nature reviews neuroscience

https://doi.org/10.1038/s41583-023-00684-y

Review article

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Shaping the brain vasculature in development and disease in the single-cell era

Thomas Wälchli[1,2,3,4] ✉, Jeroen Bisschop[1,2,3,4,5], Peter Carmeliet[6,7,8], Gelareh Zadeh[4,9], Philippe P. Monnier[5,10,11], Katrien De Bock[12] & Ivan Radovanovic[3,4]

Abstract

The CNS critically relies on the formation and proper function of its vasculature during development, adult homeostasis and disease. Angiogenesis — the formation of new blood vessels — is highly active during brain development, enters almost complete quiescence in the healthy adult brain and is reactivated in vascular-dependent brain pathologies such as brain vascular malformations and brain tumours. Despite major advances in the understanding of the cellular and molecular mechanisms driving angiogenesis in peripheral tissues, developmental signalling pathways orchestrating angiogenic processes in the healthy and the diseased CNS remain incompletely understood. Molecular signalling pathways of the 'neurovascular link' defining common mechanisms of nerve and vessel wiring have emerged as crucial regulators of peripheral vascular growth, but their relevance for angiogenesis in brain development and disease remains largely unexplored. Here we review the current knowledge of general and CNS-specific mechanisms of angiogenesis during brain development and in brain vascular malformations and brain tumours, including how key molecular signalling pathways are reactivated in vascular-dependent diseases. We also discuss how these topics can be studied in the single-cell multi-omics era.

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A full list of affiliations appears at the end of the paper. ✉e-mail: waelchli@irem.uzh.ch

Introduction

The human brain constitutes only 2% of body mass but receives 20% of cardiac output and consumes 20% of the body's total oxygen and glucose, underlining the crucial importance of the CNS vasculature for a properly functioning brain[1,2]. Accordingly, the human brain vasculature is composed of an extensive and complex network of blood vessels, with a total length of 400 miles and including up to 100 billion capillaries[2]. The brain vascular network is established during embryonic and postnatal development via vasculogenesis (de novo formation of blood vessels) and sprouting angiogenesis (formation of new blood vessels from pre-existing ones), driven by various pro-angiogenic and anti-angiogenic factors[3].

The endothelium of the brain vasculature displays specific properties that distinguish blood vessels in the CNS from those outside the CNS[4]. The most characteristic feature of the brain endothelium is the presence of a functional blood–brain barrier (BBB) — the highly selective semipermeable border between the vascular lumen of capillaries and the CNS parenchyma — established during embryonic and postnatal development by extrinsic cues provided by the perivascular microenvironment[3,5] and intrinsic endothelial cell (EC) regulation mediated by homeobox transcription factors[6]. Blood vessels in the brain are embedded in an anatomical or structural unit termed the 'perivascular niche' (PVN), which describes a microenvironment that, in addition to ECs, includes perivascular cells (PVCs), such as astrocytes, pericytes, perivascular fibroblasts, neurons, stem cells, microglia and vascular smooth muscle cells (vSMCs)[3,7–9]. Together, ECs and PVCs in the PVN form the neurovascular unit (NVU)[9–11], which is the functional correlate of the structural PVN[9–11]. Cellular and molecular interactions between ECs and PVCs in the NVU contribute to regulation of CNS angiogenesis[9–11].

Developmental vascular growth in the CNS involves general angiogenic mechanisms (that is, mechanisms involved in angiogenesis inside and outside the CNS[9]) and CNS-specific angiogenic mechanisms. The NVU becomes deregulated in vascular-dependent brain pathologies such as brain tumours and brain vascular malformations, in which angiogenic signalling pathways become activated and lead to the formation of leaky, tortuous and dysfunctional neovessels via various modes of neovascularization[9,12,13]. These angiogenic pathways are, at least in part, reactivated signalling cascades regulating vascularization and the NVU and PVN during brain development[9,12,13], but how these molecular mechanisms are involved in the initiation and progression of vascular-dependent brain pathologies remains poorly understood.

In this Review, we provide an overview of our current understanding of neovascularization in the developing, healthy adult and pathological brain (Fig. 1). Moreover, we describe recent insights into the human brain vasculature at the single-cell level, emphasizing the expanding knowledge of cerebrovascular cell type heterogeneity and the reactivation of developmental angiogenic signalling pathways in ECs of vascular-dependent brain pathologies. We review recent evidence regarding reactivated developmental signalling pathways in disease, focusing on molecules involved in angiogenesis and the neurovascular link (NVL), defined as the shared molecular mechanisms regulating both the vascular system and the nervous system[9,14–17] (Fig. 2). We describe the involvement of these signalling cues in glial brain tumours and brain arteriovenous malformations (AVMs), two typical vascular-dependent CNS pathologies, with special focus on the distinction between CNS-specific cues and general molecular cues. Finally, we discuss several outstanding questions and emphasize how novel technologies used in the field of single-cell multi-omics may influence our understanding of brain vascular biology.

Modes of neovascularization

The neovascularization of organs and tissues can occur via different mechanisms (Fig. 1). During physiological development, such vascularization may involve the formation of new blood vessels from pre-existing ones, defined as sprouting angiogenesis (by far the best-described mode)[9,12,15,18] (Fig. 1a), the de novo generation of blood vessels from mesodermal angioblasts or haemangioblasts (which differentiate into endothelial progenitor cells (EPCs) and subsequently into ECs) in a process called 'vasculogenesis'[19] (Fig. 1b), and/or the splitting of existing blood vessels, named 'intussusception'[12] (Fig. 1c). Three additional pathological modes of neovascularization may occur in glial brain tumours and in tissues undergoing regenerative processes (for example, following ischaemic stroke): vascular co-option, in which tumour cells co-opt blood vessels to grow along pre-existing healthy blood vessels (Fig. 1d), glioma (or glioblastoma) stem cell (GSC)-to-EC transdifferentiation or GSC-to-pericyte transdifferentiation[20–22] (Fig. 1e) and vasculogenic (or vascular) mimicry, in which tumour cells integrate into the blood vessel wall, mimicking ECs[12] (Fig. 1f). Whereas sprouting angiogenesis and vasculogenesis are primary contributors to neovascularization during brain development and in brain AVMs (Fig. 1g,i), all six modes of vessel formation have been described in brain tumours[23–26] (Fig. 1h), as discussed later herein.

Sprouting angiogenesis

On a cellular level, sprouting vessels are guided by specialized ECs that extend multiple filopodia, the endothelial tip cells (ETCs)[9,12,18]. Behind the leading ETC, proliferating endothelial stalk cells are responsible for the elongation of blood vessels and the formation of a functional lumen[3,9,12,15,18] (Fig. 1a). Subsequently, sprouting vessels anastomose and establish a three-dimensional, perfused and fully functional vascular network[9,18] (Fig. 1a,g). Quiescent endothelial phalanx cells line the newly formed lumenized vessels and can be reactivated by pro-angiogenic stimuli[3,12,18]. Sprouting angiogenesis and ETCs, stalk cells and phalanx cells are regulated by pro-angiogenic and anti-angiogenic molecules, the balance between them being thought to determine the angiogenic response[3,12,18,27] (Supplementary Table 1). Findings of recent studies have complemented this traditional view on sprouting and ETCs by suggesting a key role of venous ECs as the primary subtype of ECs — which proliferate and migrate against the flow to acquire the ETC position — that are responsible for sprouting angiogenesis and expanding vascular networks[28].

On a molecular level, the VEGF–VEGFR–DLL4–Jagged–Notch signalling cascade is a key regulator of sprouting angiogenesis in both CNS tissues and non-CNS tissues and is thought to be the central pattern generator underlying ETC, stalk cell and phalanx cell differentiation[3,9,29,30] in development and disease. The most important Notch ligands — DLL4 and Jagged 1 — have opposing roles in vessel formation, with DLL4 being anti-angiogenic and Jagged 1 being pro-angiogenic[31]. Interestingly, ETC and stalk cell specification is dynamically regulated by a feedback loop between the VEGF–VEGFR pathway and the DLL4–Jagged 1–Notch pathway[32]. Competition for the tip cell position occurs when activated ECs — expressing VEGFR1, VEGFR2, VEGFR3 and neuropilin 1 (NRP1) — upregulate DLL4 on their membrane, giving these ECs an advantage for the tip cell position[29,32,33]. DLL4 on ETCs activates Notch signalling in adjacent stalk cells, thereby downregulating VEGFR2, VEGFR3 and NRP1, upregulating VEGFR1 and restricting the ability of stalk cells to acquire the tip cell position[30,34] and limiting tip cell numbers[35]. In contrast to DLL4, Jagged–Notch signalling drives tip cell selection and sprouting angiogenesis by antagonizing DLL4–Notch signalling[31].

MPDZ and the transcription factor ERG are key regulators of endothelial Notch–DLL4–Jagged 1 signalling[36], underlining the dynamic nature of EC specification into ETCs, stalk cells and phalanx cells.

We previously described the regulatory effects of NVL molecules on peripheral and CNS angiogenesis during development, including their modes of action as either general cues or CNS-specific cues for vascular growth and their emerging molecular interactions with the VEGF–VEGFR–DLL4–Jagged–Notch pathway, and we do not comprehensively revisit this topic here[9].

Vasculogenesis and intussusception

During embryonic development, vasculogenesis gives rise to the heart and the primitive vascular plexus. The vascular system is generated from precursor cells (angioblasts or haemangioblasts), and its establishment occurs in parallel with haematopoiesis (the formation of blood cells)[37] (Fig. 1b). Angioblasts and blood cells constitute blood islets, which then fuse and give rise to a honeycomb-shaped primitive vascular plexus before the onset of heartbeats[37]. Once blood circulation has been established, primary vascular plexuses are remodelled into hierarchical networks with arteriovenous distinction[37] (Fig. 1g). Subsequently, PVCs, including vSMCs (in the case of arteries and veins) and pericytes (in the case of capillaries), are recruited and stabilize the vascular network[37,38]. Molecularly, fibroblast growth factors (FGFs) induce the formation of angioblasts, whereas VEGFA plays key roles in the differentiation and chemotaxis of angioblasts and EPCs[37].

Intussusceptive angiogenesis is defined as the invagination of the capillary wall into the lumen to split a single vessel in two[39,40] (Fig. 1c). This mode of neovascularization was first observed during the development of peripheral organs[41–44] and was subsequently characterized in CNS tissue[45,46] and in several cancers, including glioblastoma[47]. Transcapillary intraluminal tissue pillars arise by invagination of the capillary wall into the vessel lumen in four consecutive steps[40]. First, a contact zone is established between two opposing capillary walls[40]. Second, reorganization of EC junctions and perforation of the vessel bilayer allows growth factors and cells to penetrate the lumen[40]. Third, an interstitial pillar core forms between the two new vessels at the contact zone and is filled with pericytes and myofibroblasts[40]. Finally, the pillars increase in diameter[40] (Fig. 1c). Interestingly, intussusceptive angiogenesis allows reorganization of existing cells without the need for an increase in EC number, which is especially important during distinct stages of embryonic development in which the growth rate surpasses the cellular resources[40]. The molecular basis of vascular intussusception remains unknown.

ECs and PVCs in the NVU and BBB

Newly formed sprouting vessels are initially fragile and become stabilized by the recruitment of PVCs (such as pericytes, vSMCs and astrocytes)[9,12], which is important for the establishment of functional, perfused blood vessels integrated into a three-dimensional vascular network[3,9,48,49] (Fig. 3). Accordingly, ECs invading the CNS closely interact with PVCs of the surrounding parenchyma, thereby forming a functional NVU[9,15,50,51] (Fig. 3a–d). As initially postulated in 1981, the CNS parenchyma provides instructive signals regulating EC sprouting into the CNS and induction of CNS-specific properties in ECs[5,52]. These structural and functional EC–PVC interactions result in the specific properties of CNS blood vessels, most importantly the establishment of the BBB[53] (Fig. 3c,d), which is already established during embryonic development[54,55] in a process regulated by extrinsic cues provided by the local CNS microenvironment[5,9,52,56–58]. Tight junction-specific proteins, such as CLDN5 and OCLN, are present at the BBB interface directly after blood vessels invade the brain at the embryonic stage and achieve functionality to meet barrier functions (which go beyond the presence or absence of passive permeability) according to the particular stage of brain development during the early postnatal period[54,57–60]. This highly regulated physical permeability barrier can become leaky in CNS pathologies such as brain tumours, brain vascular malformations, ischaemic stroke and some neurodevelopmental and neurodegenerative disorders[4,60–63] (Fig. 4).

NVL molecules

Both the vascular system and the nervous system require coordinated guidance of their cellular and subcellular elements[9,15,61]. At the cellular level, axonal growth cones and ETCs exhibit similar lamellipodia and filopodia[9,12,16,18,64] (Fig. 2a–c). At the subcellular level, axonal growth cones consist of a central domain containing microtubules and a peripheral domain composed of an actin meshwork (in lamellipodia) and F-actin bundles (in filopodia)[9]. Fan-like filopodial protrusions sense stimulatory and inhibitory guidance signals in the microenvironment and steer both the growing axon[65,66] and the developing, newly forming blood vessels[12,16,18,64,67] (Fig. 2a,b). F-actin structures have been found in ETC filopodia[68], but the cytoskeletal organization of tip cells is less well described than that of axonal growth cones, mainly owing to technical limitations and the lack of specific ETC markers. Suggested tip cell markers – such as ESM1, APLN, RAMP3 and CLDN5 – that have emerged from microarray analysis and single-cell RNA sequencing (scRNA-seq) studies[69–76] await full validation.

At the molecular level, numerous cues have been discovered that guide both ETCs and axonal growth cones[9,12,15,16] (Fig. 2g,h). These cues include the four canonical axon guidance molecule families – netrins, semaphorins, ephrins and Slit proteins[9,12,14–16,77] – and other axon guidance molecules, such as WNT proteins, SHH, bone morphogenetic protein (BMP), Nogo-A and Nogo-B, exert similar repulsive and attractive effects on neuronal growth cones[78] and ETCs[9,14,15,79,80] (Supplementary Table 2). In addition to these neural cues guiding blood vessels, classic angiogenic factors such as VEGFA and FGF2 and their receptors, endothelin 3, artemin and the receptor complex RET–GFRα3 can direct neuronal development and axonal growth during brain development[9,14,15,79] (Fig. 2g). The NVL relies on direct cellular interactions between vascular cells and neural cells. For instance, sensory neurons and Schwann cells in the peripheral nervous system provide a template for the patterning of arteries but not veins during skin development, whereas neuronal release of VEGF induces arterial differentiation[81]. In the CNS, retinal ganglion cells and astrocytes provide a physical template for sprouting ECs while releasing pro-angiogenic and anti-angiogenic factors such as VEGFA, semaphorins and Nogo-A. Conversely, vessel-derived cues such as artemin and endothelin 3 guide growing axons in the retina[82,83]. Accordingly, ablation of radial glia[84], oligodendrocyte precursor cells[85] or astroglia[86] results in a severe reduction in developmental angiogenesis[14]. Many of the NVL molecules interact with key downstream angiogenic signalling axes, most notably the VEGF–DLL4–Jagged 1–Notch and YAP–TAZ pathways[9].

