Neuronal regulation of the blood–brain barrier and neurovascular coupling

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Abstract | To continuously process neural activity underlying sensation, movement and cognition, the CNS requires a homeostatic microenvironment that is not only enriched in nutrients to meet its high metabolic demands but that is also devoid of toxins that might harm the sensitive neural tissues. This highly regulated microenvironment is made possible by two unique features of CNS vasculature absent in the peripheral organs. First, the blood–blood barrier, which partitions the circulating blood from the CNS, acts as a gatekeeper to facilitate the selective trafficking of substances between the blood and the parenchyma. Second, neurovascular coupling ensures that, following local neural activation, regional blood flow is increased to quickly supply more nutrients and remove metabolic waste. Here, we review how neural and vascular activity act on one another with regard to these two properties.

Blood–brain barrier
(BBB). A physiological barrier formed by CNS endothelial cells to regulate the trafficking of molecules between the blood and the brain.

Neurovascular coupling
(NVC). The process by which local neural activation can rapidly increase local blood flow; it is the basis of functional MRI.

Mural cell
A collective term to describe the cell types that wrap around blood vessels, including the smooth muscle cells on arteries and pericytes on capillaries

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The brain is a highly vascularized organ, with every neuron positioned within 15 µm of a blood vessel[1]. This proximity allows ready exchange of nutrients and waste products, enabling the high metabolic activity of the brain despite its limited intrinsic energy storage. Furthermore, the flux of blood is finely targeted to active areas through selective dilation and contraction of blood vessels, even allowing the use of blood flow as a proxy for brain activity in functional MRI (through the blood oxygen level-dependent (BOLD) response). For more than a century, it has also been observed that exchange of molecules across this dense vasculature in both directions is highly restricted[2,3]. These emergent properties — the blood–brain barrier (BBB) and neurovascular coupling (NVC) — are due to the concerted action of the several cell types that together constitute the neurovascular unit (NVU).

The CNS vasculature itself is made up of different segments, each with a molecularly distinct composition of cell types[4,5]. Arteries, and the arterioles that branch off them, are the most upstream with respect to blood flow. The arterial endothelial cells (ECs) are enwrapped by arteriolar smooth muscle cells (SMCs), which can acutely constrict or dilate these vessels to control blood flow into the downstream capillary bed. Capillaries make up 85% of the vasculature of the brain[6] and are the principal contributors to BBB function. Capillary ECs are very tightly associated with pericytes, which are a type of mural cell related to, but molecularly and functionally distinct from, SMCs (TABLE 1). In the adult vasculature, capillaries are also surrounded by astrocyte end-feet. Finally, blood from the capillaries drains into venules and veins. Venules and veins represent the smallest fraction of CNS blood vessels. Although venous ECs, similarly to arterial ECs, are surrounded by SMCs, these SMCs are molecularly distinct from arteriolar SMCs[4], and the vasomotion of veins is thought to be a passive result of changes in upstream blood flow[7]. These cell types, along with the surrounding neurons, are the principal constituents of the NVU (FIG. 1). For a comprehensive overview of NVU cell types, readers may refer to several excellent reviews[8–10].

Serving as the interface between the periphery and the CNS, signalling within and across the NVU is critical in health and disease. Recent work has shed greater light on the cell types and molecular pathways that regulate BBB function and NVC in the CNS. In particular, many groups have published single-cell and bulk transcriptomic data illustrating the molecular composition of different cell types of the NVU[4,11–19]. Notably, dysfunction in these cell types often manifests in both BBB and NVC phenotypes.

In this Review, we discuss recent advances in our understanding of how the NVU mediates communication between the CNS parenchyma and the vasculature in the context of the BBB and NVC. We focus on how activity in the parenchyma can influence the vasculature, and vice versa. First, we cover mechanisms important to BBB function and how those mechanisms can be regulated by cells in the NVU. Second, we discuss the available evidence for how neural activity can rapidly communicate to the vasculature to lead to spatially

