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Developmental and pathological angiogenesis in the central nervous system

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Abstract

Angiogenesis, the formation of new blood vessels from pre-existing vessels, in the central nervous system (CNS) is seen both as a normal physiological response as well as a pathological step in disease progression. Formation of the blood–brain barrier (BBB) is an essential step in physiological CNS angiogenesis. The BBB is regulated by a neurovascular unit (NVU) consisting of endothelial and perivascular cells as well as vascular astrocytes. The NVU plays a critical role in preventing entry of neurotoxic substances and regulation of blood flow in the CNS. In recent years, research on numerous acquired and hereditary disorders of the CNS has increasingly emphasized the role of angiogenesis in disease pathophysiology. Here, we discuss molecular mechanisms of CNS angiogenesis during embryogenesis as well as various pathological states including brain tumor formation, ischemic stroke, arteriovenous malformations, and neurodegenerative diseases.

Keywords

Central nervous system; Angiogenesis; Brain tumors; Embryogenesis; Ischemic stroke

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Introduction

The central nervous system (CNS), consisting of the brain and spinal cord, possesses a highly specialized vasculature to meet the demands of this metabolically highly active tissue as well as to protect sensitive neurons from toxic metabolites and xenobiotics. The tissue-specific functions of CNS blood vessels are regulated by the neurovascular unit (NVU), which is characterized by cross-talk between neural and vascular cells. In the NVU, endothelial cells, perivascular cells (i.e., pericytes or vascular smooth muscle cells), and vascular astrocytes are closely opposed to each other and interact to form the blood–brain barrier (BBB) and provide the brain with a sufficient supply of oxygen and nutrients (Fig. 1). Endothelial cells form the inner lining of all blood vessels. The CNS endothelium differs from non-CNS endothelia in the presence of intercellular tight junctions and BBB-specific enzymes and transport proteins. Pericytes critically regulate BBB formation and CNS capillaries have a higher coverage of pericytes than capillaries of other tissues [1-3]. Astrocyte end-feet almost completely cover CNS blood vessels and regulate BBB function as well [4]. In addition, vascular astrocytes deliver nutrients from the blood to adjacent neurons [5, 6]. Furthermore, vascular astrocytes mediate neuronal regulation of blood flow in blood vessels of the brain [7].

Initial vascularization of embryonic tissues mainly occurs through vasculogenesis, the differentiation of mesoderm-derived angioblasts into blood vessels, whereas at later stages of embryogenesis and in the adult neovascularization mainly occurs through angiogenesis, the sprouting of new blood vessels from pre-existing vessels [8]. The developing CNS is exceptional as its vessels are exclusively formed by angiogenic sprouting of vessels from the perineural vascular plexus [8].

Angiogenesis is regulated by pro- and antiangiogenic factors [9, 10]. These molecules can be released by normal, tumor, endothelial, and stromal cells as well as from leukocytes and the extracellular matrix [11-15]. Proangiogenic factors include vascular endothelial growth factor A (VEGF), fibroblast growth factors, placental growth factor (PlGF), and interleukins, whereas angiostatin, endostatin, and thrombospondins 1 and 2 are putative antiangiogenic factors [13, 15-17]. In addition, certain metalloproteinases degrade extracellular matrix proteins, which can result in both induction and suppression of angiogenesis [12, 13, 15]. As long as expression of pro- and antiangiogenic factors is balanced, the “angiogenic switch” is off. If expression of proangiogenic factors is increased or expression of antiangiogenic factors is decreased, angiogenesis is induced. Induction of CNS angiogenesis contributes to pathological conditions such as brain tumor growth, neurodegenerative diseases, and arteriovenous malformations (AVMs). In contrast, CNS angiogenesis is required for embryonic development as well as recovery from ischemic stroke and brain injury.

This review summarizes our current knowledge of the vasculature of the CNS and the mechanisms underlying CNS angiogenesis during embryonic development and pathological conditions.

The blood–brain barrier

While the NVU is required to induce BBB properties in CNS blood vessels, the physical barrier of the BBB is confined to the endothelial compartment. All endothelia contain intercellular adherens junctions formed by transmembrane proteins of the cadherin, and intracellular proteins of the catenin, families. In addition to adherens junctions, CNS endothelial cells are interconnected by tight junctions similar to those found in most epithelia. Tight junctions, also known as occluding junctions or zonula occludens, are composed of a branching network of sealing strands. These strands are formed by transmembrane proteins of the claudin family and occludin as well as the intracellular ZO proteins (Fig. 2a, b). Tight junctions in the brain endothelium prevent passive diffusion of substances between adjacent cells (paracellular permeability). Only small non-polar molecules <400 Da (e.g., O_2, CO_2, steroid hormones) can passively diffuse through brain vessels. In addition to tight junctions, brain endothelial cells express specific transporters on the cell surface to transport small molecules such as glucose, amino acids, vitamins, and nucleosides across a concentration gradient from the blood into the brain parenchyma (transcellular permeability, Fig. 2c). Furthermore, CNS endothelial cells express transport proteins to actively transport large molecules such as transferrin, insulin, leptin, and LDL from the blood into the brain. On the other hand, they express transporters such as the multidrug resistance protein P-glycoprotein to actively export cell permeable xenobiotics back into the blood [18]. Brain endothelial cells also constitute a metabolic barrier eliminating chemicals that would otherwise move from the blood into the brain. Cytochrome P450-related enzymes oxidize unwanted substances within the cytoplasm of brain endothelial cells [19]. Likewise, monoamine oxidase (MAO) also contributes to the metabolic barrier to protect the brain from circulating neurotoxins and biogenic amines. In some cases, enzymatic activity within the BBB does not remove unwanted molecules but instead facilitates the transport of essential substances from blood to brain. Through its transpeptidase activity, gamma-glutamyl transpeptidase (GGT) assists in the transfer of amino acids across the BBB [20]. By modulating pyroglutamate levels, GGT indirectly stimulates a wide array of sodium-dependent amino acid transporters at the plasma membrane.

CNS blood vessels have a higher coverage of pericytes than blood vessels of other organs, suggesting that they play an important role in BBB formation and maintenance [1]. Indeed, it has been shown that brain pericytes critically regulate endothelial tight junction assembly and BBB formation [2, 3]. Furthermore, vascular astrocytes have been reported to regulate BBB function [4]. Expression of BBB-specific genes is not intrinsic to brain endothelial cells as brain endothelial cell lines gradually lose BBB properties in culture [21]. Stewart and Wiley [22] demonstrated that avascular embryonic quail brain transplanted into chick gut is vascularized by chick peripheral vessels. These vessels acquire BBB features including tight junctions and the ability to restrict molecular diffusion into the brain tissue.