Angiogenesis and brain development

Embryonic CNS angiogenesis

Cellular mechanisms during embryonic brain development. During brain development in mice at embryonic day 8.5 (E8.5), a perineural vascular plexus (PNVP) (non-CNS tissue of mesodermal origin) forms around the neuroectodermal-derived neural tube via vasculogenesis

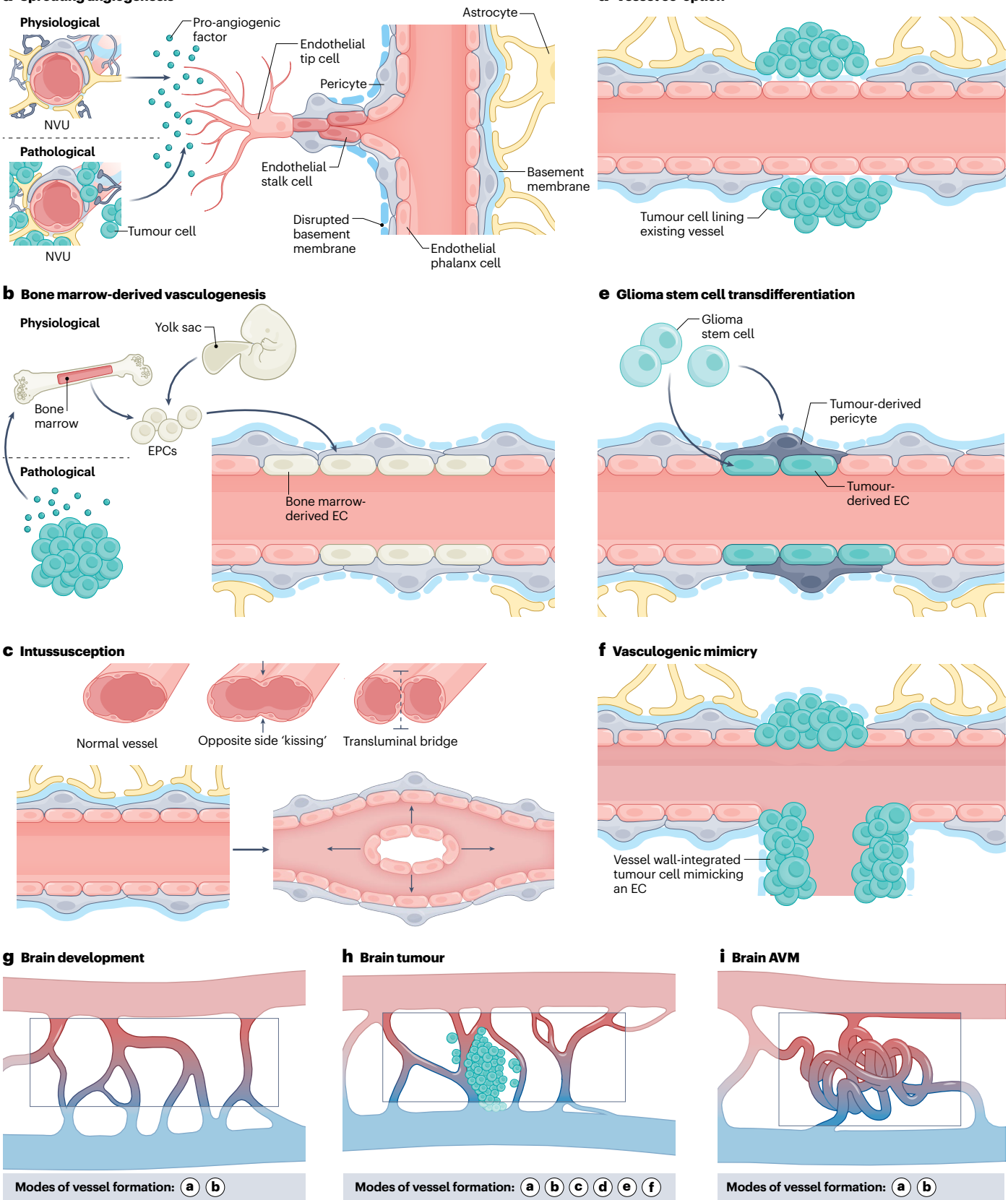

a Sprouting angiogenesis
Physiological
Pro-angiogenic factor
Endothelial tip cell
Astrocyte
Pericyte
NVU
Pathological
Endothelial stalk cell
Basement membrane
Tumour cell
Disrupted basement membrane
NVU
Endothelial phalanx cell
d Vessel co-option
Tumour cell lining existing vessel
b Bone marrow-derived vasculogenesis
Physiological
Yolk sac
Bone marrow
EPCs
Pathological
Bone marrow-derived EC
e Glioma stem cell transdifferentiation
Glioma stem cell
Tumour-derived pericyte
Tumour-derived EC
c Intussusception
Normal vessel
Opposite side 'kissing'
Transluminal bridge
f Vasculogenic mimicry
Vessel wall-integrated tumour cell mimicking an EC
g Brain development
Modes of vessel formation: a b
h Brain tumour
Modes of vessel formation: a b c d e f
i Brain AVM
Modes of vessel formation: a b

Fig. 1 | Modes of vessel formation during brain development, in brain tumours and in brain AVMs. Vascularization during brain development, in brain tumours and in brain arteriovenous malformations (AVMs) can occur via different modes of neovascularization. **a**, Neovascularization is possible via the formation of new blood vessels from pre-existing ones in response to pro-angiogenic signalling molecules secreted by components of the neurovascular unit (NVU) (defined as physiological sprouting angiogenesis) or by tumour cells (defined as pathological sprouting angiogenesis). For simplicity, the NVU (in physiological conditions) and tumour cells (in pathological conditions) are illustrated as sources of pro-angiogenic molecules for this mode of neovascularization. Note that the secretion of pro-angiogenic molecules is not limited to these sources but can also occur from brain vascular malformations and other vascular-dependent brain pathologies as well as from components of the extracellular matrix. New vessel sprouts are guided by specialized endothelial tip cells extending multiple filopodial protrusions sensing and reacting to pro-angiogenic, anti-angiogenic and hypoxia-related cues in the microenvironment. At the back of the leading tip cell, proliferating endothelial stalk cells elongate the growing blood vessel and initiate the formation of a functional lumen. Phalanx cells are the most quiescent of the endothelial cell (EC) subtypes, extend few filopodia and migrate and divide poorly in response to VEGF. Endothelial phalanx cells line vessels once the new vessel branches have been consolidated. **b**, Physiological vasculogenesis is defined as the de novo generation of blood vessels from either yolk sac-derived endothelial progenitor cells (EPCs) or bone marrow-derived EPCs, depending on the developmental time point. Pathological vasculogenesis occurs upon secretion of pro-angiogenic molecules by tumour cells that activate bone marrow to produce EPCs. Both indirect paracrine secretion of pro-angiogenic growth factors and direct luminal incorporation into sprouting nascent vessels contribute to vasculogenesis. Note that the secretion of pro-angiogenic molecules is not limited to these sources but can also occur from brain vascular malformations and other vascular-dependent brain pathologies as well as from components of the extracellular matrix. **c**, The splitting of existing blood vessels — vascular intussusception — allows the reorganization of existing cells without a corresponding increase in EC number. During this process, the opposite capillary walls invaginate into the vessel lumen in consecutive steps with the formation of a transluminal bridge of pericytes, myofibroblasts and extracellular matrix. **d**–**f**, Pathological conditions such as tumours or regenerative processes can exhibit the aforementioned modes of vessel formation and three additional ones, namely vessel co-option, glioma stem cell to EC transdifferentiation or glioma stem cell to pericyte transdifferentiation, and vasculogenic mimicry. Vessel co-option occurs when tumour cells co-opt existing vessels in response to angiopoietin 2 (ANG2) expression gradients (part **d**). In glioma stem cell transdifferentiation, glioma stem-like cells differentiate into either tumour-derived ECs or tumour-derived pericytes, induced predominantly by the TGFβ and NOTCH1 pathways in hypoxic conditions (part **e**). In vasculogenic mimicry, tumour cells (instead of ECs) are incorporated into the inner vessel wall, forming functional vessel-like structures and thereby mimicking ECs (part **f**). **g**–**i**, Modes of vessel formation involved in angiogenesis during brain development (part **g**), in brain tumours (part **h**) and in brain AVMs (part **i**).

(Fig. 3a–d and Supplementary Table 1), in which VEGFA derived from the neural tube interacts with VEGFR2 expressed on PNVP angioblasts[9,50]. This PNVP will later be transformed into arteries and veins of the pia and the arachnoid mater (leptomeninges) ensheathing the CNS tissue[87]. At E9.5, vessel sprouts from the PNVP invade the CNS parenchyma and form the intraneural vascular plexus (INVP) via sprouting angiogenesis[9,54,64,88] (Fig. 3a,b). These perforating vessels of the INVP follow a radial course towards the ventricles. Once they are inside the ventricular zone, they branch in a circumferential fashion parallel to the ependyma, giving rise to a periventricular vascular plexus[89] (Fig. 3a,b). Only after this lateral branching at the periventricular level do lateral branches from the INVP sprout at several levels throughout the cortical layers[89].

In humans, the pial capillary anastomotic plexus is considered the functional and structural analogue of the PNVP in embryonic mice[90]. The pial capillary anastomotic plexus is a meningeal layer of extracerebral or non-CNS origin and is the source of all perforating vessels entering the cerebral cortex during later embryonic and postnatal stages[67,90]. The pial capillary anastomotic plexus is already detectable in 6-week-old human embryos and is separated from the underlying cortical tissue by the brain's external glial limiting membrane[90]. Subsequently, pial capillaries perforate the external glial limiting membrane and grow into the cerebral cortex (comparable to the formation of the INVP in mice) from the eighth week of gestation onwards[90]. Whereas the CNS is, after vasculogenic formation of the PNVP, predominantly vascularized by sprouting angiogenesis[27], vascularization of non-CNS tissues mainly relies on vasculogenesis[91,92], for reasons that remain elusive.

General molecular mechanisms during embryonic brain development. Various general developmental pathways are active in both the CNS tissue and peripheral tissue, including the following: VEGFA–VEGFR–DLL4–Jagged 1–Notch signalling for appropriate vessel sprouting, patterning and vascular remodelling[34,50,93,94] (see earlier herein for a description of this signalling pathway); YAP and TAZ as essential co-transcriptional activators of the Hippo pathway in ECs[95]; angiopoietins and their receptors TIE1 and TIE2 as modulators of vessel stability[96–98]; the classic axon guidance ligand–receptor pairs SLIT2–ROBO4 (refs. [99–101]), SEMA3E–plexin D1 (ref. [102]), netrin 4–UNC5B[103] and ephrin B2–EphB4 (ref. [104]); and the non-classic axon guidance cues, namely integrin αVβ8-activated TGFβ signalling[105], WNT[78], BMP[78] and SHH[78,79] (Supplementary Table 2). Although many of these pathways are active and important in CNS angiogenesis, they were first discovered in peripheral tissues, acting through a general (non-CNS-specific) molecular mode of action.

YAP and TAZ are transcriptional co-activators regulating the Hippo pathway and have crucial roles in organogenesis and embryonic vascular brain development in a non-CNS-specific manner. The VEGF and YAP–TAZ signalling pathways converge: VEGF stimulates Rho family members, thereby altering cytoskeletal dynamics, contributing to the activation of YAP–TAZ signalling[106]. YAP and TAZ, in turn, upregulate the gene expression of Rho family members, providing actin cytoskeletal rearrangements needed for ETC migration and stalk cell proliferation during embryonic and postnatal vascular brain development[95,106].

Angiopoietin 1 (ANG1) and ANG2 bind to the tyrosine kinases TIE1 and TIE2 and directly act on ECs by modulating cell–cell and cell–extracellular matrix (ECM) communication and promoting or inhibiting angiogenesis, which is of crucial importance before E13.5 (refs. [107,108]). ANG1 and ANG2 often have complementary roles in the development of a healthy vasculature; they modulate vessel stability and can be either pro-angiogenic or anti-angiogenic depending on the context[96,98,108].

Classic axon guidance cue signalling, such as SLIT-dependent activation of the EC-specific receptor ROBO4 inhibits endothelial hyperpermeability induced by pro-angiogenic factors and enhances vascular stability[99]. ROBO4-mediated SLIT2-dependent suppression of cellular permeability occurs through inhibition of the small GTPases ARF6 and RAC[109]. In vivo, inhibition of ARF6 resembles ROBO4 activation by reducing pathological angiogenesis and vessel leakage in retinal hyperpermeability models during vascular development inside and outside the CNS[99,101,110]. The effects of *ROBO4* silencing on human brain

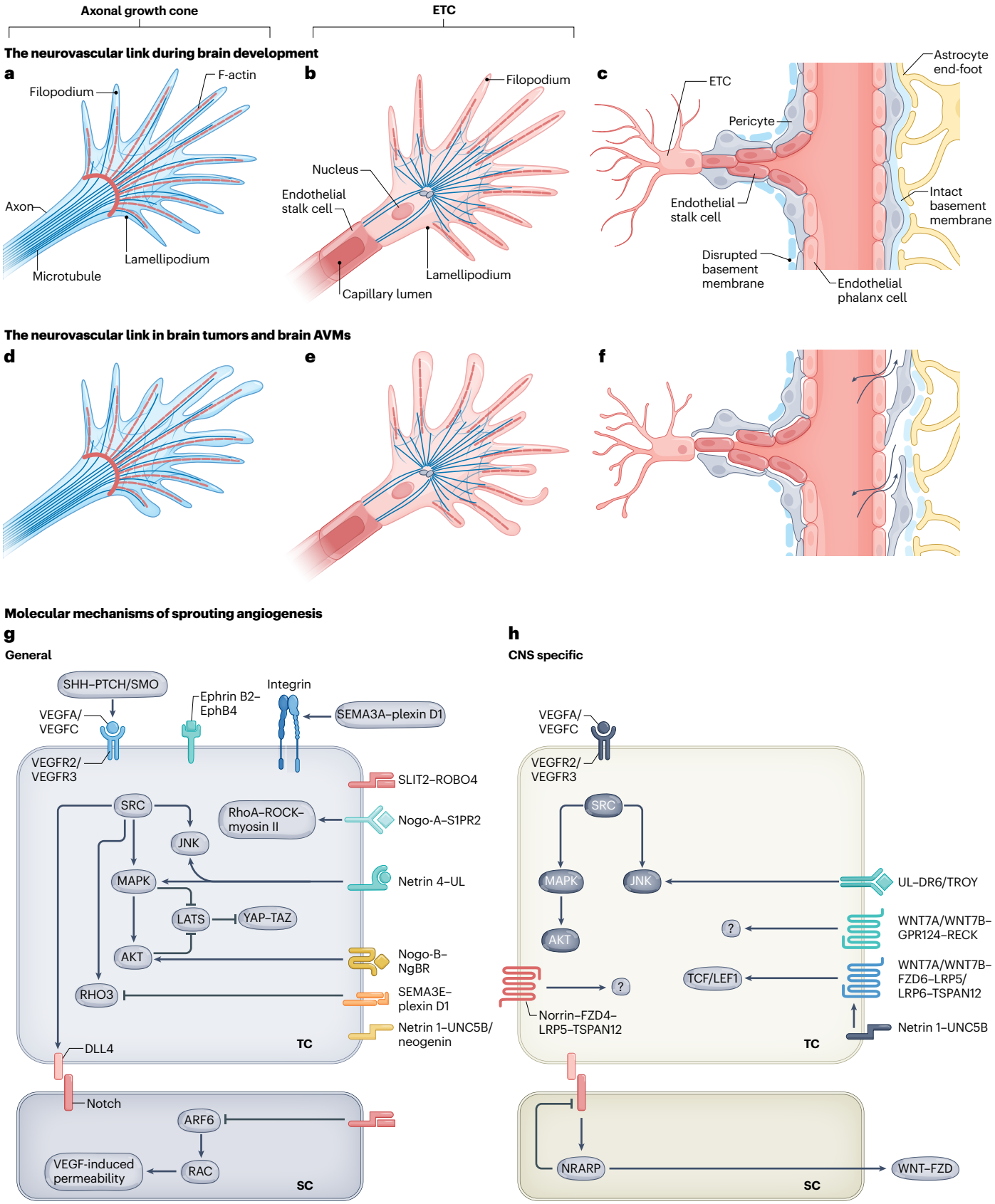

Axonal growth cone
ETC
The neurovascular link during brain development
a
Filopodium
F-actin
Axon
Microtubule
Lamellipodium
b
Filopodium
Nucleus
Endothelial stalk cell
Lamellipodium
Capillary lumen
c
ETC
Astrocyte end-foot
Pericyte
Endothelial stalk cell
Intact basement membrane
Disrupted basement membrane
Endothelial phalanx cell
The neurovascular link in brain tumors and brain AVMs
d
e
f
Molecular mechanisms of sprouting angiogenesis
g
General
SHH–PTCH/SMO
VEGFA/ VEGFC
VEGFR2/ VEGFR3
Ephrin B2–EphB4
Integrin
SEMA3A–plexin D1
SLIT2–ROBO4
SRC
RhoA–ROCK–myosin II
Nogo-A–S1PR2
JNK
MAPK
Netrin 4–UL
LATS
YAP–TAZ
AKT
Nogo-B–NgBR
RHO3
SEMA3E–plexin D1
Netrin 1–UNC5B/ neogenin
DLL4
TC
Notch
ARF6
VEGF-induced permeability
RAC
SC
h
CNS specific
VEGFA/ VEGFC
VEGFR2/ VEGFR3
SRC
MAPK
JNK
UL–DR6/TROY
AKT
?
WNT7A/WNT7B–GPR124–RECK
TCF/LEF1
WNT7A/WNT7B–FZD6–LRP5/ LRP6–TSPAN12
?
Norrin–FZD4–LRP5–TSPAN12
Netrin 1–UNC5B
TC
NRARP
WNT–FZD
SC

Fig. 2 | Neurovascular link molecules affecting endothelial tip cell sprouting during vascular brain development, in brain tumours and in brain AVMs. **a**, The axonal growth cone at the leading edge of a growing axon is a specialized, subcellular 'hand-like' structure at the tip of an extending neuron. In the axonal growth cone, lamellipodia and filopodia sense and integrate attractive and repulsive guidance cues in the local tissue microenvironment, thereby guiding the extending axon to its target. The central domain of an axonal growth cone is rich in microtubules, whereas the peripheral domain predominantly contains filopodia (composed of F-actin bundles) and lamellipodia (composed of an actin meshwork). Some microtubules extend into the peripheral domain and rarely into filopodia. **b**, The endothelial tip cell (ETC) is a specialized vascular endothelial cell type at the tip of the newly forming blood vessel, followed by proliferating endothelial stalk cells. Similarly to axonal growth cones, ETCs are specialized, 'hand-like' structures at the forefront of growing blood vessels that sense environmental cues using lamellipodia and 'finger-like' filopodia, thereby guiding the growing blood vessels to their respective targets. Endothelial phalanx cells comprise a third, mostly silent vascular endothelial cell type, lining the border of functional, established blood vessels (not shown). ETCs use actin-based lamellipodia and filopodia sensing attractive and repulsive guidance cues in the local tissue microenvironment to reach their target. Microtubules have not been detected in filopodia so far. **c**, A newly forming blood vessel sprout including a migrating ETC extending multiple filopodia, followed by proliferating endothelial stalk cells creating a newly formed capillary lumen, and quiescent endothelial phalanx cells lining an established vascular blood vessel. Pericytes, astrocytes and the basement membrane are also depicted. **d**–**f**, Schematic illustrations showing the characteristics of the axonal growth cone (part **d**), ETC (part **e**) and vessel sprouting (part **f**) in pathological conditions. Newly formed vessels often show a disrupted basement membrane, vascular leakage and a reduced pericyte coverage (part **f**). **g**,**h**, Molecularly, sprouting angiogenesis into the CNS is regulated by neurovascular link molecules that act in a non-CNS-specific way (part **g**), such as VEGFA–VEGFR2, SEMA3A/SEMA3E–plexin D1, ephrin B2–EphB4 and SLIT2–ROBO4, or a CNS-specific manner (part **h**), such as WNT7A/WNT7B–GPR124–FZD6–RECK and DR6–TROY. Of note, the VEGFA/VEGFC–VEGFR2/VEGFR3 and netrin 1–UNC5B signalling axes are shown in part **h** because even though they represent non-CNS-specific mechanisms, multiple CNS-specific mechanisms interact with these pathways downstream. AVM, arteriovenous malformation; SC, stalk cell; TC, tip cell; UL, unknown ligand.

microvascular EC proliferation, migration and tube formation remain controversial[101,110].