Table 1 | **Molecular markers of segments of the neurovascular unit**

Marker or dye	Gene	Vascular segment	Genetic construct
Smooth muscle cells			
Smooth muscle actin	*Acta2*	Arteries and venules	*Acta2*–CreER[215]
			Acta2–mCherry[216]
Myosin heavy chain 11	*Myh11* (also known as *Smmhc*)	Arteries and venules	*Myh11*–CreER[217]
Calponin	*Cnn1*	Arteries	*CNN1* gene trap LacZ reporter[218]
Transgelin (also known as smooth muscle protein 22α)	*Tagln*	Arteries	*Tagln*–Cre[219]
Pericytes			
Aminopeptidase N (also known as CD13)	*Anpep*	Capillaries and venules[24]	–
ATP-binding cassette subfamily C member 9	*Abcc9*	Capillaries and venules	*abcc9–GAL4* (in zebrafish)[220]
Genetic intersection with platelet-derived growth factor-β and chondroitin sulfate proteoglycan 4	*Pdgfrb*; *Cspg4* (also known as *Ng2*)	Capillaries	*Pdgfrb*–Flp; *Cspg2*–Frt–STOP–Frt–Cre–ER[78]
NeuroTrace 525	–	Capillaries[163]	–
Arterial endothelial cells			
Bone marrow tyrosine kinase	*Bmx*	Arteries	*Bmx*–Cre–ER[221]
			Bmx–LacZ[222]
Connexin 40 (also known as gap junction α5)	*Gja5*	Arteries[207]	*Gja5*–Cre–ER[223]
Capillary endothelial cells			
Major facilitator superfamily domain-containing 2A	*Mfsd2a*	Capillaries and venules	*Mfsd2a*–Cre–ER[224]
Arteries			
Hydrazide 633	–	Arteries[161]	–

ER, oestrogen receptor.

restricted changes in blood flow. For specific discussion of the CNS vasculature in disease, readers may consult recent reviews[6,20].

The BBB

The ultrastructural basis for the BBB was first described by Reese and Karnovsky, who showed that an intravenously injected tracer cannot pass through tight junctions (TJs) — specialized contacts between adjacent ECs that prohibit paracellular passage of water-soluble molecules —between CNS ECs[21]. The same tracer can, however, readily pass through the cell–cell junctions in the peripheral endothelium (paracellular leakage)[22]. Thus, historically, the restricted permeability of brain vasculature has been attributed to TJs. However, substances can also cross ECs by transcytosis, a process by which material enters endocytic vesicles that move across the cell and release their contents on the other side (FIG. 2). Indeed, peripheral ECs have been found to have numerous tracer-filled vesicles, whereas CNS ECs contain very few[21]. Recent evidence shows that the inhibition of transcytosis in CNS ECs is an active process to ensure BBB integrity, and that full barrier integrity requires restriction of both paracellular and transcellular leakage[10,23–29]. However, the passage of molecules across the BBB is not completely absent, as BBB ECs abundantly express nutrient transporters and efflux transporters, and have some level of receptor-mediated transcytosis, all of which enable molecule-specific exchange between the blood and the CNS[30] (FIG. 2).

There is an emerging and ever-more precise picture of the molecular players capable of modulating barrier function in the CNS vasculature. But specific barrier function also exhibits spatial and temporal heterogeneity throughout the CNS. How is neural activity influenced by these variations in BBB function? Conversely, how does neural activity influence BBB function? In the following sections, we provide a brief, inexhaustive overview of the state of our understanding of the key pathways and cell types for BBB function (for a more complete discussion, readers are referred to several recent reviews[8,10,31–34]). We then discuss several recent studies that highlight the interplay between barrier function and neural activity. These pose open questions as to how and which mechanisms that regulate barrier function may be at play.

Structural determinants

Specialized TJs. CNS ECs form specialized TJs (FIG. 2), which are regarded as the key structural feature of the BBB, sealing the blood vessel lumen from the CNS parenchyma[35–38]. TJs are characterized by dense arrays of transmembrane proteins, notably claudin family members, occludin and junctional adhesion molecules, all of

which form intercellular contacts. Classically, these transmembrane factors are scaffolded by numerous other proteins, including the zona occludens proteins (ZO-1, ZO-2 and ZO-3). Many of these TJ proteins are not unique to the CNS vasculature however, raising the question of what mechanisms distinguish the impermeability of the BBB relative to segments of peripheral continuous ECs.

A straightforward possibility is that CNS ECs have higher levels of TJ proteins, leading to denser, less-permeable TJs. Although EC TJ proteins are present in the peripheral vasculature, transcriptomics shows that the levels of transcripts encoding several TJ proteins, notably occludin, are especially high in CNS ECs[4,16,17]. It is also possible that post-translational modifications have a role in tuning TJ protein function in the CNS[38–42]. However, most of the evidence for such regulation stems from in vitro studies, which may fail to wholly replicate bone fide CNS ECs (BOX 1). Finally, it is possible that there is an as-yet uncharacterized factor present only at BBB TJs that confers unique impermeability.

