cell motility (Wälchli et al., 2013), these seemingly contradictory observations point toward differences between physiological and pathological angiogenesis, which need to be further examined.

Taken together, neuronal Nogo-A negatively regulates developmental mouse CNS angiogenesis. It is tempting to speculate that the recently identified Nogo-A Delta 20-specific receptor S1PR2 (Kempf et al., 2014) (Figures 1B, 4A, and 4B) mediates these repulsive effects on the vasculature.

In summary, current data suggest a model in which the Nogo family of proteins provides angiogenic cues with Nogo-A being a negative regulator of CNS angiogenesis and Nogo-B a positive regulator of non-CNS angiogenesis (Table 3; Table S3).

Cns-Specific Mechanisms of Angiogenesis

Wnts and Their Receptors in Angiogenesis

To date, only few molecules have been involved in CNS-specific angiogenesis (Table S4). One recent example is Wnt7a/b-β-catenin signaling that was shown to regulate CNS angiogenesis and barriergenesis (Daneman et al., 2009; Liebner et al., 2008; Stenman et al., 2008).

During mouse embryogenesis, Wnt ligands are expressed by the neuroepithelium and interact with Frizzled (Fzd) receptors expressed on CNS endothelial cells (Daneman et al., 2009; Liebner et al., 2008; Stenman et al., 2008). In the developing CNS at E10.5/E11.5, Wnt7a and Wnt7b are expressed by neural progenitors in ventral (but not dorsal) regions of the forebrain and the spinal cord (Daneman et al., 2009) and are also expressed in embryonic non-CNS tissues such as the ectoderm and the dermatome (Niswander, 2003). Interestingly, at E12.5 downstream targets of Wnt signaling such as Lef1 and Axin2 are more abundantly expressed in brain endothelial cells as compared to peripheral endothelial cells of liver and lung, thereby revealing CNS-specific expression and activity patterns. Moreover, Wnt signaling reporter mice (TOP-gal) displayed CNS-specific Wnt activation in endothelial cells (Daneman et al., 2009). Whereas Frizzled 4, Frizzled 6, and Frizzled 8 are expressed on CNS and non-CNS endothelial cells (of the lung and of the liver), Frizzled 6 expression is significantly higher in CNS endothelial cells and expressed at E11.5 mouse forebrain and spinal (Daneman et al., 2009), thereby revealing a CNS-specific expression pattern.

Genetic deletion of the Wnt ligands Wnt7a and Wnt7b (single and double mutants), delivery of a Wnt inhibitor (soluble Frizzled8-Fc) or endothelial-specific deletion of β-catenin, a Wnt effector protein involved in canonical Wnt signaling, all lead to severe disturbances of the CNS vasculature (Daneman et al., 2009; Stenman et al., 2008). Sprouting into the forebrain was almost completely abolished, resulting in a thickened PNVP. Endothelial cells stuck in the PNVP consequently formed large, malformed vessels with multiple layers of endothelial cells with a lumen only in some cases (Daneman et al., 2009; Stenman et al., 2008) and these vascular malformations showed an increased risk of hemorrhage into the nervous tissue (Daneman et al., 2009; Stenman et al., 2008). Interestingly, these effects of Wnt/β-catenin showed region specificity, as the capillary beds in the posterior regions of the cortex as well as in the hindbrain of these β-catenin mutant mice were not affected. Most importantly, angiogenesis outside the CNS, namely in the liver, in the lung, and in the heart, was not affected in all conditions lacking functional Wnt/β-catenin signaling, demonstrating a CNS-specific role for Wnt/β-catenin in angiogenesis (Daneman et al., 2009; Stenman et al., 2008).

The important pro-angiogenic role for Wnt7a on CNS endothelium was further supported by the finding that Wnt7a—but not VEGF-A—enhanced the in vitro migration of a mouse brain endothelial cell line (bEND3.0 cells) (Daneman et al., 2009). Non-CNS endothelial cells were not tested here. The finding that VEGF-A expression was not changed upon Wnt7 deletion (Daneman et al., 2009; Stenman et al., 2008) in CNS tissue suggested independence of the Wnt7- and VEGF-A pathways. However, as Wnt- and VEGF-A signaling have both been implicated in sprouting angiogenesis into the developing CNS tissue (INVP) (Bautch, 2012; Hogan et al., 2004; James et al., 2009; Mancuso et al., 2008) in vivo, a crosstalk between these pathways remains possible.