SEMA3A is a secreted protein mediating anti-angiogenesis via the NRP1 and plexin A–plexin D1 receptor complex[111]. The exact role of SEMA3A during developmental CNS angiogenesis is unknown, given the absence of a vascular phenotype in *Sema3a*$^{-/-}$ embryos[112] and in NRP1^{sema} mice[113], which express a mutated variant of NRP1 that lacks the SEMA-binding domain. At E10, SEMA3A is expressed in vascular ECs in the spinal cord and dorsal aorta[111]. Interestingly, at E12.5, SEMA3A expression is stronger on ETCs than on stalk cells during INVP sprouting into the brain parenchyma and retina, indicating that its expressed on actively sprouting endothelium[71,114]. In zebrafish, Sema3A–plexin D1 signalling negatively regulates angiogenesis through modulation of soluble Flt1 expression[115], illustrating the role of Sema3A–plexin D1 during embryonic brain vascularization in a non-CNS-specific manner.

SEMA3E–plexin D1 signalling negatively regulates angiogenesis inside and outside the CNS via interaction with the VEGF–DLL4–Jagged–Notch pathway. Plexin D1 can be detected in mouse embryos as early as E9.5 (refs. [102,116]) as well as postnatally (postnatal day 2 to postnatal day 6) in the mouse retina[102,116], where plexin D1 is expressed in ETCs and stalk cells but is absent in mature vessels, indicating that it has a role during developmental sprouting angiogenesis[117]. SEMA3E–plexin D1 signalling leads to downstream activation of the small GTPase RhoJ, with subsequent VEGF-induced DLL4 expression in retinal ETCs in vivo[118] and in human umbilical vein ECs in vitro, contributing to the ETC and stalk cell selection in both the CNS vasculature and the non-CNS vasculature[117]. Whether SEMA3A–plexin D1 signalling or SEMA3E–plexin D1 signalling regulates PNVP and INVP formation during embryonic human CNS development remains to be explored.

Netrin 1 and netrin 4 are anti-angiogenic factors that act through binding to UNC5B (in the case of netrin 1) or to neogenin with recruitment of UNC5B (in the case of netrin 4) in peripheral tissues and the CNS in a general (non-CNS-specific) manner[119–121]. Netrin 1 and netrin 4 and their receptors act as repulsive or attractive cues, partially via regulation of VEGF signalling[119], starting during embryonic developmental angiogenesis inside and outside the CNS[119,120].

Last, the Eph family of receptor tyrosine kinases interacts with membrane-bound ligands called 'ephrins'[122]. Ephrin B2, being the sole transmembrane ligand for EphB4, is specifically expressed in arterial angioblasts starting at around E9 (ref. [123]). EC and perivascular mesenchymal cell[123] interactions lead to activation of the ephrin B2–EphB4 axis, providing attractive and repulsive guidance cues for EphB-expressing cells in angiogenesis as well as regulation of migratory and invasive cellular functions in a non-CNS-specific way[122,123].

Non-classic axon guidance cues such as the five members of the αV integrin subfamily (αVβ1, αVβ3, αVβ5, αVβ6 and αVβ8) are expressed by many different cell types, notably by neurons and ECs of the brain (acting as NVL molecules) but also in other organs and tissues, and bind to RGD peptide motifs present on many shared ECM ligands, most importantly to latent TGFβ proteins[124]. The αV integrin is of particular interest in genetic studies in mice as it is an important regulator of embryonic cerebrovascular morphogenesis (although the actions of αV integrin are not exclusively CNS specific)[125,126]. Integrin αVβ8 activates ventral–dorsal TGFβ gradients in the brain, inhibiting EC sprouting and stabilizing blood vessels via downstream TGFβ1–TGFBR2–ALK5–SMAD3 signalling[105,125,127,128]. Ablation of αV integrin-coding or β8 integrin-coding genes in embryonic brain ECs causes pathological vascular phenotypes, including EC hyperproliferation and intracerebral haemorrhages[105,127]. In mice, knocking out either of the genes encoding the TGFβ signalling co-receptors — that is, ALK1 (encoded by *Acvrl1*, also known as *Alk1*) and endoglin (ENG; encoded by *Eng*) — causes embryonic lethality at E11.5 (refs. [129,130]).

Several axon guidance molecules, including the WNT proteins, SHH and BMP, guide both axonal growth cones[78] and ETCs according to the concept of the NVL[79] (Fig. 2). The specific effects of NVL molecules on ETC guidance, with the exception of the CNS-specific WNT ligands WNT7A and WNT7B (which are discussed later), are less clear than their roles in axon guidance[15].

CNS-specific molecular mechanisms during embryonic brain development. CNS-specific molecular cues that are active in developmental angiogenesis include WNT7A and WNT7B, GPR124 and its co-receptor RECK[131–137] with suggested upstream involvement of netrin 1–UNC5B[138,139], DR6 and TROY[50,140], the norrin–FZD4–LRP5–TSPAN12 complex[141–143] and the recently discovered brain EC-specific WNT regulator PPIL4 (ref. [144]) (Supplementary Table 2). Even though

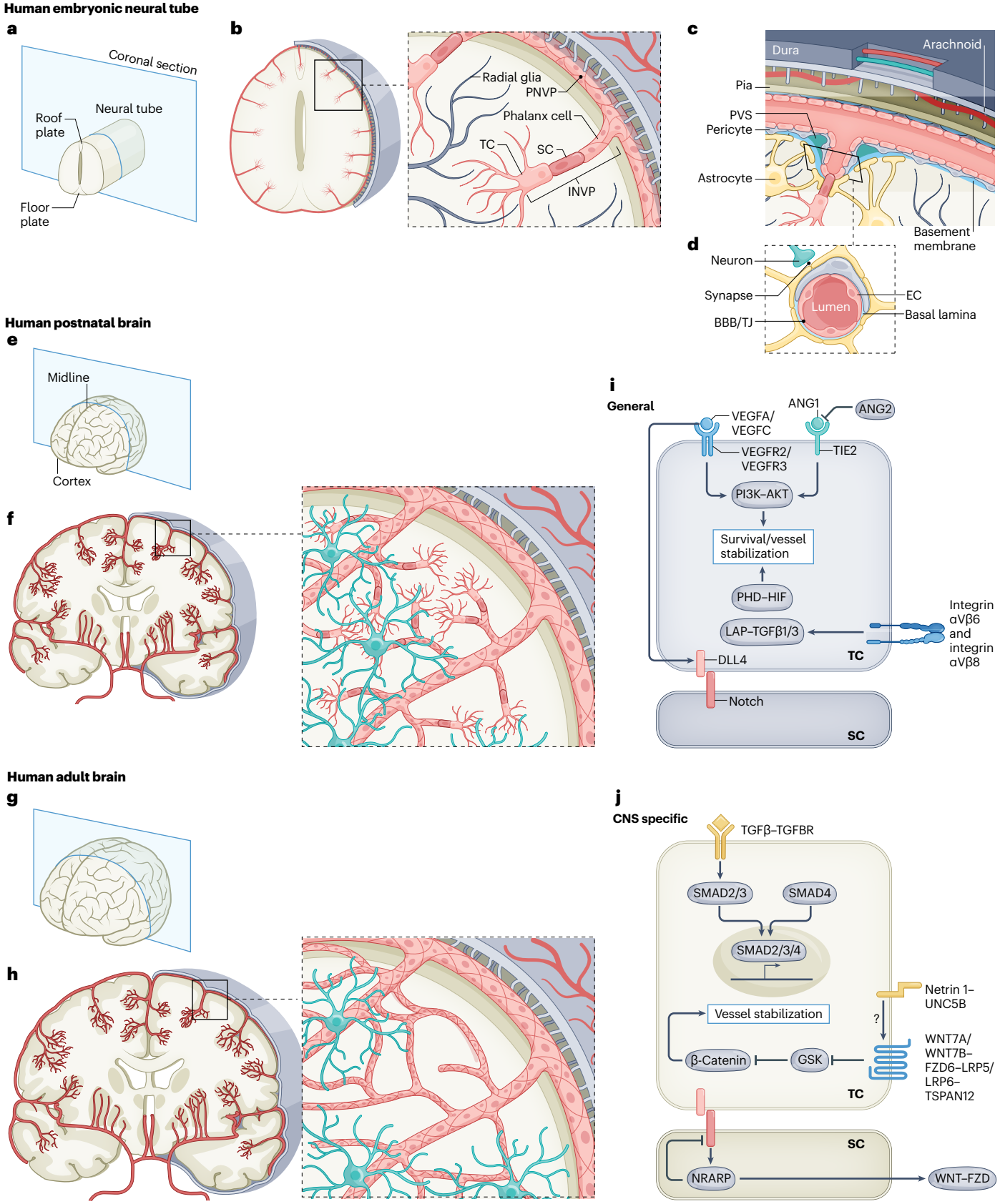

Human embryonic neural tube
a
Coronal section
Neural tube
Roof plate
Floor plate
b
Radial glia
PNVP
Phalanx cell
TC
SC
INVP
c
Dura
Arachnoid
Pia
PVS
Pericyte
Astrocyte
Basement membrane
d
Neuron
Synapse
BBB/TJ
Lumen
EC
Basal lamina
Human postnatal brain
e
Midline
Cortex
f
i
General
VEGFA/ VEGFC
ANG1
ANG2
VEGFR2/ VEGFR3
TIE2
PI3K–AKT
Survival/vessel stabilization
PHD–HIF
LAP–TGFβ1/3
Integrin αVβ6 and integrin αVβ8
DLL4
TC
Notch
SC
Human adult brain
g
h
j
CNS specific
TGFβ–TGFBR
SMAD2/3
SMAD4
SMAD2/3/4
Vessel stabilization
Netrin 1–UNC5B
?
WNT7A/ WNT7B–FZD6–LRP5/ LRP6–TSPAN12
β-Catenin
GSK
TC
SC
NRARP
WNT–FZD

Fig. 3 | Structural and molecular mechanisms of angiogenesis at the embryonic, postnatal and adult stages of vascular brain development. a, A human neural tube at the embryonic stage with the roof and floorplate illustrated on the coronal cutting plane. **b**, Sprouting angiogenesis into the neural tube during embryogenesis. The perineural vascular plexus (PNVP) is formed by vasculogenesis from mesodermal-derived angioblasts at around 7 weeks of gestational age in humans (embryonic day 8.5 (E8.5) in mice). Subsequently, at around 8 weeks of gestational age in humans (E9.5 in mice), angiogenic sprouts of the intraneural vascular plexus (INVP) are formed along radial glia via sprouting angiogenesis using endothelial tip cell (ETC) filopodia, invading the CNS parenchyma and migrating towards the ventricle, where pro-angiogenic and anti-angiogenic factors such as VEGFA and WNT proteins are produced. At the forefront of these angiogenic sprouts, ETCs guide the CNS-invading blood vessels using ETC filopodia. **c**, The anatomical organization of the meningeal layers, including dura, arachnoid and pia mater with intradural lymphatic vessels (blue) and blood vessels (red). An angiogenic vascular sprout emanating from the extraparenchymal PNVP composed of ETCs, endothelial stalk cells and endothelial phalanx cells invading the intraparenchymal INVP is shown. A perivascular space (PVS) surrounds the base of the vascular sprout. **d**, The neurovascular unit (NVU) for established blood vessels that is composed of a variety of cell types, including endothelial cells (ECs), pericytes, astrocytes and neurons. ECs and pericytes are ensheathed by a common basal lamina, the endothelial basement membrane. The blood–brain barrier (BBB) is composed of microvascular ECs that are mutually connected via complex tight junctions (TJs), thereby regulating or inhibiting paracellular diffusion of water-soluble molecules. ECs regulate the transport of molecules between the blood and the brain parenchyma via the expression of influx and efflux transporters. **e**, A coronal section of a human brain during postnatal development. **f**, At the postnatal stage, sprouting angiogenesis is the main mode of neovascularization, and vascular sprouting occurs in all directions throughout cortical layers 1–6. Endothelial sprouts invading the CNS parenchyma from week 8 of gestational age (E9.5 in mice) onwards grow along radial glia fibres towards the ventricle. **g**,**h**, In the healthy adult brain, the vasculature is almost quiescent, with only very few ECs proliferating. **i**,**j**, Molecularly, numerous pathways have been implicated in EC quiescence, survival and maintained inhibition of paracellular permeability, and the molecular cues can be either non-CNS specific or CNS specific. The TGFβ–TGFβR signalling axis is shown here because even though it is a non-CNS-specific mechanism of angiogenesis, it interacts downstream with the CNS-specific WNT7A/WNT7B–GPR124–FZD6–RECK pathway. ANG1, angiopoietin 1; ANG2, angiopoietin 2; SC, stalk cell; TC, tip cell.

absolute CNS specificity is nearly impossible to prove, most of the CNS-specific molecular mechanisms that regulate the vasculature were shown to be absent in a number of peripheral tissues.

Endothelial β-catenin signalling is crucial for the establishment and maintenance of a functional BBB during embryonic and postnatal brain development[145,146]. To activate the β-catenin pathway in a CNS-specific manner, the ligands WNT7A and WNT7B and/or norrin with its co-activator TSPAN12 (in retinal angiogenesis) is produced by glial cells or neurons to activate the co-receptors LRP5 and LRP6 on ECs[146]. Mutations in the genes encoding β-catenin, norrin, FZD4, LRP5, LRP6 and TSPAN12 can cause inherited defects in retinal vascularization, whereas targeted mutations in the genes encoding WNT7A and WNT7B cause defects in both retinal and brain angiogenesis[143]. The binding of WNT7A and WNT7B to two membrane proteins expressed on CNS ECs —GPR124 and RECK — specifically enhances intracellular β-catenin signalling and is crucial for proper vessel ingression into the CNS parenchyma and the formation of CNS-specific properties of the INVP[131,133–136,147,148]. Interestingly, in regions where the barrier function of the BBB is physiologically reduced to monitor serum osmolarity and electrolyte balance — most notably the microvasculature of the circumventricular organs, the choroid plexus and the choriocapillaris and ciliary bodies in the eye — EC WNT–β-catenin signalling is kept at low rates, resulting in strict maintenance of this high-permeability state[149,150].

Recent studies showed that EC-specific deletion of the gene encoding the non-CNS-specific receptor UNC5B in mice induces loss of BBB integrity, characterized by reduced CLDN5 levels and increased expression of the permeability protein PLVAP[138,139]. UNC5B-bound netrin 1 interacts with the CNS-specific WNT7A and WNT7B co-receptor LRP6, leading to downstream activation of the WNT–β-catenin pathway inside but not outside the CNS (for example, there are no effects on the vasculature in the lungs, heart and kidneys). This signalling might be an important CNS-specific downstream mechanism regulating BBB integrity[138].