Several groups have tested these possibilities using genetic ablation of specific TJ components. Surprisingly, knockout of the genes encoding occludin[43,44], ZO proteins[45–47], claudin 3 (REF.[48]) (whose presence at the BBB is disputed)[48] and claudin 12 (REF.[49]) does not result in gross TJ permeability. Lack of claudin 5 (REF.[50]) or lipolysis-stimulated lipoprotein receptor (LSR)[51] (FIG. 2) in mice results in increased permeability to tracers smaller than about 800 Da. Curiously, CNS TJs in both types of these knockout mice seem normal by electron microscopy, with the evident kissing points between cell membranes, in contrast to many cell–cell junctions of peripheral vasculature ECs. Consequently, TJ dysfunction in these mice would not be detected by high-molecular-weight tracers such as horseradish peroxidase that are commonly used in electron microscopy (BOX 2).

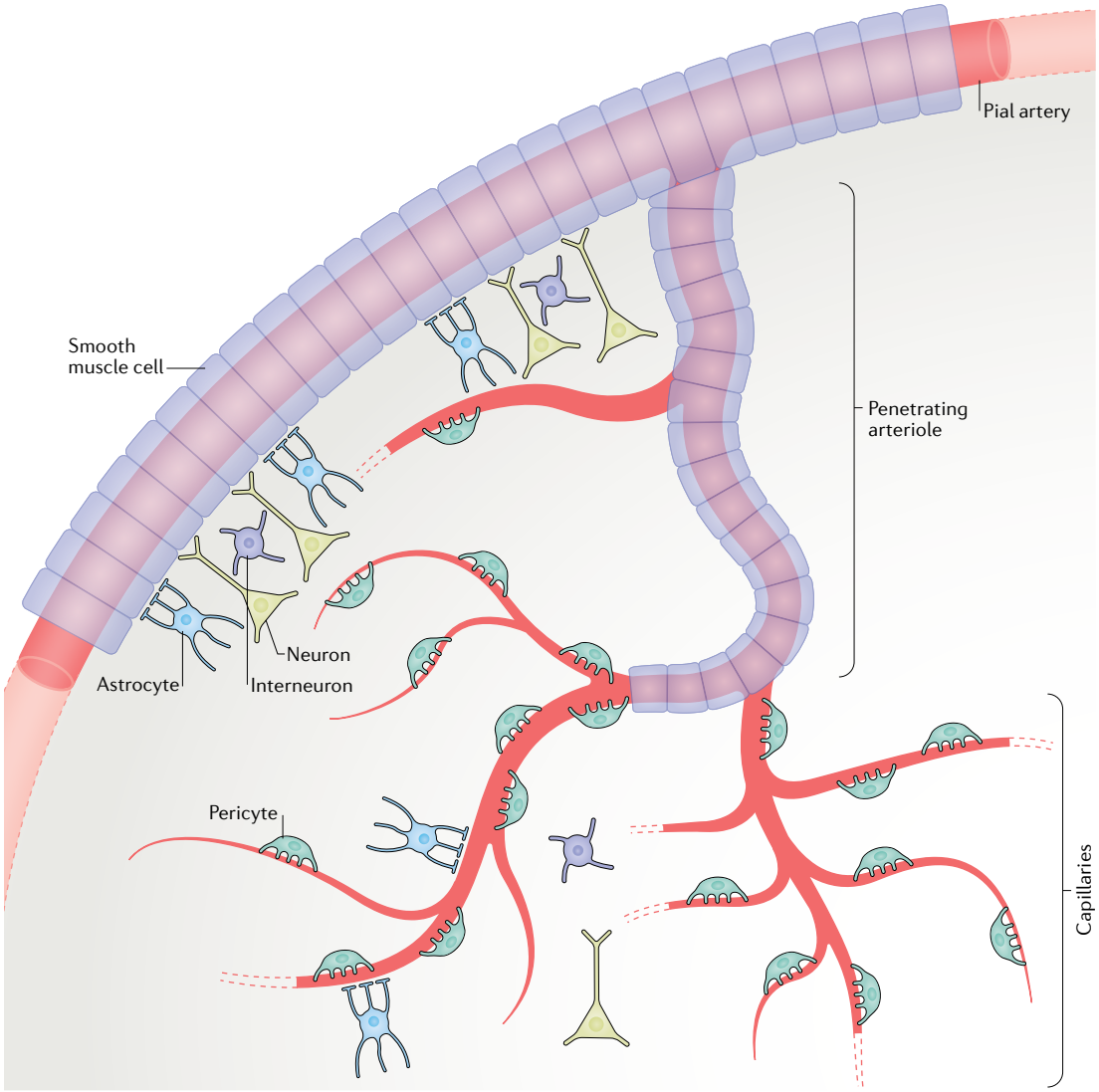

Fig. 1 | **The neurovascular unit and cerebrovascular anatomy.** Pial arteries line the surface of the brain and are ensheathed by contractile smooth muscle cells. Pial arteries then descend into the brain parenchyma, narrowing and branching to become penetrating arterioles, which then branch even more to become the dense network of capillaries. Pericytes surround capillaries and are near end-feet of astrocytes and dendrites of neurons. Together, these cells form the collective neurovascular unit.

Caveolae
Flask-shaped vesicular structures formed by caveolins, approximately 70 nm in diameter.

The at-most mild leakage levels in these knockout studies raise several questions. Is there compensation by other TJ proteins when a single TJ component is deficient? Is there some other factor that is strictly necessary for BBB TJ function? Additionally, blood-borne macromolecules can influence CNS activity if they leak out of the vasculature. Can a reduction in TJ protein levels in disease explain the paracellular leakage of these large molecules, given that deficiency of individual