In addition to its role on CNS angiogenesis, Wnt signaling is also involved in the formation and differentiation of the BBB (Table 4): Wnt7a increases endothelial expression of the BBB-specific influx transporters such as Glut-1, Cat1, and Ta1 in vitro and controls endothelial Glut-1 expression in vivo (Daneman et al., 2009) (see also Table 1). Strikingly, Wnt/β-catenin signaling can also induce BBB properties in non-brain derived-endothelial cells (Liebner et al., 2008), suggesting that Wnts are important cues in the CNS microenvironment, capable of inducing CNS-specific properties in endothelial cells. In summary, whereas Wnt7a gain of function induces a profound effect on the expression of the CNS vessel-specific transporter Glut1 in non-CNS vasculature but does not display ectopic or enhanced vessel ingression, the loss of Wnt7a/Wnt7b function exhibits severe vessel ingression defects (Stenman et al., 2008). Therefore, one intriguing possibility may be that Wnt7s can be permissive signals that are required for VEGF-A-mediated vessel ingression to proceed normally, whereas Wnt signaling is instructive for the acquisition of BBB characteristics. How and if the canonical Wnt signaling pathway interacts with VEGF signaling to control INVP formation in the brain and neural tube remains obscure.

A different, however non-CNS-specific, function for Wnt signaling is mediated by another Frizzled ligand called Norrin (Xu et al., 2004). Norrin is a small protein with the ability to activate the Wnt pathway via its interaction with Frizzled4-LRP5 receptors. Defects in these genes cause defects in the retinal vasculature in which especially sprouting into the deeper retinal layers is impaired (Wang et al., 2012; Xu et al., 2004; Ye et al., 2009). However, Norrin-Frizzled4 is also involved in inner ear angiogenesis (Xu et al., 2004) and Norrin, Wnt2, and Frizzled5 regulate placental angiogenesis (Ye et al., 2010). Norrin-Frizzled

Table 4. General- and CNS-Specific Regulators of Angiogenesis

Ligand-Receptor Pairs	Barriergenesis (BBB)	References
Global Functions in Angiogenesis and Vascular Patterning		
VEGFA/B/C-VEGFR1/2/3	? (vascular permeability)	Carmeliet et al. (1996); Ferrara et al. (1996)
Netrin-1-Unc5b	No (modulation of vascular permeability)	Bouvrée et al. (2008); Koch et al. (2011); Larrivée et al. (2007); Liu et al. (2004); Lu et al. (2004); Navankasattusas et al. (2008); Park et al. (2004)
Netrin-4-Unc5b	No (modulation of vascular permeability)	Hoang et al. (2009); Lambert et al. (2012); Larrieu-Lahargue et al. (2010, 2011); Lejmi et al. (2008); Nacht et al. (2009)
Semaphorin-3A-Plexin-D1	No (modulation of vascular permeability)	Acevedo et al. (2008); Cerani et al. (2013); Pan et al. (2007); Serini et al. (2003); Torres-Vázquez et al. (2004); Zygmunt et al. (2011)
Semaphorin-3E-Plexin-D1	No	Fukushima et al. (2011); Gu et al. (2005); Kim et al. (2011); Lamont et al. (2009)
Semaphorin-3F-unknown receptor Semaphorin-7A-unknown receptor	No (modulation of vascular permeability)	Coma et al. (2011); Morote-Garcia et al. (2012); Sultana et al. (2012); Wong et al. (2012)
EphrinB2-EphB4	No	Adams et al. (1999); Sawamiphak et al. (2010b); Wang et al. (1998, 2010b)
Slit2-Robo4	No (modulation of vascular permeability)	Jones et al. (2008, 2009)
NogoA-S1PR2?	No	Wälchli et al. (2013)
NogoB-NgBR	No	Acevedo et al. (2004); Miao et al. (2006); Zhao et al. (2010)
Sonic Hedgehog-Ptch/Smo	Yes (exception)	Alvarez et al. (2011); Pola et al. (2001)
CNS-Specific Functions in Angiogenesis and Vascular Patterning		
Wnt7a/b-(Fzd6)	Yes	Daneman et al. (2009); Liebner et al. (2008); Stenman et al. (2008)
Norrin-Fzd4	Maintenance	Wang et al. (2012); Ye et al. (2009)
Unknown ligand-GPR124	Yes	Anderson et al. (2011); Cullen et al. (2011); Kuhnert et al. (2010)
Unknown ligand-DR6/TROY	Yes	Tam et al. (2012)

signaling is—similar to Wnt7/β-catenin signaling—further involved in blood-retinal-barrier (BRB) and BBB maintenance in different brain regions, a function that was suggested to depend on Norrin-mediated endothelial cell-mural cell interactions (Ye et al., 2009).