While the BBB protects the CNS from neurotoxic substances, it also prevents entry of most drugs into the brain. The BBB constitutes a major obstacle in the development of novel neurotherapeutics [23]. Several approaches have been developed to efficiently deliver drugs

across the BBB, the most promising being drug conjugates that co-opt the transcytosis machinery of the BBB [24].

CNS angiogenesis during embryonic development

Vascularization of the developing CNS is preceded by the formation of the perineural vascular plexus by vasculogenesis. In mice, this process starts at the ventral region of the neural tube between E7.5 and E8.5 [25]. Subsequently, around E9.5, capillary sprouts invade the remaining neuroepithelium by angiogenesis. This is followed by extensive branching and arborization as capillary sprouts migrate from the pial surface to periventricular areas where angiogenic growth factors such VEGF are highly expressed and secreted by cells in the subventricular zone [26, 27]. This process continues through the remainder of embryonic development in all CNS tissues. While VEGF is a major growth factor inducing vasculogenesis and angiogenesis in CNS and non-CNS tissues, numerous other endothelial cell-autonomous and non-cell-autonomous genes regulating CNS angiogenesis during development have been identified (summarized in Fig. 3; Table 1). Endothelium-specific deletions in mice revealed that loci such as *Vegfr2, Nrp1/2, Tgfbr1/2*, β-catenin (*Ctnnb1*), and *Gpr124* (discussed below) are required to elicit proper CNS angiogenesis. However, genetic studies revealed that the CNS parenchyma also plays a crucial role in CNS angiogenesis as deletion of genes such as *Vegfa, Wnt7a/7b, Id1/3*, and integrins α_v or β_8 (*Itgav, Itgb8*) in the neuroepithelium but not the endothelium results in angiogenic defects and CNS hemorrhage [28-30]. Interestingly, a common phenotype exhibited by numerous loci controlling developmental CNS angiogenesis is the formation of glomeruloid vascular malformations at the interface between the perineural vascular plexus and the neuroepithelium representing impaired angiogenic sprouting [28-36].

VEGF signaling

Major triggers of VEGF expression during CNS development are hypoxia and glucose deprival [26, 37]. Cells respond to hypoxia by stabilizing transcription factors of the hypoxia inducible factor (HIF) family [26]. Hypoxia and glucose deprival also result in endoplasmic reticulum stress and activation of the unfolded protein response (UPR) [26, 38]. HIFs and the UPR have been shown to independently induce expression of VEGF [26, 37]. The VEGF family consists of several members including VEGF-A, -B, -C, -D, and PlGF, which bind to the VEGF receptors (VEGFR1-3) and the Neuropilin (Nrp-1 and -2) co-receptors [39]. VEGF signaling through VEGFR2/Nrp-1 is thought to be the main player in CNS angiogenesis [40, 41]. VEGF can be alternatively spliced into several isoforms, with the larger isoforms having large basic domains binding to heparin and proteoglycans found in the extracellular matrix [39]. Previous reports have implicated heparin-binding VEGFs in mediating migration of endothelial cells into avascular regions of the CNS during development [42]. Activation of VEGFR2 in endothelial cells activates several signaling pathways such as the MAP kinase and PI3 kinase/Akt pathways mediating migration, proliferation, and survival [43]. Genetic evidence showing an essential role for VEGF during vascularization of the CNS in mice originates from genetic studies in which *Nestin-Cre* was crossed into a floxed *Vegfa* background. In these studies, loss of *Vegfa* expression in the neuroepithelium leads to failure of CNS angiogenesis due to migratory arrest of

endothelial cells at the periphery of the neuroepithelium [40]. This process of VEGF-mediated endothelial migration into the CNS has been further shown to be dependent on the levels of VEGF being produced by the neuroepithelium and is endothelial cell autonomous [41]. The importance of VEGF binding to heparin during CNS angiogenesis has been demonstrated by reduced vascular branching complexity, increased microvessel caliber, and decreased endothelial filopodia in mice lacking the VEGF heparin-binding isoforms $VEGF_{164}$ and $VEGF_{188}$, and solely producing $VEGF_{120}$ [44].

Inhibitor of DNA-binding/differentiation proteins

Inhibitor of DNA-binding/differentiation proteins (Id1-4) comprise a family of proteins that heterodimerize with basic helix-loop-helix (bHLH) transcription factors to inhibit DNA binding of bHLH proteins [45]. Id proteins are key regulators of development where they function to prevent premature differentiation of stem cells [46]. *Id1/Id3* double knockout mice are embryonic lethal and exhibit premature neuronal differentiation, defective CNS angiogenesis, forebrain hemorrhage, and impaired cardiac development [31, 32]. *Id1* or *Id3* single knockout mice do not have a phenotype, indicating functional redundancy of these genes. Interestingly, mice with an endothelial-specific double deletion of *Id1/Id3* do not display CNS angiogenesis defects [47], suggesting a non-cell autonomous mechanism.

Wnt signaling

The canonical Wnt signaling pathway has been identified as an essential regulator of developmental CNS angiogenesis and BBB formation [30, 48, 49]. This pathway involves Wnt ligand-driven activation of Frizzled and LRP cell surface co-receptors, with Dishevelled-dependent stabilization of cytoplasmic β-catenin. Subsequent β-catenin translocation into the nucleus and binding to Lef-1/TCF transcription factors then induces transcription of Wnt-responsive genes [50]. In situ hybridization experiments showed that the Wnt ligands *Wnt7a* and *Wnt7b* are expressed in neural progenitor cells during CNS angiogenesis [49]. Functionally, *Wnt7a/7b* are required for developmental angiogenesis in the CNS. Invading vessels utilize paracrine Wnt signaling derived from the neuroepithelium to coordinate proper BBB development because loss-of-function of either *Wnt7a/7b* in neuroepithelium or of β-catenin/*Ctnnb1* in endothelial cells leads to angiogenic defects and subsequent vascular hemorrhage [30, 49]. These mice exhibit angiogenic defects only in the CNS and not in the periphery, consistent with the observation that Wnt signaling is not activated in non-CNS endothelium [28, 45]. Wnt signaling appears to induce not only CNS angiogenesis but also expression of BBB components Glut-1 and Claudin-3 [30, 48, 49]. Either *Wnt7a/7b* or *Cnttb1* deletion results in a lack of Glut-1 and Claudin-3 expression in the BBB. On the other hand, ectopic *Wnt7a/7b* expression induced Glut-1 expression outside the CNS, whereas addition of Wnt ligands to cultured brain endothelial cells induced both Glut-1 and Claudin-3 protein expression [30, 48, 49]. These findings emphasize the dual functional nature of Wnt-mediated vascular development in the CNS, whereby both angiogenesis and BBB formation are tightly coupled. Whether Wnts also plays a role in the final maturation of the NVU, including driving astrocytic associations with CNS blood vessels, is unclear. While the ligands required for Wnt-driven BBB development have been identified as Wnt7a/7b, the corresponding receptor(s) in CNS endothelial cells have not.