TJ proteins leads to leakage of only small molecules? Perhaps coordinated downregulation of several TJ components causes greater TJ permeability than does complete deficiency of a single component. One further possibility is that TJ proteins at the BBB have additional roles besides providing strictly structural blockade of cell–cell junctions. These may include modulating signalling or transcriptional regulation, as has been demonstrated in vitro and in epithelial tissues[52–56], but the role of such alternative pathways in CNS ECs has been poorly studied.

Suppressed transcytosis. Unlike peripheral ECs, which readily transcytose material across the endothelium, CNS ECs suppress non-specific transcytosis. The cell-biological mechanisms regulating transcytosis have been extensively studied in epithelial cells, but less is known in the context of ECs. Generally, transcytosis can proceed through either receptor-mediated transcytosis or fluid-phase transcytosis. The former is a molecule-specific transport, whereas the latter can be non-specific. Although various vesicular transcytosis pathways have been implicated at the BBB[10,27,57–59], the pathways mediated by clathrin and caveolae are the most well studied and have been extensively reviewed[60,61]. Very little is known, however, about the intracellular trafficking in the ECs that is responsible for sorting material to be transcytosed (as opposed to degraded or recycled back to the vessel lumen)[62,63]. A better grasp on the molecular mechanisms that regulate the intracellular trafficking of endosomes in CNS ECs could have important clinical applications for drug delivery across the BBB and for mitigating pathological BBB transcytosis[64].

NVU regulation of the BBB

Although barrier functionality is ultimately localized to the ECs, BBB properties are not intrinsic for CNS ECs. Instead, BBB induction and maintenance rely on the local environment — that is, signalling from other cells in the NVU onto ECs. Such interactions include