Frizzled4 was shown to be important for the maintenance of barrier function cell autonomously (Wang et al., 2012) and this function was not restricted to the retina but revealed its effect in different CNS regions including the cerebellum, the olfactory bulb and the spinal cord (Table 4). However, other CNS regions such as cerebral cortex, striatum, and thalamus showed no BBB defects in Frizzled4$^{-/-}$ mice (Wang et al., 2012). Thus, although endothelial Frizzled4 is expressed in various CNS regions (Wang et al., 2012; Ye et al., 2009), Frizzled4$^{-/-}$ shows angiogenesis and barriergenesis defects only in certain CNS regions, but how this interesting region specificity is established molecularly is not clear to date. Frizzled4 is also expressed on non-CNS endothelial cells (Wang et al., 2012; Ye et al., 2009), but its functional relevance on angiogenesis outside the CNS is unknown.

In summary, Wnt/β-catenin signaling is required for appropriate, CNS-specific and compartment-specific angiogenesis and is necessary for the establishment and differentiation of the BBB in vivo (Daneman et al., 2009; Stenman et al., 2008) and in vitro (Liebner et al., 2008) (Table 4; Table S4). The Wnt ligands and receptors thereby act as (short range) molecular cues expressed within the CNS parenchyma influencing the migration and differentiation of the invading endothelial sprouts thereby tightly coupling the regulation of CNS angiogenesis and barriergenesis, in accordance with the hypothesis formulated earlier (Stewart and Wiley, 1981).

Interestingly, Wnt/β-catenin signaling interacts with the VEGF-VEGFR-Dll4-Jagged-Notch pathway (Corada et al., 2010; Phng et al., 2009). Wnt/β-catenin upregulates Dll4-Notch signaling in vivo and in vitro, leading to defects in vascular branching and loss of venous identity in mouse and zebrafish (Corada et al., 2010). Furthermore, the Notch downstream target Notch-regulated ankyrin repeat protein (Nrarp) regulates Wnt and Notch signaling in stalk cells (Phng et al., 2009). All these effects were described in mouse retina and mouse and zebrafish ISVs and thus revealed no CNS specificity. In contrast to the CNS-specific effects of Wnt7a/7b described above, the involved ligands and receptors were not investigated here thereby suggesting that some Wnt ligands and receptors act as CNS-specific angiogenic cues, whereas others act as general cues for angiogenesis.

DR6 and TROY Receptors in Angiogenesis

Tumor necrosis factor receptor superfamily member 21 (TNFFRSF21) also known as Death receptor 6 (DR6) and Tumor necrosis factor receptor superfamily member 19 (TNFRSF19 also known as TROY) are death receptors belonging to the tumor necrosis factor (TNF) receptor family that are expressed in neurons and have been involved in axonal pruning and neuron death (Nikolaev et al., 2009). DR6 and TROY are CNS-specific regulators of angiogenesis and barriergenesis (Figures 4A and 4B; Tables 3 and 4; Table S4). In the zebrafish, DR6 is expressed in blood vessels in the brain but not in ISVs at 3 dpf, and morpholino-mediated knockdown of DR6 and TROY led to defects in

vessel arborization selectively in the brain. These CNS-specific angiogenesis defects were characterized by a reduced vessel number, length, and thus decreased vessel density of hindbrain central arteries (CtA) at 3 dpf, suggesting that DR6 and TROY are required for the initial sprouting of the CtA vessels (Tam et al., 2012).

During mouse development (E14.5, P7.5), DR6 and TROY are highly expressed in the mouse CNS (cortex) vasculature as compared to the non-CNS vasculature of the liver and the lung (Tam et al., 2012). At the functional level, DR6 global- or endothelial-specific conditional knockout mice showed reduced vessel density in E14.5 and adult brains, and in vitro sprouting of HBMECs is decreased after siRNA-mediated knockdown of DR6 or TROY (Tam et al., 2012). Thus, DR6 and TROY regulate CNS-specific angiogenesis mainly via regulation of sprouting angiogenesis but not vessel regression, vessel anastomosis, or lumen formation in vivo (Tam et al., 2012).

Mechanistically, DR6 and TROY have been shown to activate JNK signaling (Eby et al., 2000), and VEGF-mediated ERK and subsequent JNK activation is involved in sprouting angiogenesis in HUVECs (Uchida et al., 2008) (Figure 4B). Knockdown of DR6 or TROY in HBMECs reduced VEGF-mediated JNK signaling and resulted in decreased HBMECs sprouting (Tam et al., 2012) (Figure 4B; Table 2). Whether these DR6/TROY-VEGF-A interactions are also relevant for the in vivo regulation of CNS angiogenesis remains elusive.