Integrin $\alpha_v\beta_8$

Mice with a null mutation in the integrin β_8 gene (*Itgb8*) lack functional integrin $\alpha_v\beta_8$ and die either from failure of normal vasculogenesis in the yolk sac, causing death around E10, or abnormal brain angiogenesis, beginning at E11.5 and characterized by dilated vessels, endothelial cell hyperplasia and hemorrhage [51]. Mice with a null mutation in the integrin α_v gene (*Itgav*) lack integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$ and die either from early defects in the placenta or from the same brain vascular pathology seen in *Itgb8*-null embryos [52]. Interestingly, similar brain vascular abnormalities occur when integrin β_8 or α_v is conditionally deleted in neuroepithelial cells but not in endothelial cells [28, 29] indicating a non-cell autonomous vascular defect.

TGFβ signaling

The three isoforms of transforming growth factor β (TGFβ1, -2, and -3) are multifunctional cytokines involved in development, cell differentiation, immune function, and cell cycle control. While they activate the same TGFβ receptor system, the phenotypes of the corresponding knockout mice do not overlap. *Tgfb1*-null mice die either during embryogenesis or from a lymphocyte-mediated inflammatory disease a few weeks after birth [53, 54]. *Tgfb2*-null mice die perinatally with defects in several organs, including the heart, skeleton, and genitourinary tract [55] and *Tgfb3*-null mice display a cleft palate and delayed lung development without embryonic lethality [56, 57]. TGFβ signaling plays a critical role in vascular development and function by regulating endothelial cell proliferation and migration, fibronectin synthesis, and mural cell differentiation [58, 59]. This is supported by the fact that embryonic lethality in some *Tgfb1*-null mouse embryos is caused by impaired yolk sac vasculogenesis [60]. In addition, conditional deletion of the TGFβ receptors 1 (*Alk5*) and 2 in endothelial cells results in embryonic lethality and CNS hemorrhage likely caused by defective CNS angiogenesis [61, 62]. Two integrins, $\alpha_v\beta_6$ and $\alpha_v\beta_8$, affect TGFβ activity directly by activating latent forms of TGFβ1 and TGFβ3 [63-66]. TGFβ latency occurs because the cytokine remains non-covalently associated with its propeptide, latency-associated peptide (LAP), after proteolytic processing of the pro-cytokine [67]. TGFβ1-LAP and TGFβ3-LAP contain the integrin-binding motif RGD (arginine–glycine–aspartic acid). Integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ mediate the release of TGFβ1 or -3 from their respective LAPs by interacting with the RGD sequence, which either induces a conformational change in LAP, in the case of $\alpha_v\beta_6$ [68], or leads to proteolytic cleavage of LAP, in the case of $\alpha_v\beta_8$ [65]. Mice with a knock-in mutation in *Tgfb1* that inactivates the integrin-binding site in LAP (RGD → RGE) have the same developmental defects as TGFβ1-null mice [69]. Thus, RGD-binding integrins play an important role in activating TGFβ1 during development of the yolk sac vasculature. Interestingly, $Tgfb1^{RGE/RGE}; Tgfb3^{-/-}$ double-mutant mice display abnormal CNS angiogenesis accompanied by severe hemorrhage and formation of glomeruloid vascular malformations not seen in single-mutant mice, indicating that *Tgfb1* and *Tgfb3* have redundant functions in developmental CNS angiogenesis. The similarity between the phenotypes of the *Tgfb1/Tgfb3* double-mutant and integrin α_v/β_8 knockout mice suggests that integrin $\alpha_v\beta_8$ could act upstream of TGFβ1 and -3 mediating their activation. TGFβ1 and -3 are both expressed in glial cells of the developing CNS [70-72]. Whether they are also expressed in endothelial cells during CNS development is unclear. Integrin $\alpha_v\beta_8$ is

expressed in neuroepithelial but not endothelial cells [28], indicating that the molecular cross-talk in the NVU also regulates developmental CNS angiogenesis. Indeed, integrin $\alpha_v\beta_8$ expressed on astrocytes surrounding blood vessels has been proposed to act as an "angiogenic switch" in the CNS by activating TGFβ [73].

Gpr124

The orphan adhesion G protein-coupled receptor *Gpr124*/tumor endothelial marker 5 (TEM5) is mainly expressed in endothelial cells and pericytes of most vascular beds during embryogenesis and in CNS endothelial cells as well as pericytes of multiple organs in the adult [34]. *Gpr124*-null mice are embryonic lethal because of impaired angiogenic sprouting from the perineural vascular plexus into the forebrain and ventral neural tube accompanied by severe hemorrhage [34-36]. In these embryos, the telencephalon and ventral neural tube are essentially avascular. Only the typical glomeruloid vascular malformations are found at the periphery of the neuroepithelium. Furthermore, the defective *Gpr124*-null vessels fail to express the BBB marker Glut-1. On the other hand, compensatory upregulation of Glut-1 in the neuroepithelium occurs [34, 36]. Conversely, transgenic mice overexpressing *Gpr124* under the endothelial-specific Tie2 promoter display increased vascular density and hyperplasia in the CNS [34]. Endothelial-specific knockout of *Gpr124* completely phenocopies the global knockout indicating that *Gpr124* functions endothelial cell-autonomously [34, 36]. In vitro migration assays showed enhanced migration of *Gpr124*-overexpressing brain endothelial cell lines towards primary forebrain cell conditioned medium or fetal bovine serum, indicating that *Gpr124* regulates migration of brain endothelial cells [34, 36].

The striking similarity between the phenotypes of *Gpr124* single-knockout and *Wnt7a/7b* double-knockout mice suggests an intersection between Gpr124 and Wnt signaling. However, using β-catenin reporter mice (TOP-Gal) we have shown that β-catenin activation, i.e., canonical Wnt signaling, is not impaired in *Gpr124*-null mice [34]. In addition, *Gpr124* expression is not altered in β-catenin-null mice [34]. Whether *Gpr124* regulates CNS angiogenesis by activation of G proteins remains to be determined. Anderson et al. performed microarray analysis of laser-microdissected forebrain blood vessels from wild-type and *Gpr124*-knockout embryos and found that 12 out of 27 TGFβ target genes were upregulated in the knockout embryos [35]. Based on these results, the authors conclude that *Gpr124* signaling negatively regulates TGFβ signaling.