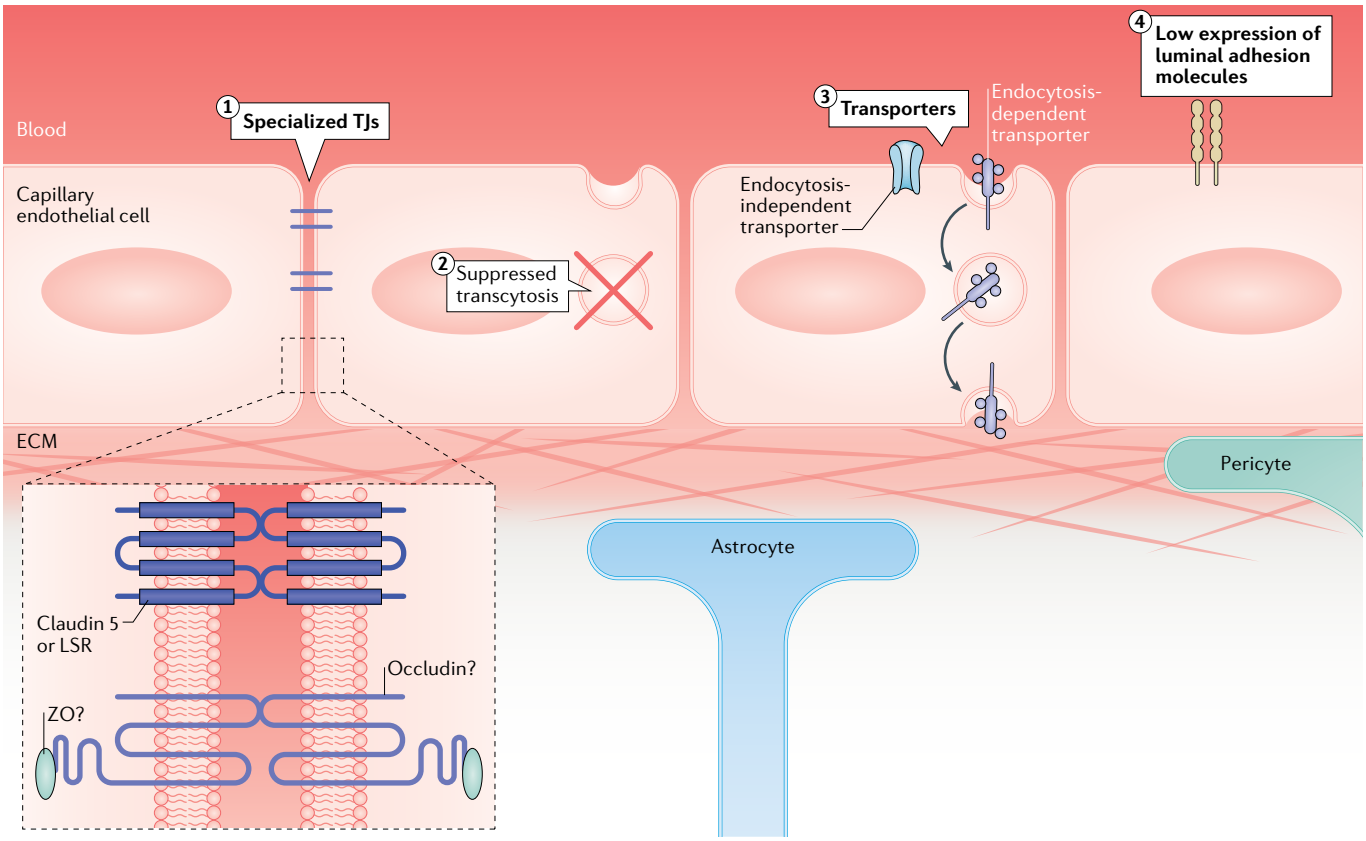

Fig. 2 | **Properties of the BBB.** The CNS capillary endothelium has four cellular properties that contribute to the function of the blood–brain barrier (BBB) by strictly regulating the molecular trafficking between the blood and the brain. Specialized tight junctions (TJs) limit paracellular flux between endothelial cells, including claudin 5 and lipolysis-stimulated lipoprotein receptor (LSR) (1). The specific contribution of occludin and zona occludens proteins (ZO) remains elusive, as knockout mice lack phenotypes of BBB dysfunction. Suppression of transcytosis limits transcellular flux through CNS capillary endothelium (2). Molecule-specific transport allows the strict passage of desirable molecules such as nutrients (3). This can be categorized into endocytosis-independent transport and endocytosis-dependent transport. Low expression of leukocyte endothelial molecules on the luminal wall of the blood vessel maintains low levels of leukocyte adhesion and thus low levels of immunosurveillance in the CNS (4). A question mark indicates some confounding evidence, as described in the text. ECM, extracellular matrix.

Box 1 | **Challenges of making in vitro BBB models**

Given the complexity of studying the blood–brain barrier (BBB) in vivo, many have attempted to develop in vitro BBB models as a more tractable platform. The major challenge of in vitro models has been to recapitulate the 'tightness' of the BBB observed in vivo, especially as BBB properties are not intrinsic to CNS endothelial cells (ECs) but are mediated by the in situ neural environment. Notably, primary CNS ECs quickly lose their BBB properties in cell culture[14,37,225,226].