Similar to Wnt7a/7b, DR6 also regulates barriergenesis (Tam et al., 2012) (Table 4): DR6 global- or endothelial-specific conditional knockout mice showed forebrain-specific hemorrhages (at E11.5) and increased leakage of transcardially perfused sulfo-NHS-biotin across the BBB at E18.5, while in the adult mouse brain, DR6 or TROY knockout increased BBB leakage assessed by Evans blue extravasation (Tam et al., 2012). The zebrafish has recently been shown to have a functional BBB from early development on (3 dpf) (Jeong et al., 2008). DR6 and TROY morphants showed leakage of cells (DAPI) and injected tracers (rhodamine-dextran) across the BBB due to BBB perturbation (Tam et al., 2012).

Based on the similar functions of DR6 and TROY, these two receptors seem to have synergistic effects on the CNS vasculature and physically and genetically interact to form a functional receptor complex important for CNS angiogenesis and barriergenesis in zebrafish (Tam et al., 2012).

In conclusion, in zebrafish and mouse, DR6 and TROY receptors are essential for proper CNS angiogenesis and barriergenesis but not for angiogenesis outside the CNS (Table 3; Table S4).

Interestingly, in HBMECs, Wnt3a stimulation or increased beta-catenin levels lead to upregulation of DR6 and TROY mRNA (Tam et al., 2012), whereas in DR6 knockout mice, Wnt expression is attenuated in mouse brain vasculature but not in the vasculature of the lung or the liver (Tam et al., 2012). Accordingly, the authors propose a model in which DR6 and TROY are downstream targets of the Wnt/β-catenin signaling in CNS (but not non-CNS) endothelial cells highlighting an interesting crosstalk of two CNS-specific angiogenic cues. Following this model, neuroepithelium-derived Wnt ligand secretion stimulates DR6 and TROY expression on CNS endothelial cells. Subsequently, DR6 and TROY reinforce endothelial Wnt/β-catenin signaling to activate JNK signaling thereby regulating angiogenesis via a JNK-mediated crosstalk with the VEGF-A/VEGFR2 pathway (Tam et al., 2012) (Figure 4B). The precise molecular mechanisms regulating CNS-specific angiogenesis and barriergenesis remain, however, elusive.

GPR124 Receptor in Angiogenesis

The orphan G protein-coupled receptor 124 (GPR124), also known as tumor endothelial marker 5 (TEM5), is highly expressed on endothelial cells and pericytes in the brain, the spinal cord, and the PNVP (Anderson et al., 2011; Cullen et al., 2011; Kuhnert et al., 2010) and expressed at much lower levels on non-CNS endothelial cells of the liver, heart, and kidney during embryonic mouse development at E10.5–E15.5 (Anderson et al., 2011; Cullen et al., 2011; Kuhnert et al., 2010). Notably, the same expression pattern is seen in endothelial cells and pericytes from human fetal brain tissue (Cullen et al., 2011). GPR124 global- and endothelial-specific knockout induced CNS-specific vascular patterning defects and was embryonic lethal at E14.5 (Cullen et al., 2011; Kuhnert et al., 2010). These patterning defects were restricted to the forebrain and ventral neural tube, thereby revealing region specificities similar to Wnt and DR6/TROY. Moreover, forebrain vessels from the PNVP did not invade the CNS tissue but showed a PNVP thickening (Anderson et al., 2011; Kuhnert et al., 2010), reminiscent of Wnt7a/b double knockout phenotype described above (Daneman et al., 2009; Stenman et al., 2008). Accordingly, migration of CNS endothelial cells to the subventricular zone (SVZ) was delayed and CNS endothelial tip cell filopodial extensions were severely disturbed (Cullen et al., 2011; Kuhnert et al., 2010), resulting in an almost avascular telencephalon, with the formation of basally localized glomeruloid vascular malformations consisting of multiple layers of endothelial cells and subsequent hemorrhages into the forebrain and along the ventral spinal cord (Cullen et al., 2011; Kuhnert et al., 2010). As these vascular malformations showed normal pericyte recruitment and radial glia development (Anderson et al., 2011; Cullen et al., 2011), the mechanisms underlying the formation of these vascular glomeruloid tufts (vascular malformations) are currently not known (Anderson et al., 2011).