Brain tumor angiogenesis

Angiogenesis is required for growth and metastasis of all solid tumors. While primary brain tumors are rare (<2 % of all tumors diagnosed each year), their location and other physiological characteristics lead to a very poor prognosis [74]. In contrast to primary brain tumors, secondary brain tumors metastasize from other cancers, such as breast, lung, and colon carcinoma as well as melanoma. Glioblastoma multiforme (GBM) is the most common brain tumor accounting for about 40 % of all primary brain tumors and 70 % of all malignant gliomas [75, 76]. GBM is among the most vascularized and deadly tumors with a 5-year survival rate of 5 % [77]. When primary brain tumors or metastases grow beyond 1–2 mm in diameter within the brain parenchyma, angiogenesis is induced and the BBB

becomes compromised structurally and functionally [78-85]. Typically, blood vessels within brain tumors are tortuous, disorganized, highly permeable, and characterized by abnormalities in their endothelial wall, pericyte coverage, and basement membrane [79-81, 86-89]. The vascular permeability of different xenograft tumors growing intracranially in mice is generally elevated [81, 90]. Some brain tumor vessels have pores in their walls as large as 550 nm in diameter [80]. Interestingly, some features of the BBB are retained in brain tumors. For example, when a tumor grows in the brain, it exhibits significantly reduced vascular permeability compared with the same tumor grown subcutaneously [79, 80]. Furthermore, although brain metastases from breast carcinomas are considerably more angiogenic than their orthotopic counterparts, the resulting tumor vessels are less permeable in the brain [91].

VEGF is a major permeability and proangiogenic factor that is highly expressed in brain tumors and is partly responsible for the loss of the BBB during tumor growth (Fig. 4). Hypoxia and acidosis have been shown to independently regulate *VEGF* transcription in brain tumors [82]. VEGF expression can also be regulated by multiple oncogenes and tumor-suppressor genes (such as Ras, Src, and p53), hormones, cytokines, the UPR, and various signaling molecules (including nitric oxide and mitogen-activated protein kinases) [14, 37, 38, 92-94]. Finally, VEGF can also be released from other cells and the extracellular matrix [11, 14]. A direct consequence of VEGF-induced vascular permeability is increased interstitial fluid pressure (IFP). This is critical in brain tumors, since increased edema and fluid pressure can cause severe complications. In brain tumors in both rodents and patients, the IFP increases with tumor size and in mice it is higher than the cerebrospinal fluid (CSF) pressure [95]. Because of the raised IFP, interstitial fluid leaks from the tumors into the surrounding tissue, raising the CSF pressure until, ultimately, it becomes equal to the tumor IFP. In addition to the co-morbidity associated with edema, high IFP forms a barrier to drug delivery (Fig. 4) [96].

Interleukin-8 is a chemokine with proangiogenic activity. High expression levels of interleukin-8 and hepatocyte growth factor have been detected in primary and recurrent glial tumors [97, 98]. Furthermore, expression of the chemokine CXCL12 and its receptor is induced in brain tumors and promotes angiogenesis [99]. Transcription profiles of gliomas from patients have shown expression of many proangiogenic factors including insulin-like growth factor 1 [100]. Stem cell factor and its receptor c-Kit have also been shown to play important roles in brain tumor angiogenesis [101]. Wnt signaling plays a crucial role in developmental CNS angiogenesis [30]. In contrast, Wnt has been shown to inhibit angiogenesis in brain tumors in the adult by inducing *PDGFB* expression and vessel normalization [102].

Inhibition of brain tumor angiogenesis represents a promising approach in the treatment of brain tumors and metastases. In 2009 the US Food and Drug Administration approved the VEGF-neutralizing antibody bevacizumab for the treatment of recurring GBM. A major mechanism by which bevacizumab improves the outcome of GBM is by reducing vascular permeability and edema, which are the major causes of morbidity and mortality associated with GBM [103]. The Notch signaling pathway is activated downstream of VEGF and mediates negative feedback and fine-tuning of VEGF signaling [104, 105]. Accordingly, it

has been reported that inhibition of the Notch ligand Dll4 leads to non-productive angiogenesis and suppression of tumor growth in a glioma model [106]. However, prolonged treatment of mice with a Dll4-blocking antibody results in formation of vascular/endothelial cell-based tumors resembling hemangioblastoma [107].

CNS angiogenesis after brain injury: stroke and TBI

The CNS vascular system in adults is extremely stable under physiological conditions. However, this system can be disrupted both structurally and functionally under acute pathological conditions such as ischemic stroke and traumatic brain injury (TBI). Numerous studies have demonstrated that angiogenesis actively occurs after ischemic stroke and TBI and contributes to functional outcome during recovery. The underlying mechanisms of this improved functional recovery by angiogenesis include not only increased oxygen and nutrient supply to injured tissue, but also involve neurogenesis and synaptogenesis [108]. Cerebral vessels are important for neuroblast migration to the ischemic boundary zone [109], where angiogenesis is actively induced, and provide neurotrophic support to newly generated neurons [110, 111]. Upon injury, activated endothelial cells secrete stromal cell-derived factor 1α (SDF-1α), which attracts CXCR4-positive neuroblasts to migrate along with cerebral vessels to the injured region [112, 113].

A variety of cellular and molecular responses in brain take place after ischemic stroke and TBI, including excitotoxicity, reactive free radical generation, inflammation, ischemia/reperfusion injury, and result in neuronal death (Fig. 4). Since ischemia plays an important role in both stroke and TBI, this review will focus on cerebral ischemiainduced vascular injury and angiogenesis.

Acute phase: vascular injury and BBB breakdown

An ischemic cascade comprised of a series of biochemical events is rapidly initiated within minutes after occlusion of a cerebral vessel, leading to excitotoxicity and oxidative stress [114]. A variety of pro-inflammatory cytokines and proteolytic enzymes that are responsible for remodeling the extracellular matrix are subsequently upregulated, including TNF-α, IL-1β, IL-6, and matrix metalloproteinases (MMPs). These pro-inflammatory cytokines and MMPs all contribute to microvascular injury and BBB dysfunction [115].

Endothelial activation and expression of adhesion molecules is important for adhesion and infiltration of leukocytes [116], which then amplify the inflammatory cascade in the infarcted region and increase brain damage [117, 118]. Intercellular adhesions molecule-1 (ICAM-1) expression increases within hours after ischemia onset in humans [119] and inhibition of ICAM-1 reduces ischemic damage in rat [120]. E- and P-selectins are upregulated and mediate leukocyte invasion into brain during the early stages of ischemia [121, 122]. Moreover, soluble ICAM-1 levels are higher in patients with acute ischemic stroke compared to controls and are associated with poor short-term prognosis [123].