Embryonically, mutations in *Gpr124* (also known as *Adgra2*) or *Reck* severely impair CNS angiogenesis and barriergenesis[133,136,148]. Endothelial-specific *Gpr124* deletion causes embryonic lethality in mice from E15.5 onwards owing to angiogenic defects in the forebrain and neural tube, whereas *Gpr124* overexpression produces CNS-specific hyperproliferative vascular malformations[135]. This forebrain (but not midbrain or hindbrain) localization pattern suggests that GPR124 mediates EC migration towards regional guidance cues in the embryonic CNS[135]. Endothelial β-catenin signalling promotes sprouting angiogenesis, ETC formation and VEGFR expression during postnatal brain and retinal vascular development[151]. Increased β-catenin levels also lead to upregulation of DR6 and TROY, which are required for vascular and BBB development and maintenance in a CNS-specific manner in zebrafish and mice[140]. *ppil4*$^{-/-}$ zebrafish exhibited a brain EC-specific phenotype, including necrosis in the dorsal midbrain and embryonic lethality 2 days after fertilization[144]. Interestingly, PPIL4 exerts brain EC-specific modes of action via a downstream effect on WNT signalling cascades[144]. Finally, the formation of arteriovenous connections during CNS development is partially mediated by the receptor–ligand pair Cxcr4–Cxcl12b in the CNS but not in the trunk of zebrafish embryos, suggesting it has a CNS-specific nature[152].

Postnatal CNS angiogenesis

Cellular angiogenic mechanisms during postnatal brain development. Sprouting angiogenesis continues postnatally and further remodels and expands the CNS vascular network[3,9,153] (Fig. 3e,f). Whereas sprouting angiogenesis and ETCs advance in a radial manner during embryonic development[88], postnatally, ETCs spread in all directions of the various cortical layers, mostly emanating from the main vessel branches established during brain embryogenesis[3,153,154] (Fig. 3e,f).

General angiogenic molecular mechanisms during postnatal brain development. Much less is known about the molecular regulation of brain angiogenesis and vascular patterning postnatally than in the embryonic stage. Many molecules and molecular pathways are probably active during both developmental stages, including the VEGFA–VEGFR–DLL4–Jagged 1–Notch pathway, YAP–TAZ, integrin αVβ8, SEMA3A and SEMA3E, and ephrin B2–EphB4 (ref. [50]) (Supplementary Table 2). We identified Nogo-A as a major negative regulator of sprouting angiogenesis, ETCs and vascular network formation in the postnatal brain[17], whereas its role during embryonic vascular brain development remains unclear. The vascular receptor for the Nogo-A

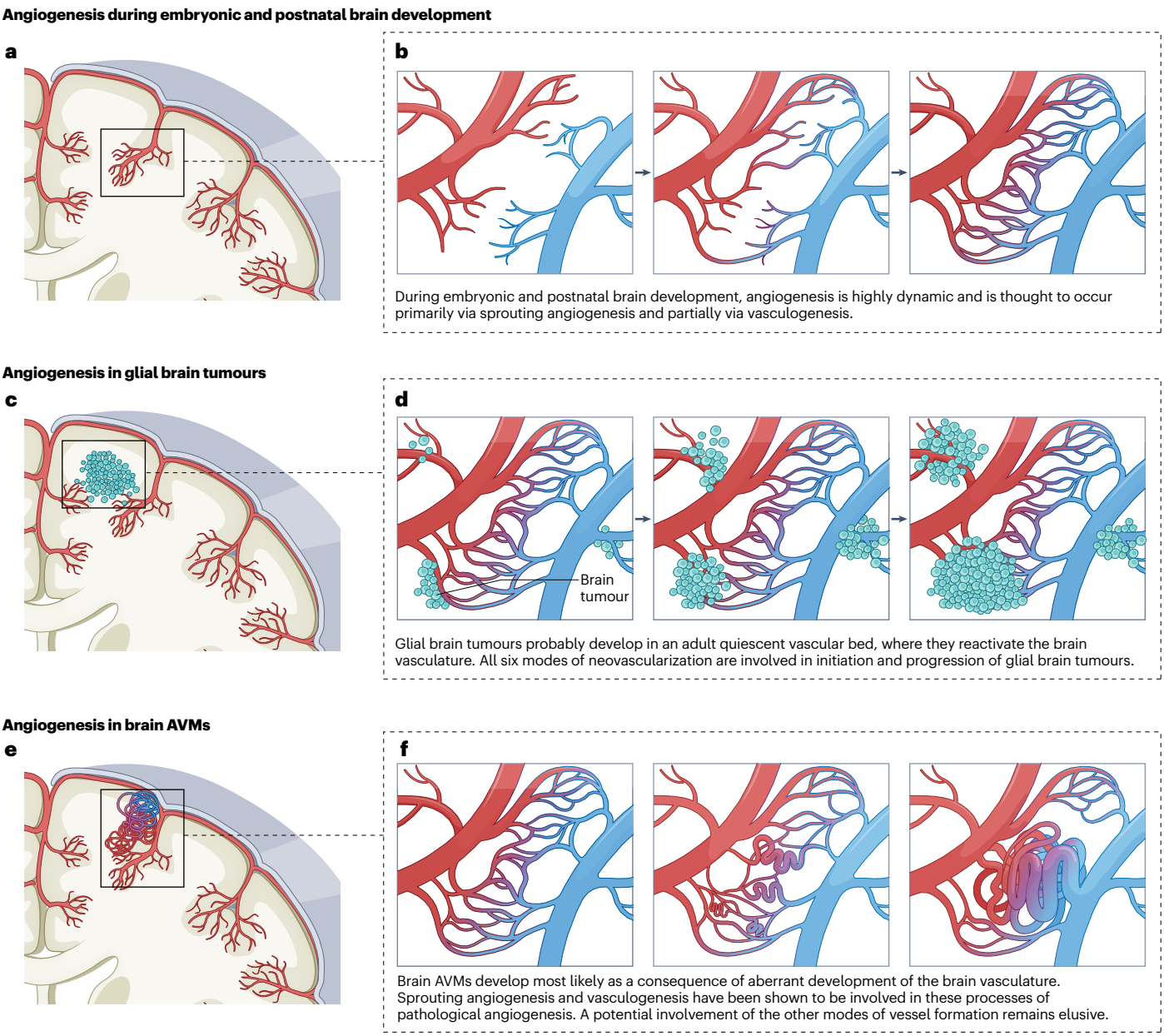


Fig. 4 | Angiogenesis during brain development, in glial brain tumours and in brain AVMs. a,**b**, Angiogenesis during embryonic and postnatal brain development is initiated by bone marrow-derived de novo vasculogenesis followed by sprouting angiogenesis with the formation and elongation of new vessel sprouts from pre-existing vessels. Newly formed vessels fuse with other vascular sprouts in a process called 'anastomosis', thereby forming a healthy capillary bed within a three-dimensional network of perfused, functional vasculature. **c**,**d**, Glial brain tumours develop in a vascular bed where they reactivate the surrounding quiescent brain vasculature but also form their own blood vessels within the tumour mass. All six modes of neovascularization are active in glial brain tumours. **e**,**f**, Brain arteriovenous malformations (AVMs) develop as a consequence of aberrant vascular development of a healthy capillary bed in which the initial formation of arteriovenous shunts leads to further progression towards brain AVMs. Sprouting angiogenesis and bone marrow-derived vasculogenesis (in the AVM nidus) play an important role during the initiation and progression of brain AVMs.

isoform Nogo-B, NgBR[155], regulates both embryonic and postnatal brain angiogenesis[9,156–159]. NgBR knockdown in zebrafish models stopped Nogo-B-stimulated EC migration and reduced VEGF-induced phosphorylation of AKT and EC morphogenesis in a general (non-CNS-specific) manner[9,156].

CNS-specific angiogenic molecular mechanisms during postnatal brain development. Similarly to observations made during the embryonic stage, postnatal deletion of *Gpr124*, *Reck* or *Ndp* (which encodes norrin) compromises angiogenesis and BBB integrity in a CNS-specific manner[133,136]. Whereas most of the CNS-specific mechanisms regulating

vascular brain development at the embryonic stage also regulate postnatal brain angiogenesis and barriergenesis, little is known about the molecular mechanisms that regulate CNS vascular development solely at the postnatal stage (Supplementary Table 2).

Summary

In conclusion, during both embryonic and postnatal brain development, sprouting angiogenesis is highly active and vascular sprouts led by ETC filopodia invade the CNS tissue to establish a functional vascular network. Molecular pathways regulating developmental brain angiogenesis in a general or CNS-specific way are increasingly being discovered, but our knowledge of these molecular processes and their interactions with the VEGF–VEGFR–DLL4–Jagged–Notch pathway and the Hippo–YAP–TAZ pathway remains incomplete[9,50] (Supplementary Table 2). In the adult human brain vasculature, most of the aforementioned developmental pathways are downregulated, keeping the vasculature in a quiescent homeostatic state[9,61,160,161] (Fig. 3g–j).

Angiogenesis in brain tumours

In contrast to the healthy adult quiescent vasculature, brain tumours are characterized by aberrant angiogenesis and alterations to the BBB[61,162], to CNS specificity and to arteriovenous specification of ECs[24], but to what extent developmental signalling axes are reactivated in brain tumours remains poorly understood. Here we focus on intra-axial glial brain tumours, which are a classic example of highly angiogenic brain tumours characterized by the crucial role of their vasculature and aberrant capillary beds in disease initiation and progression[163–166].

Glial brain tumours

Vascular proliferation is an important pathological hallmark of glioblastomas (high-grade gliomas), which have one of the most extensive vascular systems among all solid tumours and vascular proliferation is an important pathological hallmark[164–166]. However, targeting glioma vascularization using an anti-VEGF therapy[167], a combined anti-FGF–anti-VEGF therapy[168] or other approaches has resulted in disappointing results[166,169–171], probably owing to an incomplete understanding of the cellular and molecular mechanisms regulating angiogenesis and the NVU and PVN in glial brain tumours.

Modes of neovascularization

In glial brain tumours, all six mechanisms of neovascularization have been characterized[23–26,172] (Figs. 1, 4c,d and 5 and Supplementary Table 1).

Vascular co-option. Chronologically, the first mode of neovascularization in glial tumours is vascular co-option, involving the organization of tumour cells into perivascular cuffs around microvessels of the surrounding healthy brain tissue to form an early, initially well vascularized tumour mass[25] (Figs. 1d and 4c,d and Supplementary Table 1). This process mostly occurs in highly vascularized tissues but may also occur in malignancies both inside and outside the CNS, including liver cancer[173], lung tumours[174], breast-to-brain metastases[175] and glial brain tumours[176], as well as in tumour recurrence and metastatic growth following administration of anti-angiogenic therapies in glioblastoma[13,176] (Figs. 1d,h and 4c,d and Supplementary Table 1).

At the cellular level, cytoplasmic extensions of glioblastoma cells termed 'flectopodia' modify the normal contractile activity of pericytes surrounding pre-existing vessels, resulting in co-option of these blood vessels, thereby illustrating cellular interactions within the tumour NVU and PVN[177]. Molecularly, inhibition of the small GTPase CDC42, a principal regulator of cell polarity and actin cytoskeletal organization, impairs vessel co-option, thereby favouring an innate immune response against the tumour[177]. Co-opted vessels do not undergo sprouting angiogenesis as a direct next step but first regress via disruption of EC interactions and proteolysis of the basement membrane and ECM, mediated by expression of ANG2 (ref. [178]) (Supplementary Table 1). ANG2 is expressed by ECs in co-opted vessels at an early stage and appears to counter the constitutive expression of ANG1 in healthy tissues. ANG2 is upregulated through HIF1α-dependent mechanisms and contributes to the formation of the leaky, tortuous and dysfunctional vessel characteristics of glioblastoma[179]. Other molecular players in vascular co-option include bradykinin, EGFRvIII[180], MDGI[181] and ephrin B2 (ref. [182]). Ultimately, the remaining tumour is rescued by sprouting angiogenesis at the tumour borders[25,39,182] (discussed later). To date, no CNS-specific mechanisms regulating vascular co-option in glial tumours have been identified.

Sprouting angiogenesis. Glioma-associated sprouting angiogenesis begins after ANG1-mediated and ANG2-mediated breakdown of existing, co-opted vessels. In the presence of ANG2, VEGF promotes EC migration and proliferation and stimulates sprouting of pre-existing blood vessels[29]. Under hypoxic conditions characterized by high HIF1α expression, VEGF ligands and receptors are upregulated and VEGFA binds VEGFR2 and VEGFR3, resulting in MAPK (ERK)-dependent upregulation of VEGF signalling in gliomas[64]. DLL4 inhibition leads to non-productive angiogenesis with aberrantly high ETC and filopodia numbers and suppression of tumour growth in glioma models, whereas prolonged complete inhibition of DLL4 resulted in highly vascular tumours with a haemangioblastoma phenotype, illustrating this carefully balanced mechanism[183] (Figs. 1a,h, 4c,d and 5f,g and Supplementary Tables 1 and 2). Stabilization of the newly formed capillaries requires interactions between ECs, PVCs and ECM components[184–187]. For instance, during vessel lumen formation, pericytes are recruited towards the newly formed vessels in response to platelet-derived growth factor (PDGF) and matrix metalloproteinase upregulation in activated glioma ECs to stabilize the vascular sprout[53,185,187,188].

Bone marrow-derived vasculogenesis. Vasculogenesis is important in tumour biology, and involves the differentiation of three types of circulating bone marrow-derived cells: most importantly, EPCs and pericyte progenitor cells[25], and the less well characterized $CD45^+$ vascular modulatory cells[189] (Figs. 1b,h and 4c,d and Supplementary Table 1). Multiple studies showed that impaired recruitment of EPCs interferes with tumour progression in human gliomas[190,191]. EPCs, defined by the expression of progenitor markers (CD34 and CD133) and EC markers (CD31 and VEGFR2) regulate angiogenesis-mediated tumour progression indirectly via paracrine secretion of pro-angiogenic growth factors[192] and by direct luminal incorporation into nascent sprouting vessels[81,193].

In a transgenic mouse model of liver carcinogenesis, $CCR2^+$ and $CCR5^+$ EPCs were incorporated into the tumour vasculature[191]. Glioblastoma recruits $CXCR4^+$ EPCs in the process of bone marrow-derived vasculogenesis through activity of HIF1α and its target SDF1α[194]. Bone marrow-derived vasculogenesis is important in glioblastoma resistance to initial chemoradiotherapy and pharmacological VEGF inhibition[195], and clinical trials targeting inhibition of the SDF–CXCR4–CXCR7 axis combined with anti-VEGF therapy in glioblastoma are ongoing[196]. Clinically, the number of EPCs in peripheral blood of patients correlates with glioblastoma blood vessel density and angiogenic activity and