Strikingly, GPR124$^{-/-}$ did not alter sprouting angiogenesis into other regions of the embryonic CNS (e.g., diencephalon, midbrain, and hindbrain) or the vascularization in non-CNS tissues (heart, liver, intestine, and lung) at E12.5 (Anderson et al., 2011; Kuhnert et al., 2010), underlining the CNS-specific and compartment-specific effects of this molecule on angiogenesis (Figures 4A and 4B; Table 3; Table S4).

The CNS-specific mode of action was further illustrated as endothelial overexpression of GPR124 led to localized areas of hypervascularity mainly in the cortex and less frequently in the cerebellum in the adult mice (Kuhnert et al., 2010). In those animals, (micro-) vascular density was increased and abundant vascular malformations characterized by tortuous, thin-walled, and enlarged vessels were found (Kuhnert et al., 2010). Strikingly, overexpression of GPR124 in the endothelium of non-CNS organs such as the heart and the liver (which do normally not express GPR124) had no effect on vascular development (Kuhnert et al., 2010). It is tempting to speculate that the inability of GPR124 to affect non-CNS vasculature is due to the lack of

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the required ligand that is normally provided by the local microenvironment in the CNS.

The GPR124-expressing brain endothelial cell line bEND3 (Kuhnert et al., 2010) shows a directed migration toward gradients of conditioned medium from E12.5 forebrain but not hindbrain extracts (Kuhnert et al., 2010). Small interfering RNA-mediated knockdown of GPR124 abolished directed migration (Kuhnert et al., 2010), while blocking the VEGF-pathway with a recombinant soluble VEGFR1 ectodomain did not, suggesting a VEGF-independent mechanism (Kuhnert et al., 2010) (Figure 4B; Table 2). Similar results were seen for bEND3 sprouting and lumen formation (Kuhnert et al., 2010).

Mechanistically, the pro-angiogenic effect of GPR124 on directed migration is Cdc42 dependent but VEGFR2/Nrp-1 independent (Kuhnert et al., 2010) (Figure 4B; Table 2). In line with these in vitro data, expression of the vascular receptors VEGFR2, VEGFR3, Nrp-1, and Endoglin (a TGF-β co-receptor) were not altered in GPR124$^{-/-}$ embryos (Anderson et al., 2011; Kuhnert et al., 2010). However, Cullen et al. found that VEGF-A (and mainly its isoform VEGF164) was significantly upregulated in E11.5 GPR124$^{-/-}$ embryos. Given these observations, whether and how the GPR124-induced signaling pathway crosstalks with the VEGF-pathway needs further investigation.

In addition to these CNS-specific effects on angiogenesis, GPR124 also regulates the formation and differentiation of the BBB, again similar to the Wnt/β-catenin and DR6/TROY signaling pathways (Anderson et al., 2011; Cullen et al., 2011; Kuhnert et al., 2010) (Table 1). Expression of Glut1, an important marker for endothelial specialization and BBB formation (Engelhardt, 2003), was absent on vessels in E12.5/E13.5 global- or vascular-specific GPR124$^{-/-}$ mice (Anderson et al., 2011; Cullen et al., 2011; Kuhnert et al., 2010), suggesting impaired BBB formation. Indeed, the vascular glomeruloid malformations were surrounded by accumulations of extravascular fibrin, thereby indicating a BBB leakage (Cullen et al., 2011). In E18.5 GPR124$^{-/-}$ animals, intracardially injected biotin passed through the leaky blood-brain barrier into the brain parenchyma, whereas non-CNS vessels showed no permeability defects. Intriguingly, these barrier defects were again regionally restricted with leakage into the forebrain and ventral spinal cord but not into other CNS regions (Cullen et al., 2011). GPR124's regulatory role on BBB properties is further confirmed in vitro, where overexpression of GPR124 in bEnd3s enhances barrier properties (Cullen et al., 2011).

In conclusion, GPR-124 is important for angiogenic sprouting and barriergenesis in the mouse forebrain and ventral spinal cord in a highly CNS-specific and compartment-specific manner (Anderson et al., 2011; Cullen et al., 2011; Kuhnert et al., 2010). Interestingly, these findings are very similar to what has been described for the Wnt7/β-catenin signaling axis (Daneman et al., 2009; Stenman et al., 2008) and for the DR6/TROY receptors (Tam et al., 2012), thus revealing striking common features of CNS-specific angiogenic cues (Table S4).

Taken together, these observations suggest that the same molecular cues that are CNS-specific regulators of angiogenesis also regulate the differentiation and formation of the BBB, thereby representing examples of the CNS-derived instructive cues predicted by Stewart and Wiley (Stewart and Wiley, 1981) (see Table 4; Figure 4A).