Numerous animal studies have shown that ischemic injury induces a molecular cascade in the NVU, leading to disruption of the BBB and vasogenic edema/hemorrhage [124]. Degradation of tight junction proteins, junctional adhesion molecules in the endothelial cell

clefts, and basal lamina occurs a few hours after the onset of ischemia. Furthermore, there is a delayed secondary opening of the BBB occurring after 24–72 h due to a neuroinflammatory response. Experimental studies have demonstrated that expression and activation of MMPs accounts for the opening of BBB [124, 125]. MMPs in normal adult brain are either in a latent form (MMP-2) or at very low to undetectable expression levels (MMP-3 and -9). However, these MMPs can be rapidly activated or upregulated in cells of the NVU or leukocytes and released to act on multiple substrates in the extracellular space [126, 127]. In rodent stroke models, MMP-9 expression is upregulated a few hours after ischemia. Deletion of *Mmp9* preserves tight junction protein levels and reduces infarct volume [128]. In humans, plasma MMP-9 but not MMP-2 level is increased and correlated to infarct volume and final functional outcome in cardioembolic stroke patients [129]. Concomitantly, *Mmp2* knockout does not confer any protective effects against acute neural damage in mice, confounding the role of MMP-2 in BBB breakdown after stroke [130]. Cyclooxygenase-2 (Cox-2) is also involved in BBB opening but mainly in the second phase as part of a secondary inflammatory response [131].

Chronic phase: neovascularization by angiogenesis and vasculogenesis

Formation of new blood vessels plays an important role in the restoration of oxygen/nutrients and recovery phase of damaged neuronal tissue after ischemia, especially in the much larger volume of brain tissue surrounding the ischemic core, or penumbra. Intense and active neovascularization has been found in this area and is coupled with neurogenesis.

In rodent stroke models, endothelial cells in the peri-infarct brain tissue start to proliferate as early as 12–24 h following ischemic stroke and can continue proliferating for at least 21 days [132-134]. Accordingly, vessel density significantly increases in the peri-infarct region 3 days after ischemia [133, 134]. This angiogenic process occurs robustly and in a long-term way because vessel numbers in the ischemic area continue increasing even 21 days after ischemic injury [134, 135]. Importantly, studies on postmortem brain samples from patients with stroke demonstrated that cerebral ischemia induces active angiogenesis in the penumbra, which may contribute to longer survival of stroke patients [136].

New blood vessel formation is an intricately regulated and step-wise process. Important steps include degradation of surrounding matrix, proliferation and migration of endothelial cells, recruitment of pericytes, and stabilization and cessation of newly formed vessels [137]. As a result, an enormous variety of molecules including pro-angiogenic factors and molecules that are involved in other steps of angiogenesis are promptly induced after the onset of cerebral ischemia. A study by Hayashi et al. [134] analyzed the temporal expression profile of 96 angiogenesis-related genes in a mouse transient cerebral ischemia model. They found that 42, 29, and 13 angiogenesis-related genes are increased at 1 h, 1 day, and 21 days after ischemia, respectively. Moreover, some genes may demonstrate early or delayed induction or biphasic expression pattern, depending on their specific roles in angiogenesis. For example, VEGF, the most important mitogen for angiogenesis [39], and its receptor Flk1/VEGFR2 are rapidly upregulated at 1 h and reach the peak levels at 3 h after ischemia. Angiopoietin-1, which is involved in blood vessel maturation and stabilization [138], shows a delayed upregulation starting at 1 day and culminating at 7 days after stroke. On the other

hand, vessel-stabilizing thrombospondins are rapidly induced at 1 h after ischemia but reduced at 1 day, and then increase again 3 days after stroke, indicating a shift from vascular protection to vascular remodeling. There have been many studies investigating the expression profile of different angiogenesis-implicated genes and their specific roles in post-ischemia angiogenesis (summarized in Table 2).

In addition to angiogenesis, the outgrowth of pre-existing vessels, formation of vessels by circulating bone marrow-derived endothelial progenitor cells (EPCs), or vasculogenesis [139-141] may also contribute to neovascularization after cerebral ischemia. Animal studies demonstrated that these circulating EPCs could home to infarcted brain tissues, differentiate into ECs in situ, and incorporate into the vasculature [142-144].

Angiogenesis in neurodegenerative diseases

Capillary loss and endothelial activation

Previous studies have shown that cerebral microvascular pathology precedes and accompanies age-related cognitive dysfunction and neurodegeneration. Capillary density decline has been found in aging, Alzheimer's disease (AD), and leukoaraiosis (LA) [145]. Cerebral hypoperfusion triggers cognitive and degenerative changes in the brain and contributes to the pathologic process of AD [146]. A study on atherosclerosis revealed that severe circle of Willis atherosclerosis is associated with sporadic AD [147]. Mechanistically, impaired induction of HIF-1 and VEGF by hypoxia is proposed to be a major mechanism underlying capillary loss during aging [148-152]. Moreover, HIF-1 reduction is associated with neuronal loss [153]. In AD, amyloid β (Aβ) also plays an important role in the reduced capillary density by exerting both direct and indirect effects. Firstly, Aβ accumulates on capillaries and inhibits angiogenesis via anti-angiogenic activity [154, 155]. Secondly, it can bind to VEGF and co-deposit in plaques, resulting in decreased availability of VEGF [156], which is even upregulated in AD brains presumably due to hypoxia [157].

Decreased vascularity in the brain leads to hypoxia, which in turn stimulates the upregulation of proangiogenic factors and endothelial activation [158]. In a transgenic mouse model of AD, endothelial cell activation occurs in an age-dependent manner and is associated with Aβ deposition [159]. Activated endothelial cells elaborate a number of proteases, inflammatory factors, and other products with biologic activity that may promote neuronal death (Fig. 4) [158].

BBB disruption

Disruption of the BBB exists in many neurological disorders, including AD, multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS) [160]. In a mouse AD model, compromised BBB occurs long before other disease pathology occurs, such as consolidated amyloid plaques [161]. BBB disruption is also found in AD patients manifested by magnetic resonance imaging (MRI) [162]. The exact cause of BBB dysfunction in AD remains unclear but accumulation of Aβ and neuroinflammation have been suggested to play a role [163-165]. Interestingly, Aβ immunization restores the disrupted BBB in a mouse AD model, demonstrating that Aβ may directly affect the BBB [166]. Similar to AD, disruption of BBB is observed in mouse ALS models before motor neuron degeneration and the

neurovas-cular inflammatory response occurs [167]. MS is an autoimmune disease seen in young adults where the immune system erroneously attacks oligodendrocytes forming the insulating myelin sheath of axons. Disruption of BBB is an early event in MS, evidenced by in vivo gadolinium uptake on MRI and post-mortem evidence of focal microvascular leakage. While the exact etiology of MS remains unknown, it is generally believed that leukocyte activation and transendothelial migration play an important role in initiation of CNS inflammation in MS [168]. Treatment of MS with the humanized monoclonal antibody natalizumab targeting the α4-chain of α4β1 integrin, also known as very late antigen-4 (VLA-4), expressed on circulating immune cells, inhibits leukocyte adhesion and extravasation into the CNS, effectively reducing CNS inflammation and secondary BBB breakdown [169]. The presence and mechanisms of BBB disruption in other neurological diseases, such as epilepsy and neuromyelitis optica, have been recently reviewed by Obermeier and colleagues [115].