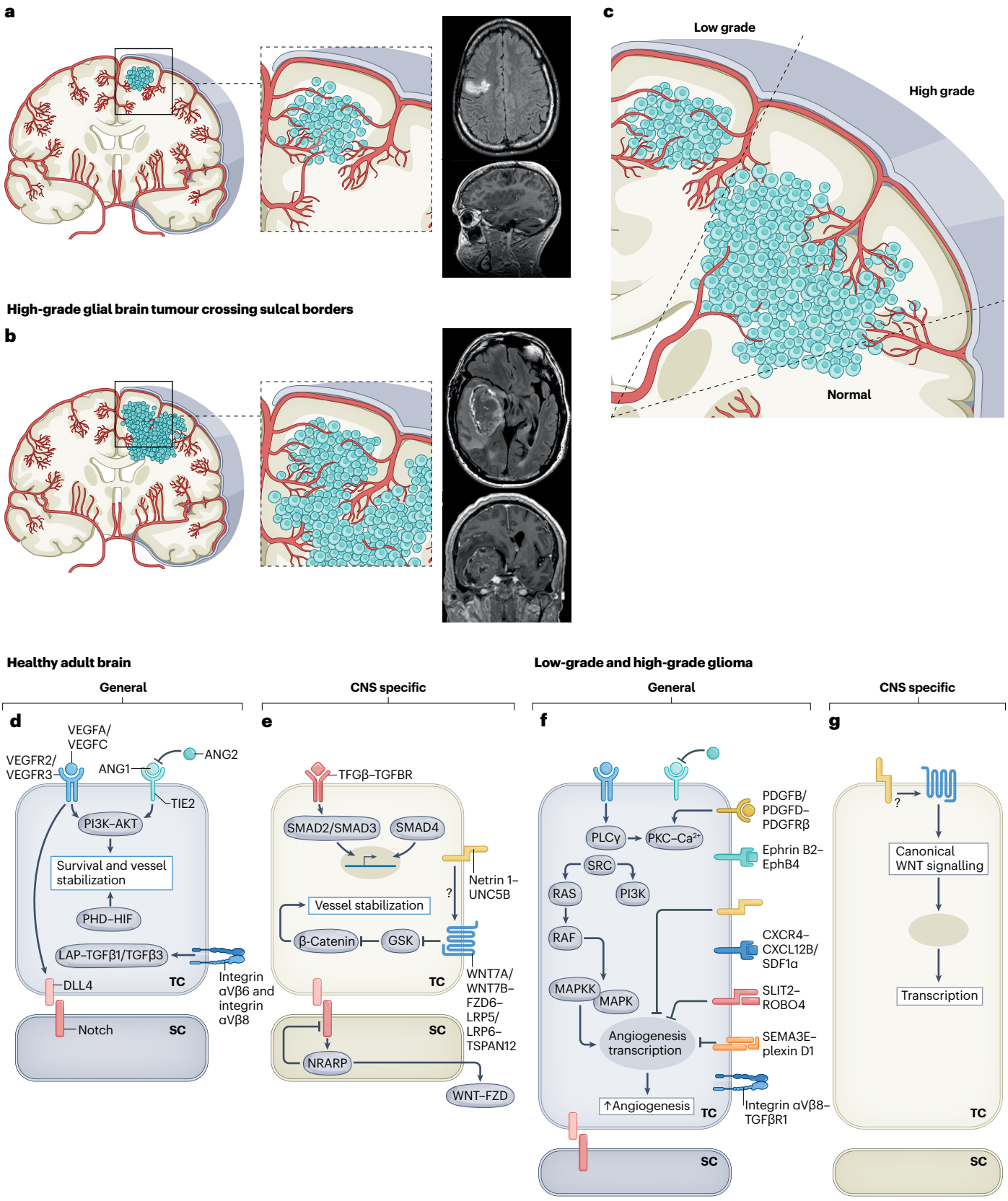

Low-grade glial brain tumour respecting sulcal borders
a
High-grade glial brain tumour crossing sulcal borders
b
c
Low grade
High grade
Normal
Healthy adult brain
Low-grade and high-grade glioma
General
CNS specific
General
CNS specific
d
VEGFA/
VEGFC
VEGFR2/
VEGFR3
ANG1
ANG2
TIE2
PI3K–AKT
Survival and vessel
stabilization
PHD–HIF
LAP–TGFβ1/TGFβ3
DLL4
TC
Notch
SC
Integrin
αVβ6 and
integrin
αVβ8
e
TFGβ–TGFBR
SMAD2/SMAD3
SMAD4
Vessel stabilization
β-Catenin
GSK
TC
SC
NRARP
WNT–FZD
?
Netrin 1–
UNC5B
WNT7A/
WNT7B–
FZD6–
LRP5/
LRP6–
TSPAN12
f
PLCγ
PKC–Ca2+
SRC
RAS
PI3K
RAF
MAPKK
MAPK
Angiogenesis
transcription
↑Angiogenesis
TC
SC
PDGFB/
PDGFD–
PDGFRβ
Ephrin B2–
EphB4
CXCR4–
CXCL12B/
SDF1α
SLIT2–
ROBO4
SEMA3E–
plexin D1
Integrin αVβ8–
TGFβR1
g
?
Canonical
WNT signalling
Transcription
TC
SC

Fig. 5 | Molecular mechanisms regulating the vasculature during initiation and progression of glial brain tumours. Figure illustrating the hypothetical concept postulating the gyral confinement and respect of sulcal borders during progression from low-grade glial brain tumours to high grade glial brain tumours based on radiological observations and the concept of sprouting angiogenesis and recruitment of blood vessels from the adjacent brain parenchyma. **a**–**c**, Cross sections of the adult human brain in the coronal plane showing pathological angiogenesis in glial brain tumours. Illustrations and T1-weighted coronal and sagittal MRI scans with gadolinium show that low-grade gliomas are often confined to one gyrus, thereby respecting sulcal borders (parts **a**,**c**). Illustrations and T1-weighted coronal and sagittal MRI scans with gadolinium show that invasive high-grade gliomas do often not respect gyral confinement and cross sulcal borders (parts **b**,**c**). **d**,**e**, Molecularly, numerous signalling pathways have been implicated in the adult healthy brain, regulating endothelial cell quiescence, survival and maintained inhibition of paracellular permeability. Molecular cues can be either non-CNS specific (part **d**) or CNS specific (part **e**). These signalling pathways are thought to be of importance during both embryonic and postnatal vascular brain development, as well as to contribute to the maintenance of the quiescent healthy adult brain vasculature. **f**,**g**, Molecularly, different non-CNS-specific and CNS-specific angiogenic molecular mechanisms have been implicated in glioma initiation and progression, and they include the reactivation of developmentally active ligand–receptor pairs. ANG1, angiopoietin 1; ANG2, angiopoietin 2; SC, stalk cell; TC, tip cell. Images in parts **a**,**b** courtesy of P. Nicholson.

might serve as a biomarker for the identification of patients who may benefit from anti-angiogenic therapy[197]. The contribution of pericyte progenitor cells to pathological glioblastoma angiogenesis is a matter of debate, given that the pericyte progenitor cell population varies dramatically depending on the stage of disease and that glioblastoma shows a relatively low pericyte coverage of 10–20% (with substantial interpatient variability), compared with 67% in mammary carcinomas and 65% in colon carcinomas[198].

Molecularly, EPC migration and proliferation are regulated by VEGFA–VEGFR2–VEGFR3–MAPK signalling, with VEGFR2 and VEGFR3 being expressed on EPCs[199], whereas EPC homing is regulated by key angiogenic chemokines (CXCL1, CXCL7, CXCL12 and CCL2), their respective receptors (CXCR2, CXCR4 and CCR2) and the TGFβ–SDF1α–CXCL12 axis[200]. CNS-specific molecular mechanisms involved in vasculogenesis remain to be discovered.

Intussusception. Intussusceptive angiogenesis has been characterized in several cancers[39], including glioblastoma[47]. Nico et al. detected a number of connections of intraluminal tissue folds with the opposite vessel walls (corresponding to a key step in the process of intussusception (Fig. 1c)), thereby suggesting the existence of this mode of neovascularization in human glioblastoma[47]. The relevance of intussusception to human brain development and brain disease remains unknown, as do its underlying molecular mechanisms and whether it displays a CNS-specific or general mode of action.

Glioma stem cell to EC and glioma stem cell to pericyte transdifferentiation. Located in the glioblastoma PVN, GSCs are closely associated with microvascular ECs, and studies have proposed that soluble factors secreted by ECs — including VEGFA[201], IL-8 (ref. [202]), SHH[203] and CD9 (ref. [204]) — and adhesive connections between ECs and GSCs control the fate and survival of GSCs, thereby affecting the aggressiveness of glioblastoma (Figs. 1e,h and 4c,d and Supplementary Table 1). A subpopulation of glioblastoma-derived ECs harbours the same somatic mutations (for example, mutation in the gene encoding EGFRvIII and chromosome 7 amplification) as GSCs, indicating that a notable portion of the vascular endothelium has a neoplastic origin and GSCs can transdifferentiate into functional ECs, thereby contributing to tumour vascularization[20,21,205]. Recently, the P4HA1–COL6A1 axis was identified as a modulator of GSC-to-EC transdifferentiation[206]. Additional candidate modulators of this process include ETV2, a master regulator of EC development, and the transcription regulator TWIST1, and their expression positively correlates with malignancy grade[207,208].

Mechanistically, treatment with the chemotherapeutic drug temozolomide increases the expression of GSC-specific markers in glioblastoma ECs and induces the transdifferentiation of GSCs to glioblastoma ECs, thus identifying chemotherapeutic stress as a driver of this mode of neovascularization[209]. Ionizing radiation has also been shown to initiate GSC-to-EC transdifferentiation through the previously described TIE2 pathway[210,211]. Interestingly, GSCs can also give rise to tumour pericytes supporting vessel function and tumourigenesis[22]. In vivo cell lineage tracing in a glioblastoma xenograft model demonstrated that GSCs generate the majority of glioblastoma pericytes (predominantly via TGFβ signalling) and revealed that selective cell arrest of GSC-derived pericytes led to vessel wall disruption in vivo[22]. Transdifferentiation of GSCs to pericytes along with stem cell plasticity and angiogenic properties of GSCs are regulated predominantly by the NOTCH1 pathway in hypoxic conditions[212]. The observation that GSC-derived pericytes bear tumour-specific genetic alterations distinguishing them molecularly from normal pericytes (for example, mutations in the gene encoding EGFRvIII, chromosome 7 amplification, or *PTEN* or chromosome 10 deletion) provides possibilities to specifically target these tumour-derived pericytes[22].

Clinically, pericyte coverage of tumour vasculature inversely correlates with response to chemotherapy and survival in individuals with glioblastoma, suggesting that pericytes with a neoplastic origin in glioblastoma may regulate the brain tumour barrier, which impacts the efficiency of drug delivery[213]. Tumour vascular endothelium and GSC-derived pericytes have been suggested as novel targets for anti-angiogenic therapy[165,166,214]. Cancer stem cell to EC or pericyte transdifferentiation is a non-CNS-specific process that has been described in non-CNS tumours[215].

Vasculogenic mimicry. 'Vasculogenic mimicry' (VM) refers to the ability of tumour cells to form functional vessel-like networks[216,217] (Figs. 1f,h and 4c,d). Tumour cells lining these erythrocyte-containing 'vascular' channels, which are devoid of ECs, continue to express tumour cell markers. First identified in melanomas[216], this mode of neovascularization has been reported in various cancers inside and outside the CNS[218–220] and in glial brain tumours[221].

Molecularly, hypoxia promotes VM through expression of VE-cadherin (also known as CD144) on tumour ECs and tumour cells[222]. In glioblastoma, tumour cells lining the vasculature display an undifferentiated embryonic-like biological and molecular phenotype, suggesting the involvement of GSCs and reactivation of neurodevelopmental signalling programmes[223]. Several molecules and ligand–receptor pairs associated with anaplastic properties of these GSCs are associated with VM formation, including TGFβ, Nodal, EphE2 and VE-cadherin[224]. The incidence of VM was markedly higher in high-grade gliomas than in lower-grade gliomas[225]. Overall survival was notably lower and

microvascular density was higher in people with VM-positive high-grade gliomas than in individuals with VM-negative high-grade gliomas, indicating a notable contribution of VM channels to glioma blood supply[225]. IGFBP2 (ref. [226]), leptin receptor ObR[227], the RNA-binding protein ZRANB2 (ref. [228]) and several specific long non-coding RNAs[229] and microRNAs[230] stimulate VM, whereas histone deacetylase inhibitors impair the process of VM in human glioblastoma[231]. CNS-specific mechanisms of VM have not been discovered to date.

Developmental pathways in glial tumours

General molecular mechanisms reactivated in glial brain tumour angiogenesis. Typical examples of developmentally active general mechanisms that are reactivated in pathological glial brain tumorigenesis include VEGF–VEGFR, DLL4–Jagged–Notch, YAP–TAZ, PDGF–PDGFR, SLIT2–ROBO4, semaphorin–plexin, semaphorin–neuropilin, ANG2–TIE1, ANG2–TIE2 and ephrin B2–EphB4 signalling (Fig. 5f and Supplementary Table 2). An increase in VEGFA expression has been associated with an increase in glioma malignancy and poor prognosis[232]. A frequent hallmark of glioma-associated angiogenesis is the activation of the developmentally active RTK signalling pathways[233], most commonly caused by amplifications of, mutations in or overexpression of *EGFR* in GSCs and ECs or pericytes[234], contributing to sprouting angiogenesis and stem cell to EC transdifferentiation or stem cell to pericyte transdifferentiation[233]. Mutations in *EGFR*, in particular mutations encoding the EGFRvIII variant, lead to ligand-independent and constitutive activation of the EGFR signalling pathway[235]. This prolonged activation leads to tumour progression and stimulation of angiogenesis via secretion of proteases, which degrade the ECM and enable ECs to proliferate in the surrounding matrix via upregulation of unidentified pro-angiogenic molecules[235].

The Notch pathway is linked to several glioblastoma-specific responses to hypoxia, angiogenesis and tumour growth[183,236,237]. Combined targeting of EGFR signalling and Notch signalling results in decreased cell viability and EC sprouting compared with use of either of the monotherapies, supporting an important role of Notch–EGFR signalling crosstalk in glioblastoma[238]. However, inhibition of both the EGFR signalling pathway and the Notch signalling pathway is not sufficient to fully stop EC sprouting in human glioblastoma cell cultures, despite almost complete inhibition of VEGF secretion upon combined treatment, suggesting that VEGF-independent pro-angiogenic factors contribute to sprouting angiogenesis[238]. Indeed, VEGF-independent YAP–TAZ upregulation was observed in glioblastoma on both glial tumour cells and tumour-associated ECs, and this correlated with malignancy grade[95,239].

PDGFs, which have several critical roles in physiological embryonic development, are also known to have an important role in sprouting angiogenesis in human glial brain tumours[240,241]. Five different PDGF isoforms (PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD) activate cellular responses through two different receptors (PDGFRα and PDGFRβ; the latter is mainly involved in tumour ECs)[242]. PDGF-mediated endothelial-to-mesenchymal transition induces EC resistance to anti-angiogenic therapies that target VEGF pathways by downregulating VEGFR2 expression in ECs that were isolated from human glioblastoma samples[241].

Among the reactivated general molecular mechanisms regulating glial brain tumour vasculature, signalling by the classic axon guidance cue ephrin B2–EphB4 regulates ETC guidance in brain tumour angiogenesis, and ephrin B2–EphB4 expression is associated with accelerated glioma progression and a worse clinical prognosis in patients with glioblastoma[123,243]. Sawamiphak et al. found a reduction of tumour volume of up to 25% in an intracranial glioma model in ephrin B2-deficient mice[104]. Furthermore, ephrin B2 activation in ETC filopodia regulates VEGFR2 internalization, which is required for downstream signalling and VEGF-induced tip cell filopodial extension and sprouting angiogenesis[104]. Additionally, in a glioblastoma EphB4 overexpression model, reactivation of this developmentally active ephrin B2–EphB4 receptor–ligand pair in glial brain tumours and subsequent overexpression of EphB4 leads to a stabilization of pericyte–EC interactions, intact pericyte coverage and cellular proliferation, all hallmarks of anti-angiogenic therapy-resistant tumour vessels[244].

Active during physiological embryonic and postnatal vascular development, TIE1-bound ANG2 and TIE2-bound ANG2 were also detected in tumour cells and ECs in high-grade gliomas (they are present at negligible levels in low-grade gliomas)[245,246]. Reactivation of TIE receptor signalling during ectopic overexpression of ANG2 in glioblastoma accelerates tumour progression and compromises the benefits of anti-VEGFR treatment in murine glioblastoma models[247]. Dual inhibition of ANG2 and VEGF receptors normalizes tumour vasculature and prolongs survival in glioblastoma models[247].

SLIT2–ROBO4 signalling constitutes another classic axon guidance cue regulating vascular development[99,248]. ROBO4 is markedly downregulated in ECs cultured in glioma-conditioned medium, and binding of SLIT2 to ROBO4 suppresses glioma-induced EC proliferation, migration and tube formation in vitro by inhibiting VEGFR signalling[249].

Among the five members of the αV integrin subfamily, αVβ8 – expressed in neurons, ECs and PVCs – is of particular interest as an important regulator of angiogenesis in the developing brain[125,126]. In mosaic mouse models of astrocytoma, xenografts and cell culture systems of human glioblastoma, αVβ8 integrin-activated TGFβ proteins suppress pathological angiogenesis and differentially regulate glioblastoma (vessel) growth via autocrine activation of TGFβ signalling pathways[250].

Other classic axon guidance cues such as netrin 1 and semaphorins (for example, SEMA3D, SEMA3E, SEMA3F and SEMA4D) play important roles in glioblastoma tumorigenesis and progression by affecting infiltration patterns and the aggressiveness of GSCs[251–253].

Recently, we identified nucleolin, a neurodevelopmental regulator of angiogenesis in the human fetal brain vasculature, as a reactivated, positive regulator of sprouting angiogenesis in glioblastoma[254]. In our own scRNA-seq dataset, we have identified various reactivated fetal signalling pathways in human low-grade and high-grade glioma or glioblastoma with a general (non-CNS-specific) mode of action, including, cell–ECM interaction-related and cell–cell interaction-related signalling pathways, as well as WNT, BRAF, Notch, VEGF–VEGFR1 and VEGF–VEGFR2, IL-8–CXCR1, PI3K–AKT, PDGF–PDGFR, Hedgehog, angiopoietin–TIE1, angiopoietin–TIE2, ephrin and integrin signalling cascades[163].