Nevertheless, in the past decade, many laboratories have developed various in vitro BBB models through the use of co-cultures with pericytes and astrocytes[227], induced pluripotent stem cell differentiation[228–230], brain organoids[231] and 'organ-on-a-chip' approaches[232] in an effort to circumvent this issue. Validation of these in vitro models is typically done by measuring transendothelial electrical resistance (TEER) — a measure of tight junction function — and the expression of BBB markers. However, non-BBB tissues, including epithelial cells[233], can exhibit high TEER, and TEER is a proxy for only paracellular, not transcellular, permeability. Additionally, measurement of a few BBB markers can result in false positives owing to antibody cross-reactivity with related non-BBB proteins[48,49].

There is an urgent need to validate whether the ECs from these in vitro BBB models share similar transcriptomic profiles with CNS ECs in vivo. This is now possible given the increasing number of data sets describing the brain vasculature transcriptome[4,11,12,16–18,234]. Thus, basic requirements of in vitro BBB models should include transcriptomic validation and functional readouts that reflect in vivo barrier permeability.

secretion of WNT ligands by neurons and astroglia[65,66], processing of transforming growth factor-β (TGFβ) by astrocytes[67,68], signalling through extracellular matrix (ECM) factors secreted by pericytes and astrocytes[69,70] and direct cell–cell contacts between ECs and mural cells[71]. The influence of the neural niche on BBB induction has been classically demonstrated in studies showing that transplanting CNS neural tissue into the periphery can drive ectopic BBB formation[72,73]. Here we highlight the contributions of just astrocytes and pericytes, owing to recent investigations into their roles in BBB maintenance and NVC. Readers may consult recent reviews for the contributions of other NVU cell types, including microglia and oligodendrocyte precursor cells[9,10,74].

Pericytes. Pericytes are recruited to the CNS vasculature early in development as ECs invade the neural tube. Early studies demonstrated that reducing pericyte recruitment by antagonizing platelet-derived growth factor receptor-β (PDGFRβ) signalling during development prevents functional BBB formation. These animals show substantial increases in transcytosis, as well as TJ abnormalities[23,24,75].

When pericyte recruitment is constitutively inhibited throughout development, adult mice continue to have leaky CNS vasculature[24,75,76]. Although these studies demonstrate that pericytes are required for BBB formation, they do not address whether pericytes are required for BBB maintenance after BBB formation. Correlative evidence suggests they are. First, reductions in the pericyte coverage of CNS capillaries with age coincide with increased BBB permeability as well as decreased cerebral blood flow[75]. Second, across different regions even in the CNS, vascular impermeability correlates well with pericyte coverage[77].

Two recent studies directly tested the role of pericytes in adult mice using pericyte ablation. First, Park et al. conditionally expressed diphtheria toxin subunit A in mural cells, severely depleting both SMCs and pericytes throughout the entire mouse[71]. Remarkably, they did not observe BBB leakage even after 2 weeks, suggesting pericytes are important for initial BBB formation but not maintenance. However, Nikolakopoulou et al. used an intersectional genetic approach to specifically ablate roughly 60% of pericytes, sparing SMCs[78]. Using this approach, they observed BBB disruption, although it was much less severe than the leakage observed in models of pericyte deficiency through development. On this pericyte depletion, Nikolakopoulou et al. also did not observe the increases in transcytosis that are apparent in mice with constitutive pericyte depletion[23,24].

A possible reason for this discrepancy may be methodological differences between the two studies. First, different molecules were used as proxies for BBB permeability (BOX 2). Park et al. examined leakage of intravenously injected 70-kDa dextran by post-mortem staining. By contrast, Nikolakopoulou et al. monitored leakage of intravenously injected gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA; molecular mass ~500 Da) with MRI and infiltration of endogenous plasma proteins into the parenchyma by post-mortem staining. It may be possible that pericyte ablation makes the BBB susceptible to passage of specific molecules and not others. Second, the pericyte ablation techniques differed: whereas Park et al. directly expressed the cytotoxic subunit of diphtheria toxin in all mural cells[71], Nikolakopoulou et al. targeted the expression of the diphtheria toxin receptor and then administered diphtheria toxin systemically[78]. Although both of these approaches to cell ablation should be equivalent, subtle differences may exist between them[79]. Finally, it is notable that Nikolakopoulou et al. found more barrier disruption despite ablating a lower percentage of total pericytes. In light of this, other recent studies manipulating specific pericyte genes, including *Foxf2* and *Rbpj*, have observed pronounced effects on BBB permeability without reduction in pericyte coverage of CNS blood vessels[80,81]. This illustrates the potential for dysfunctional pericytes to drive signalling pathways in ECs that reduce BBB integrity.