Arteriovenous malformations in the brain

AVMs are a collection of abnormally clustered blood vessels resulting from shunting from arterial to venous circulation. AVMs in the brain have a high risk of rupture with subsequent intracerebral and subarachnoid hemorrhage due to the fragility of these improperly formed vessels in the absence of an intervening capillary bed (Fig. 4). Most AVMs are sporadic, but the genetic component of AVM pathogenesis has been studied in hereditary hemorrhagic telangiectasia (HHT, Osler-Weber-Rendu disease), an autosomal dominant disorder presenting with high frequency of AVMs with hemorrhage from superficial AVMs in the nose and GI tract, and larger AVMs in major organs including liver, brain, and lung [170]. Cerebral AVMs and pulmonary AVMs occur in 15–20 % of HHT cases [170]. HHT is the most frequent cause of hemorrhagic stroke in young adults [171, 172].

The TGFβ superfamily plays a critical role in vascular development [173]. TGFβ is necessary for formation and remodeling of the primary vascular plexus through regulation of endothelial cell proliferation, migration, and differentiation. It also plays a role in recruiting pericytes and vascular smooth muscle cells to newly formed blood vessels. HHT is frequently associated with mutations in the *SMAD4*, endoglin, and *ALK1* (*ACVRL1*) genes of the TGFβ pathway [174-180]. Heterozygous mutations in endoglin lead to HHT type 1 [170]. Endoglin encodes a homodimeric integral membrane glycoprotein that exhibits high expression on vascular endothelial cells [181, 182]. Endoglin has been demonstrated to interact with the ligand-binding receptors of multiple members of the TGFβ superfamily, including activin and bone morphogenic proteins (BMPs) [183]. Heterozygous mutations in *ALK1*, an activin-like kinase receptor type I, lead to HHT type 2, which has a later disease onset and reduced penetrance in comparison to HHT1 [184, 185]. Patients with mutations in the *SMAD4* gene and juvenile polyposis may also develop HHT [186]. Environmental causes may also play a role in AVM development in HHT patients, which is supported by a study showing that excisional skin wounding induces AVMs in *Alk1*-deficient mice [187].

Targeted deletion of TGFβ signaling pathway components *Alk1*, *Tgfrb1* (*Alk5*), *Tgfbr2*, and *endoglin* in mice results in AVMs similar to HHT [173]. The Alk1 knockout phenotype has been elucidated in both the zebrafish and the mouse model and both demonstrate dilation of

cranial vessels with an abnormal direct connection to veins [188, 189]. Additionally, Alk1 deletion decreases expression of the arterial marker ephrin-B2 [189]. Mice with *endoglin* deletion develop AVMs and fail to confine intraembryonic hematopoiesis to arteries, without demonstrating a phenotype of vessel dilation or downregulation of arterial marker ephrin-B2, suggesting a role for endoglin as an accessory coreceptor modulating Alk1 signaling [189]. Endothelial cell-specific inducible deletion of endoglin results in an abnormal increase in endothelial cell proliferation observed in veins of adult skin and all vessels of neonatal retina [190]. Expression of molecular regulators of arterial and venous identity, including *Jagged-1*, *ephrin-B2*, apelin receptor (*Aplnr*), and *EphB4*, is preserved in the vasculature of the retina in *endoglin* mutants, arguing against loss of arterial and venous molecular identity as the primary cause of AVM formation in HHT pathogenesis [190]. However, *endoglin* deletion in mouse embryos increases arterial expression of chicken ovalbumin upstream promoter (COUP) transcription factor II, a venous-specific marker [191].

Conclusions

CNS capillaries differ from the vasculature of other organs through the presence of the BBB, which is critically regulated by a NVU containing endothelial cells, pericytes, and astrocytes. The balance of pro- and antian-giogenic factors maintaining vascular homeostasis in the CNS may be disrupted in pathological conditions and acute vascular injury. Insult to the BBB can occur as a result of ischemia, leading to the upregulation of proinflammatory cytokines, metalloproteinases, and other proteolytic enzymes that remodel the extracellular matrix. The importance of proper regulation of angiogenesis and BBB integrity is attested to by both the pathophysiology of disorders as diverse as brain AVMs, CNS neoplasms, stroke and MS, as well as the therapeutic efficacy of VEGF inhibitors such as bevacizumab for brain tumors and leukocyte adhesion inhibitors such as natalizumab for MS. Although numerous mouse and human genetic models have identified crucial regulatory molecules governing CNS angiogenesis, the further inquiry into the regulation of vascularization and cerebrovascular integrity during physiological and pathological states is critical to the future development of pharmacologic therapies in a broad variety of neurological disorders.

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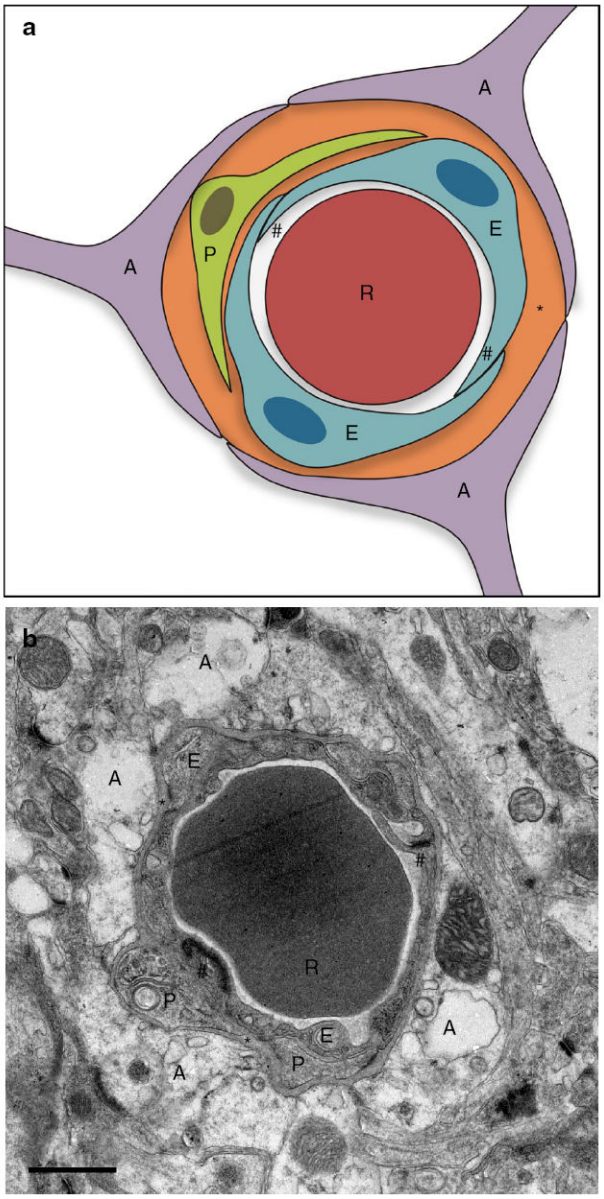

Fig. 1.