CNS-specific molecular mechanisms reactivated in glial brain tumour angiogenesis. Only a few studies have been published to date relating to the CNS-specific regulation of angiogenesis in primary glial brain tumours[12,132] (Fig. 5g and Supplementary Table 2). WNT7A/WNT7B–β-catenin signalling, regulating embryonic and postnatal developmental angiogenesis in a CNS-specific manner via the co-activator GPR124, also regulates pathological angiogenesis in mouse models of glioblastoma and ischaemic stroke[132,145,146]. Mice in which *Gpr124* was conditionally knocked out in ECs (*Gpr124*-CKO mice) exhibited decreased vessel density and increased loss of CNS microvascular

integrity, measured by BBB leakage, compared with heterozygous control animals in both the model of stroke[255] and the model of glioblastoma[132]. To investigate whether GPR124 functions via downstream WNT–β-catenin signalling to regulate BBB function, primary cultured brain ECs from adult *Gpr124*-CKO mice and the *Gpr124*-heterozygous control group were transduced with *Wnt7b*-expressing adenovirus. Upregulation of WNT7B signalling resulted in increased BBB integrity in glioblastoma by positively regulating tight junction proteins, pericyte coverage and cell–ECM interactions in the ECs from adult global *Gpr124*-heterozygous mice but not in those from *Gpr124*-CKO mice[132], indicating a crucial role for WNT7A/WNT7B–GPR124–RECK–FZD–LRP signalling in brain tumour BBB integrity and identifying this molecular signalling pathway as a possible therapeutic CNS-specific target in glioblastoma[132,256]. More recently, engineered WNT7A ligands were shown to enable BBB repair in mouse models of stroke and glioblastoma by selectively binding the WNT7A/WNT7B-specific GPR124–RECK co-receptor complex, thereby acting as BBB-specific WNT activators to induce WNT signalling[257]. It remains to be determined whether WNT–GPR124 signalling also affects pathological vascularization in non-CNS tumours or whether this signalling axis keeps its developmental CNS specificity in vascular-dependent CNS pathologies such as brain AVMs.

Other regulators of developmental brain angiogenesis such as norrin, DR6 and TROY have been reported to have effects in brain tumours such as medulloblastoma (mainly on neuronal migration, not on angiogenesis)[258,259], but their potential regulatory roles in angiogenesis in glial brain tumours and other non-CNS tumours remain to be investigated. Similarly, in light of the recently identified CNS-specific UNC5B–netrin 1-mediated interaction with LRP6 (ref. [138]), it would be interesting to see whether intravenous injection of netrin 1 could increase WNT–β-catenin signalling in the BBB and repair CNS endothelial barrier breakdown in glial brain tumours.

From the findings taken together, reactivation of the VEGF–VEGFR–DLL4–Jagged–Notch signalling axis, along with the YAP–TAZ pathway, is of crucial importance in the initiation and progression of angiogenesis in glial brain tumours. Many of the discussed classic axon guidance cues of the NVL are reactivated in glial brain tumours in a general way. Besides possible involvement of netrin 1 and semaphorins in glioblastoma vascularization[252], the role of additional classic and non-classic axon guidance cues and CNS-specific cues in this process remains to be explored.

Molecular mechanisms in glial brain tumour vasculature at the single-cell level. scRNA-seq is a powerful approach to study brain tumour (including low-grade and high-grade glioma) biology[260–264]. Single-cell techniques enable the study of genetic heterogeneity[265,266], developmental cellular lineages and hierarchies, and stem cell programmes[261,262,264,267], as well as the investigation of the various cell types in the tumour microenvironment[266]. Until recently, however, single-cell sequencing had not been applied to the study of the glioma vasculature. Xie and colleagues used scRNA-seq to study freshly isolated ECs from human glioblastoma tissues, gaining molecular insight into the heterogeneity of the human BBB and the pathological neovascularization in glioblastoma[265]. They identified distinct EC clusters that represent different states of angiogenesis and EC activation and impairment of the BBB in both the tumour centre and the tumour periphery, thereby highlighting the importance of different regions within the tumour with regard to the tumour vasculature.

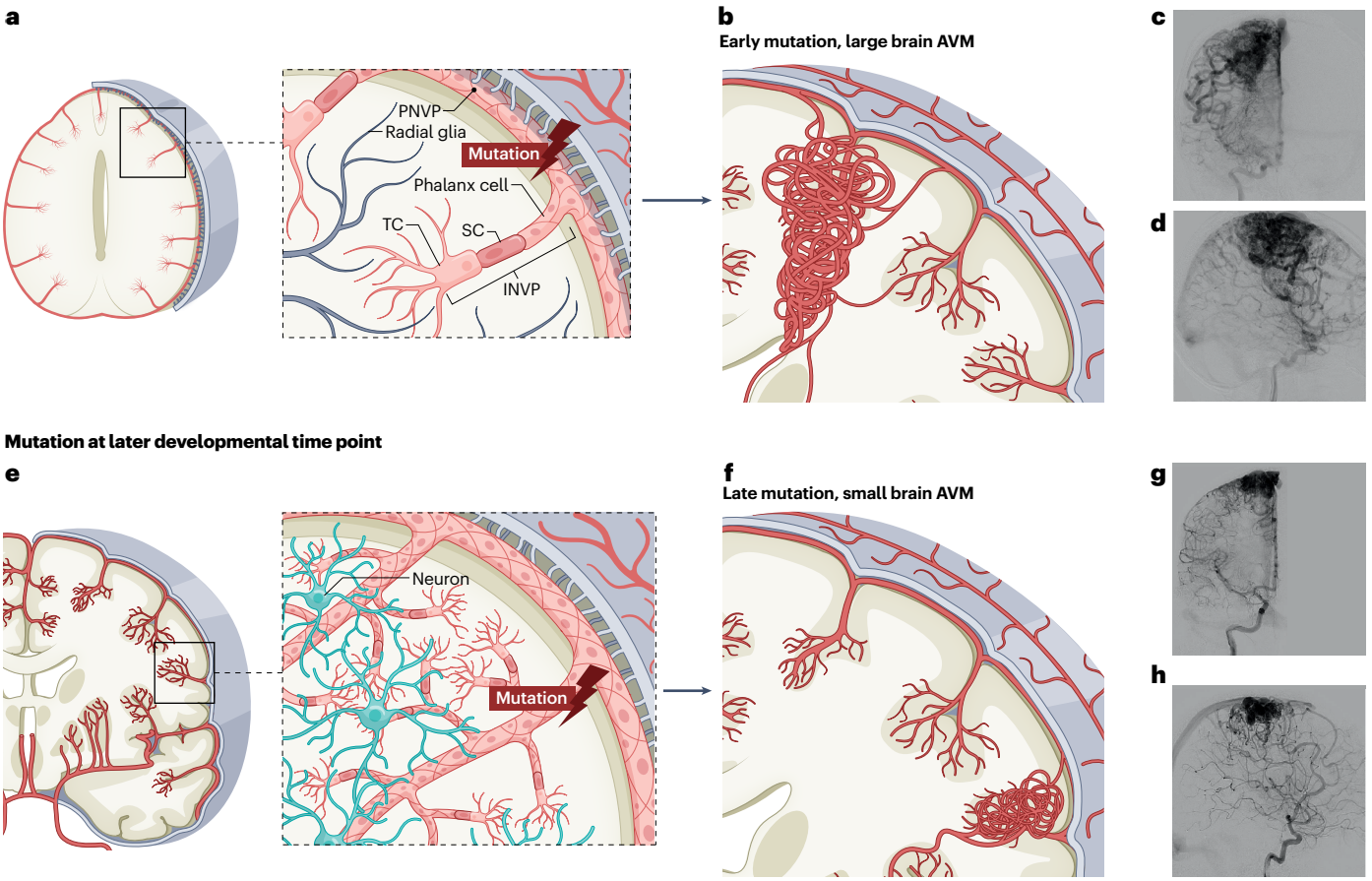
To address the molecular heterogeneity of brain ECs (and PVCs) across development and disease, we recently created the first large-scale single-cell molecular atlas of the developing fetal, healthy adult and diseased human brain vasculature, focusing on brain vascular malformations and brain tumours, including AVMs and low-grade and high-grade gliomas[163]. We performed scRNA-seq on approximately 600,000 freshly isolated ECs and PVCs from 47 fetuses and adult patients[163]. This unprecedented insight into EC and PVC heterogeneity and functional specialization of the human brain vasculature in development, health and disease at the single-cell level revealed alterations in arteriovenous differentiation and CNS-specific properties, upregulation of major histocompatibility complex class II molecules and a central role for ECs in the brain NVU in pathological ECs across different brain diseases, including brain tumours and brain vascular malformations. Notably, we observed a marked increase in the angiogenic capillary EC cluster in glioblastoma (and lung cancer brain metastases) and to a lesser extent in lower-grade gliomas as compared with the adult control brain, indicative of the angiogenic nature of lower-grade and especially high-grade brain tumours. Moreover, these findings unravelled the top differentially regulated pathways (belonging to five major groups, namely angiogenesis-related pathways, development and NVL molecules, cell–cell and cell–ECM interactions, immune-related processes and metabolism) in both fetal and pathological brain ECs as compared with healthy adult brain ECs. Most interestingly, more than half of the differentially regulated pathways in pathological brain ECs also showed differential regulation in fetal brain ECs[163]. This observation was also made in both low-grade and high-grade gliomas, with the reactivated pathways belonging to the five canonical groups listed above.

In summary, these results showed that, in the human brain, pathological ECs share common hallmarks across various diseases, including brain tumours and brain vascular malformations. Comparison of fetal and pathological ECs also suggested that signalling pathways regulating vascular growth during fetal brain development are silenced in adulthood and subsequently activated again in the vasculature of brain tumours and brain vascular malformations, thereby highlighting the potential importance of developmental pathways in various vascular-dependent brain pathologies. Notably, the observed similarities between fetal and pathological brain ECs at the level of active signalling pathways (for example, reactivated developmental pathways versus persistence of a less differentiated cell type) as well as their functional importance are currently incompletely understood and warrant further investigation.

A developmental look at glial brain tumours

From surgical and neuroradiological observations, glial brain tumours are frequently confined to specific brain regions (Fig. 5), as illustrated by gliomas largely having a gyral or subgyral location[268,269]. Low-grade gliomas (from which many high-grade gliomas arise) are typically confined to a gyrus while respecting pial borders, rarely crossing sulci[268,270] (Fig. 5a,c), but the cellular and molecular mechanisms underlying these observations are unknown. In light of compartment-specific embryonic vascular development[6,56], it is intriguing to speculate that the restriction of the brain tumour extension within defined gyri might, at least partially, be due to its territorial vascular supply. Interestingly, upon malignant transformation of a low-grade glioma to a high-grade glioma, the tumour mass often spreads on a radial axis, crossing sulci and extending to adjacent gyri[270] (Fig. 5b,c).

Strikingly, this brain tumour extension or progression looks comparable to the axis of brain AVM growth towards the ventricle, with infiltration along white matter tracts, such as the corpus callosum and subgyral short association fibres[270,271] (Fig. 6). As long as glial tumours are


Mutation at early developmental time point
a
PNVP
Radial glia
Mutation
Phalanx cell
TC
SC
INVP
b
Early mutation, large brain AVM
c
d
Mutation at later developmental time point
e
Neuron
Mutation
f
Late mutation, small brain AVM
g
h

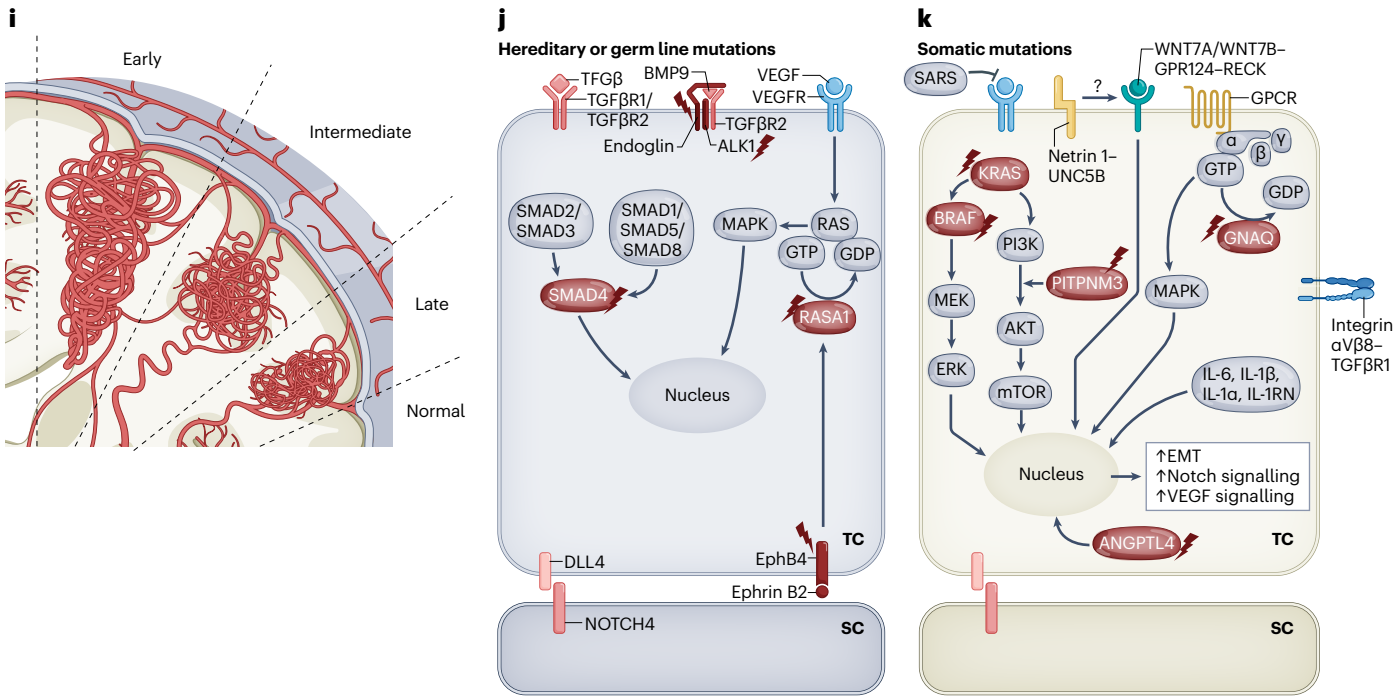

Brain AVM extension depending on developmental time point of mutation
i
Early
Intermediate
Late
Normal
j
Hereditary or germ line mutations
TFGβ
TGFβR1/
TGFβR2
BMP9
Endoglin
TGFβR2
ALK1
VEGF
VEGFR
SMAD2/
SMAD3
SMAD1/
SMAD5/
SMAD8
SMAD4
MAPK
RAS
GTP
GDP
RASA1
Nucleus
TC
DLL4
EphB4
Ephrin B2
NOTCH4
SC
k
Somatic mutations
SARS
?
WNT7A/WNT7B–
GPR124–RECK
GPCR
Netrin 1–
UNC5B
α
β
γ
GTP
GDP
KRAS
BRAF
PI3K
GNAQ
PITPNM3
MAPK
MEK
AKT
ERK
mTOR
IL-6, IL-1β,
IL-1α, IL-1RN
Integrin
αVβ8–
TGFβR1
Nucleus
↑EMT
↑Notch signalling
↑VEGF signalling
ANGPTL4
TC
SC

Fig. 6 | Molecular mechanisms regulating the vasculature during initiation and progression of brain AVMs. Figure illustrating the hypothesis stating that the timing of mutation influences the size and location of the arteriovenous malformation (AVM). **a**,**b**, Cross section of the human brain in the coronal plane illustrating that mutations occurring in progenitor endothelial cells (ECs) at an early developmental time point will 'trace' the future developmental territory of their daughter cells, resulting in a large lesion spreading along a radial axis from the pial cortical surface to the ventricles. **c**,**d**, Anterior–posterior (**c**) and lateral (**d**) digital subtraction angiography of the right intracarotid artery showing a large AVM. **e**,**f**, Cross section of the human brain in the coronal plane illustrating that mutations at later developmental time point result in smaller lesions restricted to a local vascular territory. Note that these smaller AVMs are located around the pial, sulcal and cortical areas or alternatively in the ventricular, ependymal and subependymal zones (that is, choroidal AVMs) but do not occur isolated midway in the white matter without reaching either the cortical surface or the ventricular surface. **g**,**h**, Anterior–posterior and lateral digital subtraction angiography of the right intracarotid artery showing a smaller AVM. **i**, AVM extension as result of early, intermediate and late time points of mutation. **j**,**k**, Various molecular pathways have been implicated in AVM initiation and progression. The mutations shown belong to either hereditary or germ line mutations (part **j**) or somatic mutations in genes in the endothelial tip and stalk cells (part **k**). The proteins encoded by mutated genes are indicated with a flash symbol. Additional molecules and ligand–receptor pairs involved in regulating the vasculature during initiation and progression of brain AVMs can be found in Supplementary Table 2. BMP9, bone morphogenetic protein 9; EMT, endothelial-to-mesenchymal transition; GPCR, G protein-coupled receptor; INVP, intraneural vascular plexus; SARS, seryl-tRNA synthetase 1; SC, stalk cell; TC, tip cell. Images in parts **c**,**d**,**g**,**h** courtesy of P. Nicholson.

localized within gyri and respect the sulcal borders, their blood supply is thought to be provided by neovessels forming via sprouting angiogenesis from pre-existing arteries running within the sulci[271]. High-grade gliomas crossing these borders may find ways to break those boundaries and recruit neovessels from adjacent sulci or gyri (for example, via CNS-specific and/or general reactivated NVL molecules or endothelial metabolism cues) via sprouting angiogenesis and other modes of vessel formation (Fig. 1), but this intriguing hypothesis needs further testing.