Astrocytes. Astrocytes, like pericytes, are critical NVU constituents implicated in BBB and NVC regulation. Astrocyte end-feet tile CNS capillaries, and secrete trophic factors and ECM proteins. Indeed, just the steric coverage of the vasculature by astrocyte end-feet may provide a degree of barrier function[82]. Furthermore, the role of astrocytes in regulating neural function is now quite well appreciated[83]. These simultaneous direct interactions with neurons and the rest of the NVU make astrocytes attractive candidates for relaying signals between the parenchyma and the vasculature. Unlike pericytes, however, they mature postnatally after the barrier has formed[84]. Determining their precise role in barrier maintenance and regulation has remained elusive, partly because the diversity of astrocyte populations can make them experimentally difficult to address[12].

Astrocytes are one of the principle sources of ECM critical to maintaining BBB function. Thus, mice in which the gene encoding laminin (*Lamc1*) is conditionally knocked out of astrocytes exhibit changes in NVU

Diphtheria toxin
A toxin that inhibits protein synthesis, leading to cell death.

Angiogenesis
Growth of new blood vessels from existing blood vessels.

Circumventricular organs
(CVOs). Midline brain structures with permeable vasculature allowing ready exchange of molecules between neurons and the blood.

characteristics. These include reduced coverage by aquaporin 4-positive end-feet, a change in pericyte differentiation and considerable BBB permeability[85,86]. Secretion of soluble factors such as apolipoprotein E by astrocytes has also been shown to prevent BBB dysfunction[70].

Analogously to pericytes, several groups have attempted to investigate astrocyte influence on NVU function via diphtheria toxin-mediated ablation. Diphtheria toxin-mediated reduction of the numbers of either *Gfap*$^+$ (REF.[87]) or *Aldh1l1*$^+$ (REF.[88]) astrocytes had profound effects on neuron function and survival, but curiously had limited observable effects on BBB function, as assayed by measuring the leakage of the endogenous plasma proteins. Specific elimination of astrocyte–vascular contacts is possible in a low-throughput manner[89], but a systemic method to ablate such contacts without affecting the ability of astrocytes to provide trophic support to neurons is needed to clarify their role in BBB maintenance.

Box 2 | **Experimental methodology for assessing BBB function**

Experimental assessment of blood–brain barrier (BBB) function requires measuring the degree of leakage of a molecule (that is, a tracer) from the blood to the CNS parenchyma. The choice of which molecule to use and which modality to use to measure leakage is important to consider when evaluating BBB integrity.

Detection modality

Most commonly used assays of BBB integrity involve killing the experimental animal and measuring the amount of tracer that has leaked into the parenchyma. These approaches are versatile and allow high spatial resolution, as with electron microscopy. By their nature, however, they do not allow sampling of the same subject over time and are not possible in humans. One of the most powerful non-invasive detection modalities is MRI with contrast agents (for example, longitudinal dynamic contrast with gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA)[235,236]), which allows measurement of the permeability of the BBB throughout the whole CNS[139,167]. However, MRI-based imaging has relatively low spatial resolution and limited molecular capabilities, which can make it difficult to draw mechanistic conclusions.

Less common in animal models, but useful in humans[237], sampling cerebrospinal fluid (CSF) for the presence of molecules from the blood can be used to approximate BBB function, as normally the BBB would ensure separation of these fluids. However, this method can be ambiguous, as the presence of blood-derived molecules in the blood reflects a function of leakage as well as CSF production and clearance. This method also does not resolve precisely where potential leakage may be occurring from the blood into the CSF. Finally, some groups have used functional readouts of BBB permeability by injecting neuroactive but BBB-impermeable compounds, whose effects on neural activity would manifest themselves only if there is BBB breakdown. An example is penicillin, which can act as a GABA receptor antagonist; with a leaky BBB, it can access the parenchyma, block inhibitory neuron activity and result in net increases in neural activity.

Tracer size

Even when compromised, tight junctions (TJs) at the BBB typically allow leakage of only relatively low-molecular-weight molecules (up to ~1 kDa). Many common larger tracers, such as Evans blue, high-molecular-weight dextrans and horseradish peroxidase, may therefore show no leakage across the BBB even if TJ integrity is compromised. Common low-molecular-weight tracers include microperoxidase for electron microscopy and cadaverine for fluorescence-based detection. Differential leakage of high- and low-molecular-weight tracers can discriminate between paracellular and transcellular BBB leakage.