The neurovascular unit. Schematic representation (**a**) and electron microscopic image (**b**) of the neurovascular unit in a brain capillary of an adult mouse. *A* astrocyte end-foot, *E* endothelial cell, *P* pericyte, *R* red blood cell, *asterisk* basement membrane, *hash symbol* tight junction. *Scale bar*: 1 μm

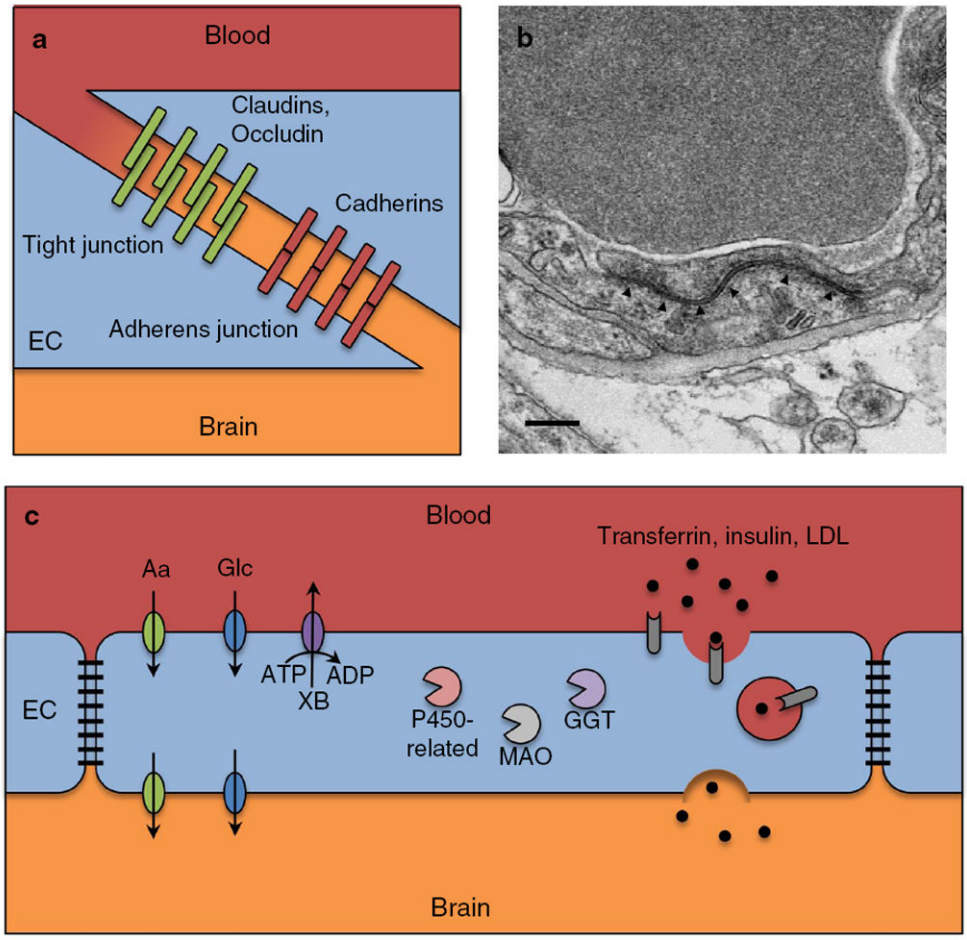

Fig. 2.

The blood–brain barrier. Schematic representation (**a**) and electron microscopic image (**b**) of tight and adherens junctions found in brain endothelium. **b** High-magnification electron microscopic image showing a tight junction (*arrowheads*) in a brain capillary of an adult mouse. *Scale bar* 200 nm. **c** Schematic representation of the cellular and molecular mechanisms regulating transcellular permeability of the blood–brain barrier. *EC* endothelial cell, *Aa* amino acids, *Glc* glucose, *XB* xenobiotics, *MAO* monoamine oxidase, *GGT* gamma-glutamyl transpeptidase, *LDL* low-density lipoprotein

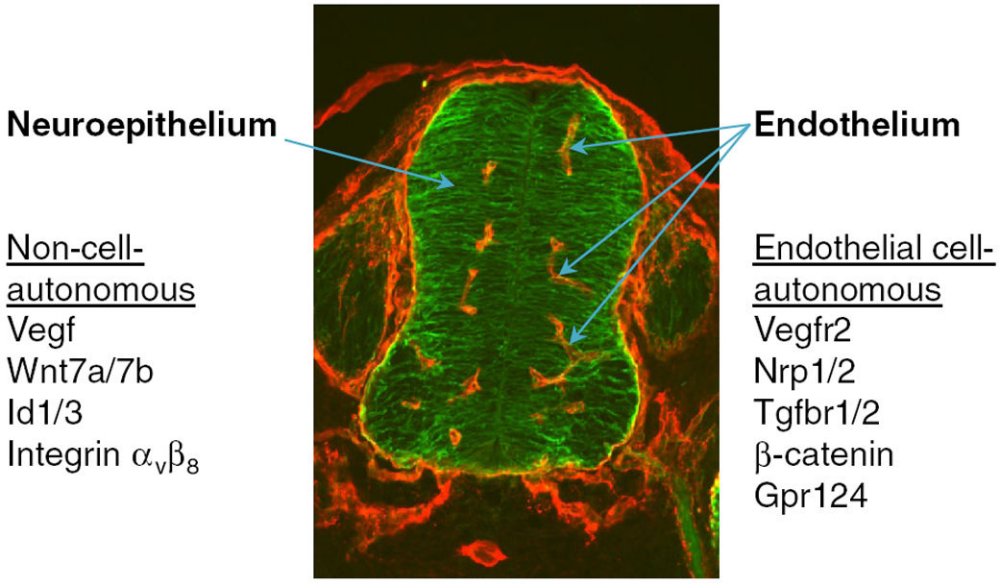

Fig. 3.