Angiogenesis in brain AVMs

Brain vascular malformations are characterized by abnormal blood vessel growth and altered maturation of the vessel wall[61,162]. Here, owing to space limitations, we focus on brain AVMs, which are one of the most commonly encountered brain vascular malformations and are a leading cause of haemorrhage in children and young adults[272]. Brain AVMs are characterized by aberrant angiogenesis and a malformed capillary bed, thereby representing an exemplar pathology to understand brain vascular biology across arteriovenous zonation[273] (Figs. 4e,f and 6). For in-depth discussions of other types of brain vascular malformations, we refer readers to review articles on cerebral cavernous malformations[274–276], vein of Galen malformations[277] and dural arteriovenous fistulas[278].

Brain AVMs

High-pressure arterial blood from feeding arteries shunts directly into the low-pressure outflow veins, rendering brain AVMs prone to rupture[273]. Regarding their potential developmental origin, brain AVMs so far not been detected in utero (via either ultrasound or MRI techniques). As the same detection methods are capable of detecting similarly sized vein of Galen vascular malformations in utero[279], brain AVMs might not develop during embryonic or fetal stages of development. Moreover, the existence of more than ten case reports of de novo formation of brain AVMs in children (for example, they are not present on initial postnatal imaging after trauma but are present on subsequent postnatal imaging[280]) suggests a postnatal rather than a fetal or embryonic origin.

During normal vascular (brain) development, arteries and veins follow a parallel and countercurrent course without direct communication[273]. They are separated by capillary networks in the respective tissues, and premature arteriovenous connections are prevented by specific developmentally active molecular control systems (involving, for example, COUP transcription factor 2, NRP2, VEGFR3–FLT4 and EphB4 (refs. [273,281,282])). CNS and peripheral AVMs are thought to occur as a consequence of a failure in these control systems[273]. Whereas the molecular basis of this aberrant arteriovenous separation leading to AVM formation is unclear, genetic AVM syndromes have provided insight into some crucial signalling pathways that govern arteriovenous patterning[273,283–285].

Hereditary brain and peripheral AVMs

Hereditary haemorrhagic telangiectasia. Hereditary haemorrhagic telangiectasia (HHT), or Osler–Weber–Rendu syndrome, is an autosomal dominant disorder characterized by germ line mutations in genes encoding components of the TGFβ signalling pathway[27,273,286]. As TGFβ is required in embryonic and postnatal development for the establishment and remodelling of the INVP via molecular regulation of EC proliferation, migration and differentiation as well as of pericyte and vSMC recruitment to newly formed blood vessels, it can be considered an important developmentally active signalling cascade that is reactivated in AVMs[14,27,124] (Supplementary Table 2).

Mutations in *ENG*, encoding a TGFβ co-receptor that potentiates TGFβ signalling[27,50], cause HHT type 1 (refs. [287,288]) (Fig. 6j). *Eng*$^{-/-}$ mice die at E11.5 owing to defective (both CNS and non-CNS) vascular development, caused by a lack of functional vSMCs and arrested vascular remodelling[130]. Thus, ENG is required for both CNS and peripheral vasculogenesis and angiogenesis[289]. Mutations in *ALK1*, encoding a type 1 TGFβ receptor that stimulates kinase activity[290], cause HHT type 2 (ref. [288]) (Fig. 6j). *Alk1*$^{-/-}$ mice die at E11.5 owing to comparable non-CNS-specific vascular defects such as AVMs in the intra-embryonic aortic endothelium, decreased vSMC coverage and disrupted arterial identity[129,291]. Mutations in *SMAD4*, encoding a downstream effector of TGFβ signalling[290], lead to the combined syndrome of HHT and juvenile polyposis[292] (Fig. 6j).

In addition, BMP9 and BMP10, which are important for vascular brain and retinal development[293] and vessel normalization in breast cancer[294], bind ALK1 with high affinity and induce downstream SMAD signalling, and their genes are mutated in a vascular anomaly syndrome that has phenotypic overlap with HHT[295–297] (Fig. 6j). Increasing evidence shows that the BMP9–TGFBR–ENG–ALK1 signalling axis is a developmental (and non-CNS-specific) angiogenic pathway crucially involved in the formation of hereditary AVM syndromes[298] (Fig. 6j). Whereas ENG and ALK1 are involved in sprouting angiogenesis during development in a non-CNS-specific manner[299], BMP9 and BMP10 are critical for postnatal retinal vascular remodelling and embryonic vascular development inside and outside the CNS[293,300].

Differences between mouse models of brain AVMs in adult mice versus developing mice might be due to the dynamic vessel remodelling

Glossary

Blood–brain barrier
(BBB). A physiological barrier formed by the brain endothelium to regulate trafficking of most compounds from the blood to the brain.

Brain arteriovenous malformations
High-flow low-resistance vascular malformations characterized by a loss of vascular organization, a network of tortuous, dysplastic vascular channels (termed 'nidus') in between one or multiple feeding arteries and one or multiple draining veins in lieu of a normal intervening capillary network.

Brain vascular malformations
Malformations characterized by abnormal blood vessel growth and altered maturation of the vessel wall, including brain arteriovenous malformations, cerebral cavernous malformations, developmental venous anomalies, dural and pial arteriovenous fistulas, capillary telangiectasias, vein of Galen malformations and carotid-cavernous fistulae.

Glial brain tumours
Primary brain tumours originating from neuroglial stem or progenitor cells, accounting for almost 30% of all primary brain tumours and for 80% of all malignant primary brain tumours.

Glioma (or glioblastoma) stem cell
(GSC). A subpopulation of tumour cells with stem cell-like properties that contribute to tumour initiation, progression and resistance to anticancer therapies.

Neurovascular link
(NVL). The similar appearance and coordinated guidance of the cellular and subcellular elements of both the vascular system and the nervous system.

Neurovascular unit
(NVU). The functional unit of the complex crosstalk between endothelial cells and perivascular cells in the perivascular niche.

Perivascular niche
(PVN). The microenvironment around a blood vessel; it includes endothelial cells and perivascular cells such as astrocytes, pericytes, neurons, stem cells, microglia and vascular smooth muscle cells.

Reactivated developmental signalling pathways
Molecular signalling cues and pathways that are active during embryonic and/or postnatal vascular brain development, are silenced in the adult healthy brain vasculature and might be reactivated in vascular-dependent CNS diseases, including brain tumours and brain vascular malformations.

Single-nucleotide polymorphisms
A somatic mutation characterized by a single nucleotide change in the DNA sequence that can modulate biological mechanisms. Somatic mutations do not occur in the germ line but occur in a postzygotic progenitor or differentiated cell and are well described in both CNS and non-CNS cancer development.

and highly angiogenic character of the vascular bed during development versus the relatively stable and quiescent nature of the vasculature at the adult stage. Accordingly, in adult mice, regional or tissue-specific CKO of *Eng* or *Alk1* produced AVMs in the lung, brain and gastrointestinal tract but only if angiogenesis was simultaneously stimulated by VEGF[301–303]. This 'second hit' theory[304] postulates that a genetic predisposition (the first hit) in combination with an angiogenic trigger (for example, a repetitive injury; the second hit) leads to the reactivation of several developmental angiogenic pathways (for example, the TGFβ pathway)[303]. As HHT-related mutations involve loss of function in TGFβ pathway-linked genes in ECs but AVMs occur in only certain organs affected by these mutations, it may be that TGFβ haploinsufficiency is not sufficient to initiate a brain AVM in adulthood and requires another somatic mutation (a 'second hit') affecting the TGFβ pathway.

Accordingly, whereas in the adult mouse, with a stable or quiescent brain vasculature, this second hit is required to initiate brain AVM formation, in the developing (embryonic or postnatal) mouse, with a dynamic or active brain vasculature, brain AVM formation can occur without a second hit[303]. In about 15% of patients with clinical features of HHT, no mutations in genes encoding components of the TGFβ pathway are found and the origin of the malformation is unknown[305].

Capillary malformation–AVM syndrome. Another hereditary genetic syndrome is capillary malformation–AVM syndrome type 1, caused by heterozygous germ line mutations in *RASA1*, encoding the cytoplasmic protein RasGAP, a negative regulator of the RAS–MAPK signalling pathway crucial for growth regulation and EC and PVC proliferation in various tissues[306–309]. RasGAP inactivates RAS by hydrolysing GTP to GDP, thereby negatively regulating the RAS–MAPK signal transduction pathway, with a loss of RasGAP activity resulting in the excessive activation of RAS and downstream signalling pathways[295,307,309,310] (Fig. 6j). Mechanistically, RasGAP acts downstream of the endothelial receptor EphB4, a marker of venous endothelial identity and a regulator of developmental and brain tumour angiogenesis, by promoting venous differentiation[311]. Accordingly, *RASA1* mutations result in dysregulation of arteriovenous patterning (with a shift from venous to arterial differentiation) and formation of AVMs inside and outside the CNS[310,312]. Germ line mutations in *EPHB4* have been identified in CM–AVMs that are negative for *RASA1* mutations and are therefore categorized as capillary malformation–AVM type 2 (ref. [313]).

Sporadic brain and peripheral AVMs

Somatic mutations are increasingly being reported in studies investigating the genetic basis of sporadic (brain) vascular malformations[314–317]. Many of these mutations are common non-coding single-nucleotide polymorphisms. For example, non-CNS-specific venous malformations are associated with somatic mutations in *PIK3CA* and *TIE2* (refs. [315,316]), lymphatic malformations are associated with mutations in *PIK3CA*[318], Sturge–Weber syndrome, capillary malformations and congenital haemangiomas are linked to *GNAQ* mutations[319,320], verrucous venous malformations are linked to *MAP3K3* mutations[321], extracranial AVMs are associated with *MAP2K1* mutations[322] and brain AVMs were recently associated with activating somatic mutations in *KRAS*[162,323–325]. Other groups studying brain AVMs have reported single-nucleotide polymorphisms located in *ALK1* (refs. [326,327]), *ENG*[328], *IL1B*[329], *ITGB8* (ref. [330]), *ANGPTL4* (ref. [331]), *GPR124* (ref. [332]), *VEGFA*[333], *MMP3* (ref. [334]) and *MMP9* (ref. [317]) (Fig. 6k; see Supplementary Table 2 for additional candidate genes for brain AVM initiation and progression).

Sturge–Weber syndrome is caused by non-hereditary somatic mutations in the protein GNAQ, characterized by port wine stains on the face and leptomeningeal angiomatosis with brain vascular malformations, indicating an underlying general/non-CNS-specific molecular mechanism[319]. Mutations in GNAQ decrease GTPase activity and increase signalling of associated G proteins, leading to increased

MAPK activity[319,335] (Fig. 6k and Supplementary Table 2). It remains to be investigated whether genetic risk factors in the context of hereditary AVM syndromes render individuals more susceptible to developing sporadic AVMs.

A key future step in the improvement of the clinical management of brain AVMs would be the development of novel anti-angiogenic therapies[336,337], for instance targeting the pathways downstream of *KRAS* mutations with MEK inhibitors (which are already approved for the treatment of brain tumours[338,339]) or other targets emanating from single-cell studies[75,163,265]. For explorations of the future clinical and pharmacological treatment of brain AVMs, we refer readers to recent reviews on this topic[336,337].

Developmental pathways in brain AVMs

General molecular mechanisms reactivated in brain AVMs. Interestingly, most of the mutations associated with vascular malformations characterized so far are linked to the RAS–RAF–MAPK and PI3K–PTEN–AKT–mTOR pathways, both of which have pivotal roles in physiological (CNS and non-CNS) vascular development (Fig. 6j,k and Supplementary Table 2). In particular, high-flow AVMs are associated with the latter, as most brain and spinal AVMs have mutations in *KRAS*[162,323–325], whereas low-flow vascular malformations are often associated with activating mutations affecting the PI3K pathway[314,316]. These observations strongly suggest that the RAS–RAF–MAPK pathway is a central signalling node for the development of AVMs in the brain and spinal cord as well as in non-CNS organs. It remains, however, unclear whether and how the BMP9–TGFβ–SMAD pathway involved in HHT-related AVMs (but also somatic mutations, for example, found in *ITGB8*) and genes affecting the RAS–RAF–MAPK pathway overlap or interact during normal brain vascular development and (CNS and non-CNS) AVM initiation and progression.

Currently, the downstream effector signalling pathways that are required for AVM development are not well characterized in humans but they are hypothesized to be crucial regulators of arteriovenous specification and zonation[273]. Several AVM mouse models have elucidated underlying molecular mechanisms driving brain AVM initiation and progression[340]. In particular, manipulation of the developmentally active DLL4–Jagged–Notch pathway resulted in the development of (CNS and non-CNS) vascular malformations in mice[340]. Whereas genetic ablation of both *Notch1* and *Notch4* resulted in embryonic lethality, haploinsufficiency of *Dll4* induced AVM-like brain (and non-CNS, including dorsal aorta and cardinal veins) lesions at the embryonic stage that were characterized by the lack of a capillary bed between feeding arteries and draining veins[341].

At the postnatal stage, endothelial-specific inducible postnatal expression of constitutively active NOTCH4 induced brain AVMs in mice[342], which resulted from the increase in length and calibre, and not the absence, of brain capillaries[343]. Strikingly, these AVMs were reversible upon normalization of NOTCH4 expression[342]. vSMCs and ECs in human brain AVMs exhibited upregulated DLL4–Jagged–NOTCH1 signalling compared with healthy cerebral vessels[344], indicating that NOTCH1 signalling contributes to the development of human brain AVMs. In arteriovenous differentiation of ECs during development, NOTCH1 and NOTCH4 are major determinants of arterial fate choice, associated with expression of the arterial markers ephrin B2, CXCR4 and connexin 40 (ref. [345]). Lack of Notch signalling results in a default phenotype characterized by venous markers such as COUP transcription factor 2, NRP2 and VEGFR3 and the receptor EphB4 (refs. [281,282]). Furthermore, activating mutations in RAS–RAF–MAPK pathway genes would result in constitutively active and VEGF-independent activation of the Notch pathway. Indeed, expression of mutant active KRAS in ECs results in overexpression of the Notch pathway and angiogenic cascades downstream of VEGF[162] along with endothelial-to-mesenchymal transition. At a cellular level, mutant KRAS induced a migratory phenotype of brain (and peripheral) ECs, loss of tight junctions and disorganization of cytoskeletal actin with intact proliferation[162].

A better understanding of the signalling downstream of the RAS–RAF–MAPK and PI3K–PTEN–AKT–mTOR pathways during normal vascular development in CNS and non-CNS tissues and in AVMs may help to develop a more comprehensive picture of arteriovenous morphogenesis. A recent study addressed endothelial aberrancy in brain AVMs at the single-cell level, linking the transcriptional state of ECs isolated from human brain AVMs to a dysregulation of arteriovenous zonation, evidenced by a strong enrichment of arterial and venous transcriptional identity but not of capillary or venule transcriptional identity[75]. That study further found an upregulation of PLVAP (a marker of fenestrated endothelium[75]) and the pro-angiogenic protein PGF in the AVM nidus[75].

Along those lines, in our own scRNA-seq dataset, we found upregulated PLVAP predominantly in angiogenic capillary ECs of brain AVMs as well as reactivated fetal signalling pathways in human AVMs with a general (non-CNS-specific) mode of action, involving the integrin, TGFβ, angiopoietin–TIE, epithelial-to-mesenchymal transition-related, inflammatory-related and IL4-mediated signalling cascades[163].

CNS-specific molecular mechanisms in brain AVMs. Most of the molecules involved in vascular brain development that are reactivated in brain AVMs act via a general (non-CNS-specific) mechanism of action (Fig. 6j,k and Supplementary Table 2). Interestingly, somatic mutations in the gene encoding the CNS-specific angiogenesis regulator GPR124 were identified in human brain AVMs. This finding, however, could not be substantiated in a replication cohort or meta-analysis of individuals with brain AVMs[332].