Outlook

Over the last decade, our understanding of how the vascular network of tissues and organs is established has significantly increased, especially with regard to the process of sprouting angiogenesis (Carmeliet and Jain, 2011; Potente et al., 2011; Wacker and Gerhardt, 2011). In parallel, molecular interactions between the nervous and the vascular system are increasingly discovered (Eichmann and Thomas, 2013; Quaegebeur et al., 2011) and thereby contribute to our understanding of angiogenesis and the neurovascular link.

However, several outstanding questions regarding the molecular basis of vascular morphogenesis and the nature of neurovascular interactions remain unanswered. (1) How abundant are CNS-specific and general cues and how do they interact molecularly to govern CNS angiogenesis in health and disease? (2) How comparable are the mechanisms governing angiogenesis during development and in pathology models such as tumors or ischemic conditions? (3) How strong is the molecular link between CNS angiogenesis and barriergenesis and are there cues that only regulate barriergenesis? (4) Do most of the angiogenic cues in angiogenesis in- and outside the CNS interfere with the canonical VEGF pathway and, if yes, at which level? Moreover, how important are VEGF-independent pathways and how do these pathways correlate with the mechanisms of vascular development in different organ systems? (5) Given the current focus on sprouting angiogenesis, how important are other modes of vessel formation, how do they differ between distinct vascular beds, and how are they regulated molecularly? (6) Finally, is a co-patterning of vessels and nerves also apparent in the CNS?

CNS-Specific and General Mechanisms of Angiogenesis

How Abundant Are CNS-Specific and General Cues and How Do They Interact Molecularly to Govern CNS Angiogenesis in Health and Disease? As highlighted in this Review, one central question is whether a protein regulates angiogenesis in a general (e.g., non-organ specific) or in an organ-specific manner (Figure 4), with effects restricted to one or only some organs and tissue beds. This aspect is especially interesting in the CNS with its highly specialized vasculature (Figure 3). Notably, the presently known CNS-specific cues for angiogenesis additionally display remarkable region-specific effects within the CNS (e.g., between hind- and forebrain) (Daneman et al., 2009; Kuhnert et al., 2010; Stenman et al., 2008; Tam et al., 2012; Vasudevan et al., 2008) (Table 1). This is a very interesting finding in light of the highly regionalized functions of the brain (deCharms, 2008). As described in this Review, classical molecules regulating nerve- and vessel morphogenesis such as Netrins and Semaphorins, as well as Nogo proteins regulate angiogenesis in various tissues including the CNS. Only recently, CNS-specific regulators of vascular development such as Wnt7a/b, DR6/TROY, and GRP124 have been discovered. In order to address these questions of CNS-specific angiogenesis more systematically, we propose a novel conceptual framework for future studies: for instance, the effects of certain gene knockouts on in vivo angiogenesis should be studied in CNS (brain and retina) and

in non-CNS tissues/organs (e.g., where the protein is expressed). Moreover, corresponding in vitro studies should use isolated endothelial cells from the organ studied in vivo whenever possible.

How Comparable Are the Mechanisms Governing Angiogenesis during Development and in Pathology Models Such as Tumors or Ischemic Conditions? Organ and tissue specificity could also provide opportunities for selective vascular targeting in CNS pathologies involving angiogenesis such as brain tumors or stroke. The rationale for drug development that targets neoangiogenesis in pathology is classically based on exploiting the differing molecular signature and status of the quiescent endothelium present in most healthy organs in the adult and of the activated endothelium where new vessel growth occurs (Carmeliet and Jain, 2011; Jain and Carmeliet, 2012). However, the emerging insights into the profound heterogeneity of the endothelium (Herbert and Stainier, 2011; Nolan et al., 2013) raises the prospect of identifying selective cell-surface targets and signaling pathways that act specifically within the organs and vascular beds affected by the pathology. Such selective targeting may increase efficacy and minimize unwanted side effects, for example, on the vasculature of the surrounding CNS parenchyma and/or of peripheral organs.

Moreover, interacting with an angioneurin-related pathway always has the potential danger of affecting not only the targeted system (i.e., the vascular) but also the related system (i.e., the nervous system). This is, for instance, illustrated by the recent finding that glioblastoma patients treated with the anti-VEGF-A antibody bevacizumab (Avastin, Roche) may have a higher incidence of dementia than the control group (AVAglio study, 2013, ASCO, abstract), even though the molecular mechanisms responsible for this finding remain obscure (direct effect on neurons versus effect on endothelial cells outside the tumor region). Therefore, the differences in expression patterns of angiogenic ligand-receptor pairs between organs and between pathological and physiological tissue is of outstanding importance. Targeting, e.g., vascular-specific receptors such as Unc5b (Larrivée et al., 2007; Lu et al., 2004), Robo4 (Jones et al., 2008, 2009), or NgBR (Miao et al., 2006) or tissue-specific receptors such as GPR124 (Anderson et al., 2011; Cullen et al., 2011; Kuhnert et al., 2010) or DR6/TROY (Tam et al., 2012) may allow to minimize those side effects.