Endothelial cell-autonomous and non-cell-autonomous genes regulating developmental CNS angiogenesis. Immunofluorescence staining of an E10.5 mouse neural tube showing the two major compartments, neuroepithelium and endothelium, regulating developmental CNS angiogenesis. Neuroepithelial (*green*) and endothelial (*red*) cells were visualized by staining for nestin and CD31, respectively

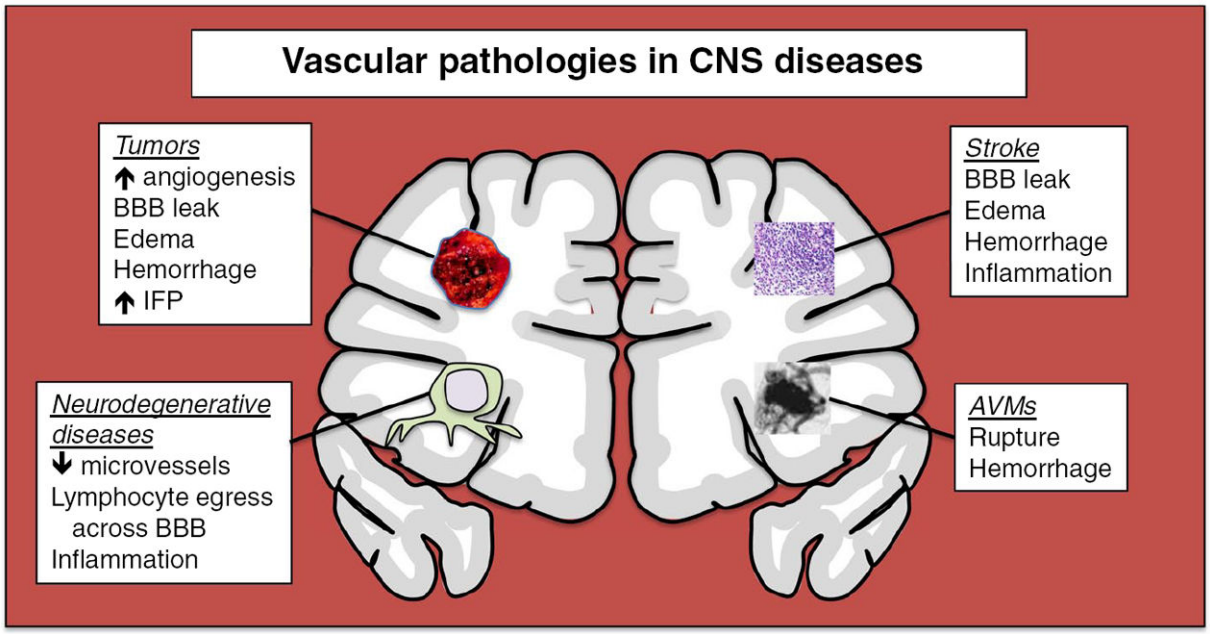

Fig. 4.

Vascular pathologies in CNS diseases. Tumors, neurodegenerative diseases, stroke, and arterio-venous malformations are major classes of CNS disorders that significantly alter the brain vasculature, with accompanying pathophysiologic consequences

Table 1

Genes regulating developmental CNS angiogenesis

Knocked-out gene/s	Compartment	Angiogenesis defects		CNS hemorrhage	CNS glomeruloid vascular malformations	References
		Non-CNS	CNS			
Vegf	Neuroepithelium	−	+++	−	−	[40]
Id1 + Id3	Global	Xenograft tumors[a], heart	++	++	++	[31, 32]
Id1 + Id3	Endothelium	Heart	−	−	−	[47]
Wnt7a + Wnt7b	Embryo proper, neuroepithelium	−	++	++	++	[30]
β-catenin	Endothelium	−	+++	+++	−	[30]
Tgfβ1[b]+ Tgfβ3	Global	Yolk sac	++	++	++	[33]
Tgfbr1	Endothelium	Heart	?	++	?	[61]
Tgfbr2	Endothelium	Heart	?	++	?	[62]
Integrin α_v	Neuroepithelium[c]	−	+	+	+	[28]
Integrin β_8	Neuroepithelium[c]	−	+	+	+	[29]
Gpr124	Global, endothelium	−	++	++	++	[34-36]

[a] Since $Id1^{-/-}$; $Id3^{-/-}$ mice are embryonic lethal, experiments were performed using $Id1^{+/-}$; $Id3^{-/-}$ mice, which are viable

[b] The TGFβ1-RGE mutant, which phenocopies the TGFβ1 null allele was used

[c] The conditional knockout in endothelial cells was also investigated but had no phenotype

Table 2

Molecules involved in angiogenesis post-brain injury

Name	Role in angiogenesis	Expression level and induction time after ischemia	Cellular localization	References
VEGF	EC proliferation/migration; vascular permeability	Up; 1 h to 7 days	ECs; astrocytes; neurons; microglia/macrophages	[133, 134, 192-200]
VEGFR-1 (Flt-1)	Receptor of VEGF	Up; 48 h to 14 days	ECs	[133, 192, 197]
VEGFR-2 (Flk-1)	Receptor of VEGF	Up; 48 h to 7 days	ECs; neurons; microglia	[133, 195, 198]
PLGF	Ligand of VEGFR1; potentiating VEGF	Up; 3 days	Vessels	[201]
Neuropilins	Co-receptor of VEGF/PLGF	Up; 24 h to 28 days	ECs	[201, 202]
Angiopoietin-1/2	Ligands of Tie2; vascular maturation and stabilization	Up; 1 h to 28 days	ECs; neurons	[132, 134, 135, 203, 204]
Tie1/2	Vascular maturation and stabilization	Up 0 to 1 h→down→Up 3–14 days	ECs	[203, 205, 206]
PDGF-B	Pericyte recruitment; vascular stabilization	Up; hours to 7 days	ECs; neurons; macrophages	[207-209]
PDGFRβ	Receptor of PDGF-B	Up; 48 h to 14 days	Pericytes; neurons	[207-209]
Erythropoietin	EC proliferation	Up; 1–7 days	ECs; astrocytes; microglia	[210]
eNOS	EC proliferation/migration; vascular tone	Up; 1–7 days	ECs	[211]
TGFβ	Cellular migration; extracellular deposition	Up 12 h→down up 7 days	Vessels; astrocytes;	[212-215]
FGFs	EC growth	Up; 1–14 days	ECs; neurons; astrocytes; macrophages	[216-219]
Thrombospondins	Vessel stabilization	Up 1 h→down 1 day→up 3 days	ECs; neurons	[134]