Molecular mechanisms in brain AVM vasculature at the single-cell level. scRNA-seq allows the study of the biology of brain vascular malformations (including brain AVMs) at the single-cell level[75,346,347], yielding insights into EC and PVC heterogeneity, their interactions in the blood vessel microenvironment, the intermediate cell types that arise during blood and lymphatic vessel development, and cell type-specific responses to disease[347].

Recently, Winkler and colleagues presented a human cerebrovascular cell atlas that compared isolated cells from the adult human brain with cells isolated from resected human brain AVM tissue[75]. They uncovered a previously unknown heterogeneity in PVCs, revealed transcriptional variation within SMCs and perivascular fibroblasts, and identified SMC-like cells known as fibromyocytes[75]. In addition to a loss of physiological arteriovenous zonation, which is characteristic of brain AVM pathology, they reported a distinct transcriptomic state in a subset or cluster of ECs relating to heightened angiogenic potential and immunogenicity, indicating that this subset of ECs may originate from the AVM nidus[75].

In our molecular single-cell atlas, we found an increase in the number of venous EC clusters in brain AVMs and cavernomas compared with adult control brain tissue[163], suggesting an involvement of venous ECs in the pathophysiology of brain vascular malformations, as reported for cavernomas in mice[346]. Similarly to the situation observed in glial brain tumours, we identified alteration of arteriovenous differentiation and CNS-specific properties, upregulation of major histocompatibility complex class II molecules and reactivated developmental pathways

in brain AVMs (although these were less numerous than those in brain tumours) belonging to the aforementioned five major groups of pathways[163], indicating some common mechanisms across brain tumours and brain vascular malformations[163].

Although shared signalling pathways seem to regulate vascular growth in brain pathologies (including brain tumours and brain vascular malformations) and in the fetal brain, it remains to be clarified whether the pathways observed in brain pathologies are reactivated developmental pathways or rather reflect the persistence (for example, the presence since development) of a less differentiated cell type (or even a combination of these two). Moreover, the functional relevance of these developmental pathways in vascular-dependent brain pathologies is not clear, and further studies will be needed to elucidate their translational potential in terms of developing therapies targeting the vasculature in brain tumours and brain vascular malformations. Single-cell atlases such as those discussed above will inform such endeavours[347].

A developmental look at brain AVMs

On the basis of neuroradiological and surgical observations, most brain AVMs occupy a defined segment of the brain's vascular tree and do not grow after diagnosis[273,348,349]. It is currently thought that AVMs develop during early postnatal life, at highly active developmental stages, as mentioned earlier herein (Fig. 6a–i). Postnatal development of brain AVMs is supported by the lack of cases reported in utero (which indicates an embryonic AVM development). However, this does not exclude the possibility that somatic mutations and brain AVM initiation occur during embryonic development but remain undetectable until later stages of postnatal life. *KRAS* mutations seem restricted to the endothelium in brain AVMs, suggesting that somatic mutations occurring in progenitor ECs will 'trace' the future developmental territory (for example, the vascular network field) of their daughter ECs. Accordingly, large brain AVMs would result from somatic mutations occurring early in development (spanning larger vascular territories) (Fig. 6a–d), whereas small AVMs may reflect later mutations spanning a restricted vascular territory (Fig. 6e–h). Strikingly, many brain AVMs spread preferentially along a radial axis extending from the ventricles to the pial cortical surface. When small, they can be constrained in and around the pial, sulcal and cortical areas or alternatively in the ventricular, ependymal and subependymal zones (for example, choroidal AVMs), but they do not occur isolated midway in the white matter without reaching either the cortical surface or the ventricular surface (Fig. 6a–i). These observations prompt a comparison with the radial ventriculocortical axis of the radial glia and cortical neuron migration as well as of sprouting angiogenesis during embryonic and postnatal brain vascular development and maturation (Fig. 3). Could somatic mutations in EC progenitors actually be genetic tracers of the migrating and dividing EC progenitors recruited in sprouting angiogenesis and could brain AVMs, consequently, be an aberrant, dysmorphic and oversized capillary network occupying a developmentally defined vascular zone? This tempting but speculative hypothesis may clarify the temporo-spatial organization of sprouting angiogenesis in the developing CNS vascular network and developmental morphogenesis of brain AVMs.

Perspectives and conclusion

Several outstanding questions exist regarding the cellular and molecular mechanisms and the EC and PVC heterogeneity that underlie the brain vasculature during brain development, in the adult healthy brain and in vascular-dependent CNS pathologies and the shared angiogenic pathways between brain development and pathologies. First, how do CNS-specific and general cues interact molecularly to govern CNS angiogenesis during embryological and postnatal brain development and in vascular-dependent CNS pathologies? The CNS-specific cues that are known to regulate developmental angiogenesis show striking region specificity (for example, between the hindbrain and the forebrain)[133,135,136,148]. Moreover, brain region-specific intrinsic transcription factors were shown to govern embryonic brain angiogenesis in a spatially regulated manner[6]. These are interesting observations that lead to the question of whether region-specific vascular growth might be linked to region-specific brain function during development and in disease. Furthermore, both glial brain tumours and brain AVMs are most often confined to specific brain regions, but whether CNS-specific and region-specific regulators[6] of angiogenesis participate in the molecular mechanisms underlying these observations, suggestive of another link between the developmental brain vasculature and the pathological brain vasculature, remains unknown.

All currently known molecules and signalling pathways underlying hereditary AVM syndromes and sporadic brain AVMs characterized by somatic mutations are non-CNS-specific regulators of angiogenesis[162,287,288,323,325,328] (although the CNS-specific signalling receptor GPR124 is expressed in brain AVM ECs[332], a functional role for it in brain AVM has not been established to date). The lack of CNS specificity in this signalling is in line with the fact that multiple organs are affected by AVMs in these syndromes. Regarding sporadic AVMs, *KRAS* and *BRAF* mutations in ECs cause brain and spinal cord AVMs (peripheral AVMs were not reported)[162,323,325], whereas RAS and MAPK variants cause sporadic brain AVMs and skin vascular malformations[350], indicating specificity for neuroectodermal-derived tissues. Given the highly specialized vasculature of the CNS[9] and the observed alteration of the CNS-specific gene profile in pathological brain ECs (for example, pathological brain ECs partially acquiring a gene profile that is characteristic of peripheral or non-CNS ECs)[163], we think that the role of CNS-specific and general regulators of angiogenesis in brain tumours, brain AVMs and other CNS pathologies warrants further investigation. For instance, conducting single-cell multi-omics studies of the vasculature in different compartments of the developing brain as well as of brain region-confined pathologies (for example, brain tumours in defined gyri, for instance superior temporal lobe glioblastoma[351]) will be an important step forward to elucidate these very exciting concepts.

The second question is how different or comparable are the mechanisms governing angiogenesis during brain development, in brain tumours and in brain AVMs, and how can this be addressed by single-cell analyses in the multi-omics era? Currently, it remains incompletely understood to what extent developmental signalling pathways reactivated in pathologies differ from those active during (brain) development. Regulatory effects of neurodevelopmental programmes in glioblastoma cells[267] as well as oncofetal reprogramming of ECs in hepatocellular carcinoma have been reported in single-cell studies[352], but the relevance of fetal pathways in the pathological brain vasculature has not been described so far. Therefore, direct comparison between ECs derived from developing (fetal or embryonic or postnatal) brains, ECs derived from healthy adult control brains and ECs derived from vascular-dependent CNS pathologies at single-cell resolution is of crucial importance.

Recently, the power of single-cell analyses enabled us to unravel key signalling pathways in brain ECs active during development that were reactivated in brain tumour and brain vascular malformation ECs[163]. Our finding that more than half of all regulated pathways in pathological ECs are of developmental origin confirm a paradigm

in which signalling axes driving vascular growth during fetal human brain development are silenced in the adult human control brain and (re)activated across various human brain pathologies, including various brain tumours and brain vascular malformations[163]. The crucial importance of developmental pathways in vascular-dependent brain pathologies and the suggested functional plasticity of ECs[353,354] across developmental and disease states will need to be functionally validated using emerging novel techniques such as single-cell genomics[355], spatial transcriptomics (for example, Slide-seq[356] or other spatial transcriptomics techniques[357]) and single-cell proteomics (for example, imaging mass cytometry[358], single-cell cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq)[359], and single-cell western blotting[360]). These techniques provide exciting novel avenues allowing direct measurement of RNA and protein expression in isolated human brain ECs (and PVCs of the NVU), thereby providing insights into the molecular and genetic basis while retaining spatial information of both developmental and pathological CNS angiogenesis using an unbiased approach. These cellular and molecular insights at single-cell precision leading to the identification of novel molecular angiogenic signalling cascades then need to be studied using in vivo models of angiogenesis for both CNS (brain, spinal cord and retina) and non-CNS tissues/organs using xenograft models and other strategies[361–363].

The third crucial question for future studies is can single-cell multi-omics techniques be used to further clarify the role of inflammatory or immune-related processes in pathological angiogenesis beyond what is currently known (for example, inflammation-induced pro-angiogenic effects on the vasculature in brain tumours and brain vascular malformations[364–366])? Notably, recent single-cell studies have further emphasized additional roles of inflammatory processes in pathological ECs across various brain diseases, including brain AVMs[75,163], brain tumours[163] and neurodegenerative diseases[367–369]. Interestingly, pathological ECs show upregulation of inflammatory or immune-related pathways and of major histocompatibility complex class II molecules in brain tumours (including glial brain tumours) and brain vascular malformations (including brain AVMs)[75,163] as well as elevated levels of immune cell–EC interactions in brain tumours (including glial brain tumours) and brain vascular malformations (including brain AVMs)[163] as well as in Alzheimer disease and Huntington disease[367,368]. These very interesting observations warrant further investigation at both the single-cell level and the functional level, with potentially crucial implications for both basic biological understanding and translational settings.

The fourth question is how important are NVL-related developmental pathways for pathological brain angiogenesis, and are they of a CNS-specific nature or a general nature? We anticipate that addressing the role of NVL molecules in ECs and PVCs within the developing and the pathological CNS (for example, through leveraging single-cell multi-omics techniques[163,370–372]) will provide important insights that have to be characterized at multiple organizational levels.

At the molecular level, NVL-related pathways are of crucial importance during vascular brain development, and many are reactivated in vascular brain pathologies, as evidenced by our recent single-cell atlas[9,12,14,79,373]. As the cellular and molecular interaction and bilateral crosstalk between neuronal and vascular tissue are especially tight in the CNS[9,14,374], we reason that NVL molecules (being of a general or a CNS-specific nature) are of crucial importance in the healthy and the diseased brain and need to be studied in more detail in the future.

Regarding the layered organization of the human brain[375], neurovascular interactions might be fundamentally different in distinct CNS compartments, with predominantly neuron-to-EC interactions in CNS grey matter and mainly oligodendrocyte-to-EC interactions in the CNS white matter, both involving classic and non-classic axon guidance cues[9].

Members of the classic axonal guidance and NVL molecule families such as netrins, semaphorins, ephrins and Slit proteins, and their receptors, as well as non-classic axon guidance and NVL molecules such as WNT proteins, SHH and BMPs are implicated in arteriovenous differentiation[14,15,163,201,374,376]. NVL molecules interact with the Notch pathway[9], and interactions between NVL molecules and Notch are important in arteriovenous differentiation[9,14,201]. Most notably, NOTCH1 and NOTCH4 are major drivers of arterial fate, associated with expression of the arterial markers ephrin B2, CXCR4 and connexin 40 (ref. [345]). Lack of Notch signalling results in upregulation of venous markers such as COUP transcription factor 2, NRP2, VEGFR3 and the receptor EphB4 (refs. [75,281,282]). It remains to be explored how NVL molecules contribute to these phenomena, and analyses of NVL molecules and pathways in distinct arteriovenous compartments at the single-cell level[75,163,367,368] comprise a promising approach.

The final question is as follows: given the current focus on sprouting angiogenesis, how important are other modes of vessel formation during brain development, in brain tumours and in brain AVMs, how do they differ between distinct vascular beds inside and outside the CNS and how are they regulated at the molecular level? Modes of vessel formation other than sprouting angiogenesis probably have roles in both brain development and vascular-dependent brain pathologies[24]. Vasculogenesis is important during PNVP formation, whereas the INVP is predominantly vascularized by sprouting angiogenesis[27], but the involvement of other modes of vessel formation during these developmental stages remains to be determined. In tumours located inside and outside the CNS, GSC transdifferentiation into tumour ECs[20,21,215] or tumour pericytes[22] directly involves PVCs of the NVU. Similarly, vascular co-option and mimicry in liver cancer exemplifies PVC–EC interactions outside the CNS[173]. The molecular mechanisms underlying vascular co-option, vascular mimicry and vascular intussusception remain largely unexplored. A more thorough investigation of the influence of PVCs and cellular interactions within the NVU in the setting of these additional modes of neovascularization using single-cell sequencing (for example, scRNA-seq[377] and CITE-seq[359]) and imaging (for example, fluorescence light sheet microscopy and high-throughput microscopy[378]) techniques is key for future progress in developmental and pathological settings.

In conclusion, it has become increasingly evident that the remarkable cellular heterogeneity and molecular heterogeneity of the human brain vasculature within and between individuals across development and disease as well as its specific characteristics, such as CNS specificity and arteriovenous zonation, require thorough characterization at the single-cell level. Furthermore, a clearer mechanistic understanding of the silencing of developmentally active angiogenic processes in the healthy adult brain and subsequent reactivation in disease at the single-cell level will be crucial for the development of future therapies aimed at targeting vascular pathology. We anticipate that the constantly evolving multi-omics approaches (including scDNA-seq, single-cell assay for transposase-accessible chromatin with sequencing (scATAC-seq), imaging cytometry by time of flight and spatial transcriptomics) will enable various long-standing questions in the field of neurovascular biology to be answered and will continue to increase our knowledge of the human brain vasculature in development, adulthood and disease.

Published online: 20 March 2023

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Acknowledgements
The authors thank N. Chu Ji for help with the illustrations, P. Nicholson for providing neuroradiological images of the brain AVMs and brain tumours, J. Fish, M. Ghobrial, H. Zhong and F. Farnhammer for discussion regarding the scRNA-seq data and A. Thomson for help with English proofreading. T.W. was supported by the OPO Foundation, Swiss Cancer Research (KFS-3880-02-2016-R and KFS-4758-02-2019-R), the Stiftung zur Krebsbekampfung, the Kurt und Senta Herrmann Foundation, Forschungskredit of the University of Zurich, the Zurich Cancer League, the Theodor und Ida Herzog Egli Foundation, the Novartis Foundation for Medical-Biological Research and the HOPE Foundation.

Author contributions
T.W. had the idea for the Review, wrote the manuscript with J.B., designed the figures and, with J.B., created the figures. T.W. and J.B. researched data for the article, provided substantial contributions to discussion of its content, and reviewed and edited the manuscript before submission. P.C., G.Z., P.P.M., K.D.B. and I.R. provided substantial contributions to discussion of the article's content and reviewed and edited the manuscript before submission. I.R. also helped write the article.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41583-023-00684-y.

Peer review information *Nature Reviews Neuroscience* thanks S. Liebner; J. Siegenthaler; R. Wang, who co-reviewed with S. Yuan; and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

[1]Group of CNS Angiogenesis and Neurovascular Link, Neuroscience Center Zurich, and Division of Neurosurgery, University and University Hospital Zurich, Swiss Federal Institute of Technology (ETH) Zurich, Zurich, Switzerland. [2]Division of Neurosurgery, University Hospital Zurich, Zurich, Switzerland. [3]Group of Brain Vasculature and Perivascular Niche, Division of Experimental and Translational Neuroscience, Krembil Brain Institute, Krembil Research Institute, Toronto Western Hospital, University Health Network, University of Toronto, Toronto, ON, Canada. [4]Division of Neurosurgery, Department of Surgery, Toronto Western Hospital, Toronto, ON, Canada. [5]Department of Physiology, Faculty of Medicine, University of Toronto, Toronto, ON, Canada. [6]Laboratory of Angiogenesis and Vascular Metabolism, Center for Cancer Biology, VIB & Department of Oncology, KU Leuven, Leuven, Belgium. [7]State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, People's Republic of China. [8]Laboratory of Angiogenesis and Vascular Heterogeneity, Department of Biomedicine, Aarhus University, Aarhus, Denmark. [9]Princess Margaret Cancer Centre, University Health Network, Toronto, ON, Canada. [10]Donald K. Johnson Research Institute, Krembil Research Institute, Krembil Discovery Tower, Toronto, ON, Canada. [11]Department of Ophthalmology and Vision Sciences, Faculty of Medicine, University of Toronto, Toronto, ON, Canada. [12]Laboratory of Exercise and Health, Department of Health Science and Technology, Swiss Federal Institute of Technology (ETH) Zurich, Zurich, Switzerland.