CNS-Specific Angiogenesis and Barriergenesis

How Strong Is the Molecular Link between CNS Angiogenesis and Barriergenesis and Are There Cues that Only Regulate Barriergenesis? As outlined above, CNS-specific cues also act on the formation and differentiation of the BBB, whereas general cues for angiogenesis do not (Figure 4A; Table 1). Intriguingly, CNS angiogenesis and barriergenesis are regulated by the same CNS-specific cues and hence are tightly linked. Given that the processes of CNS sprouting angiogenesis and endothelial tip cell biology on the one and of barriergenesis on the other hand are quite different, the observation that they are regulated by at least some common signaling pathways is very interesting.

Notably, some general angiogenic cues such as VEGF-A, Semaphorin-3A, Semaphorin-3F, and Semaphorin-7A, Netrin-1, and Netrin-4 as well as Slit2-Robo4 affect BBB maintenance by increasing vascular permeability (Figures 4A and 4B; Table 1). Despite this modulation of BBB physiology, these molecules are—in contrast to the above-described CNS-specific angiogenic cues—not involved in barriergenesis or BBB differentiation. The exception to this rule seems to be the Sonic hedgehog pathway, which is known to be important for various aspects of brain development and function (e.g., axonal guidance or adult hippocampal neurogenesis) (Ferent and Traiffort, 2014). Sonic hedgehog affects angiogenesis in a non-CNS specific manner but—in contrast to the other non-CNS specific cues—also barriergenesis (Alvarez et al., 2011; Nagase et al., 2008).

Based on these concepts and given the predominant role of VEGF and its receptors on angiogenesis (Carmeliet and Jain, 2011; Potente et al., 2011), it seems likely that tissue vascularization is achieved by a combination of VEGF, general-, and organ-specific cues for angiogenesis.

A combination of general and tissue-specific cues is also at work in other examples of highly specialized vasculature. The blood vessels of the kidney glomeruli and the liver sinusoids, for instance, are lined by specialized, fenestrated endothelial cells (Rocha and Adams, 2009). This vascular bed-specific, fenestrated endothelial cell phenotype is generated by a combination of VEGF-A and the organ-specific factor plasmalemmal vesicle-associated protein-1 (PV-1 also known as PLVAP) (Rocha and Adams, 2009). In the peripheral nervous system, the nerve-blood barrier is established (Weerasuriya and Mizisin, 2011) and is—although similar to the BBB—less tight with regard to the cell-cell connections. It would therefore be interesting to understand whether different cues regulate barrier formation in peripheral nerves as compared to CNS angiogenesis and barriergenesis.

Interaction with the VEGF-VEGFR-Dll4-Jagged-Notch Pathway

How Important Are VEGF-Independent Pathways and How Do These Pathways Correlate with the Mechanisms of Vascular Development in Different Organ Systems? At the molecular level, one outstanding question is whether and how the general and CNS-specific cues for angiogenesis interact with the VEGF-VEGFR-Dll4-Jagged-Notch pathway (Figure 4). Recent findings suggest that most of the guidance pathways that are shared between the neural and vascular system, such as Netrin/Unc5b, Semaphorin/Plexin, Ephrin/Eph, and Slit/Robo, all exert direct effects on the cytoskeleton but also function in the endothelium by modulating VEGF-VEGFR signaling (Jones et al., 2008, 2009; Kim et al., 2011; Koch et al., 2011; Lu et al., 2004; Sawamiphak et al., 2010b; Wang et al., 2010b; Zygmunt et al., 2011) (Figure 4B). Conceptually, these observations reinforce the central role of VEGF-VEGFR signaling, and its feedback mechanisms including Notch, as central pattern generators in angiogenesis (Blanco and Gerhardt, 2013). However, they also raise additional questions as to how the endothelial cells, singly or as a collective, can integrate the disparate inputs into guided angiogenesis. Moreover, recent evidence indicates that there is at least the possibility that some CNS-specific cues (GPR124 [Anderson et al., 2011; Cullen et al., 2011; Kuhnert et al., 2010], Wnt7a/7b [Daneman et al., 2009; Stenman et al., 2008] as well as Nogo-A [Wälchli et al., 2013]) may signal independently of the VEGF-axis to regulate angiogenesis. Further work along these lines will reveal whether these pathways are

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indeed VEGF independent or just converge to the VEGF-axis at another—yet unknown—molecular level. This concept referring to a central pattern generator for angiogenesis that is modulated by other angiogenic pathways may emerge as a fundamental difference to neuronal guidance, where no such concept has been described so far and where direct signaling to the cytoskeleton steers the axonal growth cone.

Future work will need to establish whether the moderate clinical success of approaches blocking the VEGF-VEGFR-Dll4-Jagged-Notch pathway (Carmeliet and Jain, 2011; De Bock et al., 2011; Hanahan and Weinberg, 2011; Jain and Carmeliet, 2012) can be enhanced, supplemented or surpassed by targeting additional modulators or VEGF-independent pathways.

Cellular Interactions in the Neurovascular Unit and Different Modes of Vessel Formation

Given the Current Focus on Sprouting Angiogenesis, How Important Are Other Modes of Vessel Formation, How Do They Differ Between Distinct Vascular Beds, and How Are They Regulated Molecularly? Angiogenesis occurs within a complex microenvironment composed of endothelial cells, the extracellular matrix, and the different cell types of the corresponding tissue (Figure 3). In the CNS, interactions of the endothelium with neurons, neuronal stem cells, astrocytes, oligodendrocytes, myelin, and pericytes (Hjelmeland et al., 2011; Quaegebeur et al., 2011; Storkebaum et al., 2011) during embryonic and postnatal development are just beginning to be unraveled. Intriguingly, these cellular interactions can display region-specific differences even within the CNS as, for instance, highlighted by the recent finding that BBB properties are altered at the SVZ where dividing neural stem cells contact vascular endothelial cells at BBB sites that lack the usually present astrocyte endfeet and pericyte coverage (Abbott et al., 2006; Tavazoie et al., 2008). This mutual crosstalk between cells emerges as a central concept also in pathologies. For instance, glioblastoma stem-like cells have been suggested to de-differentiate into tumor endothelial cells (Ricci-Vitiani et al., 2010; Wang et al., 2010a) or into tumor pericytes (Cheng et al., 2013), and endothelial tip cells can activate metastasis of breast cancers (Ghajar et al., 2013), and these fascinating yet poorly understood phenomena offer great therapeutic potential (Butler et al., 2010; Carmeliet and Jain, 2011; Herbert and Stainier, 2011; Potente et al., 2011; Quaegebeur et al., 2011). A more thorough investigation of the influence of perivascular cells—which significantly differ between organs including CNS and non-CNS tissues (Quaegebeur et al., 2011)—on angio- and arteriogenesis—is key for future progress in developmental and pathological settings.

Besides the current focus on sprouting angiogenesis, other modes of physiological (vasculogenesis, intussusception) and pathological blood vessel formation (vascular mimicry, vascular co-option, differentiation of stem cells into endothelial cells or into pericytes) (Carmeliet and Jain, 2011; Jain and Carmeliet, 2012; Potente et al., 2011) require further attention.

Co-patterning of Vessels and Nerves in the CNS?

Finally, Is a Co-patterning of Vessels and Nerves Also Apparent in the CNS? The initial observation of the co-patterning and alignment of vessels and nerves at different scales in the periphery has initiated research in the field of the neurovascular link and has led to the discovery of angioneurins (Carmeliet and Tessier-Lavigne, 2005). As highlighted in this Review, angioneurins affect axonal guidance and angiogenesis also in the CNS. Axonal guidance cues are known to steer neuronal growth cones to their appropriate targets by attraction and repulsion along an expression gradient. For instance, Semaphorin-3A shows a graded expression pattern in cortical layers of the postnatal brain thereby guiding radial migration of pyramidal neurons (Chen et al., 2008). On the other hand, a Netrin-1 gradient in the ganglionic eminence in the embryonic forebrain (around E14.5–E15.5) is important for the correct projection of thalamocortical axon tracts (Powell et al., 2008). Interestingly, however, nothing is known about possible effects of these gradients on late embryonic or postnatal vascular sprouting and vessel density in the CNS. It will therefore be interesting to investigate how the common molecular cues acting on axonal growth cones and endothelial tip cells affects the morphogenesis of nerves and vessels within different compartments of the brain.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four tables and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2015.06.038.

AUTHOR CONTRIBUTIONS

T.W. had the idea for the review, wrote the manuscript and made the figures. A.W. helped with manuscript writing and figure illustration. H.G. and B.E. gave critical inputs. All authors edited and approved the final manuscript.

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