Endogenous versus exogenous tracers

Molecules such as immunoglobulin G and fibrinogen are naturally in the blood and cannot permeate the BBB under normal conditions. Monitoring the CNS content of these endogenous molecules can be a useful alternative to delivering exogenous tracer, but does not provide kinetic information on leakage. Furthermore, most readily detectable endogenous molecules are too large to rigorously report on TJ functionality.

EC molecular pathways

Many signalling pathways in CNS ECs are crucial for barrier formation and maintenance. These including the WNT–β-catenin, TGFβ[68,90], Hedgehog[91], Notch[92], angiopoietin[71] and retinoic acid[93] pathways. These pathways tend to also be important for CNS angiogenesis and vascular patterning, so it can be challenging to disentangle their barrier-specific effects. Systematic reviews of BBB-relevant pathways can be found in other reviews[9,94,95]; here, we focus on two of the pathways with clear implications in barrier function in development and adult animals, WNT and TGFβ.

WNT signalling. WNT signalling is crucial for CNS-specific angiogenesis and barriergenesis[96]. WNT pathway activation in ECs is detectable at early stages of CNS vascularization, starting around embryonic day 9.5 (E9.5) in mice[97], before formation of a functional barrier[23,25]. Although WNT activity in ECs drops substantially in postnatal stages, after completion of vascular patterning, it remains necessary for barrier function throughout life[97–99]. In this pathway, one of several WNT ligands is secreted in the local environment, binds to Frizzled receptors on ECs and signals to prevent β-catenin degradation. This enables β-catenin translocation into the nucleus, where it induces gene expression. Notably, astrocytes and neurons are important sources of WNT ligands[66].

Several proteins important for cell-autonomous barrier function are downstream of WNT, including claudin 5 and the glucose uniporter GLUT1, which are upregulated by canonical WNT signalling[15]. WNT signalling also downregulates plasmalemma vesicle-associated protein (PLVAP), a marker associated with fenestrated vessels that do not possess a barrier. Furthermore, recent studies suggest that canonical WNT signalling influences suppression of transcytosis: antagonizing canonical WNT signalling by deleting the gene encoding the WNT co-receptor low-density lipoprotein receptor-related protein 5 (LRP5) decreases the expression of *Mfsd2a* (which encodes a lipid transporter that suppresses transcytosis)[100], whereas β-catenin gain-of-function mice display upregulation of *Mfsd2a*[15]. Although WNT signalling seems critical to barrier function throughout the CNS, different CNS regions have a principal dependence on different WNT ligands (for example, Norrin in the cerebellum and retina, but WNT7A and WNT7B in the cortex)[101].

As WNT signalling regulates many BBB genes at once, could inducing WNT signalling in non-barrier ECs be sufficient for barrier formation? Recently, two studies explored this question in the circumventricular organs (CVOs). CVOs are regions in the brain that have vasculature that naturally has a leaky BBB[102] to allow neurons to sense systemic signals, such as blood osmolarity[103]. β-Catenin gain of function in the CVOs results in upregulation of BBB-related genes and decreased permeability to intravenously injected tracers[15,104]. Intriguingly, β-catenin stabilization alone is not as effective at inducing barrier properties in tissues that are not developmentally part of the CNS, such as the anterior pituitary gland, liver or lung[15,17]. Furthermore, fine regulation of

β-catenin activity with other secreted factors such as WNT inhibitory factor 1 (WIF1)[15,105] may be an additional means for the neural environment to tune barrier function. It will be interesting to further explore the extent to which WNT signalling regulates endothelial barrier function generally.

TGFβ signalling. TGFβ signalling is important for cell-fate determination for every cell type of the NVU[81,92]. The ligand is subjected to multiple post-translational regulatory steps, and the pathway is pleiotropic, particularly in ECs. Binding of TGFβ to the receptor ALK1 leads to phosphorylation of SMAD1 and SMAD5 and a leaky, proliferative state in ECs, whereas binding to ALK5 (also known as TGFβ receptor type 1) leads to phosphorylation of SMAD2 and SMAD3 and a stable quiescent state[106,107]. These multiple points of regulation make TGFβ signalling an important factor in BBB activity modulation.

Knocking out different components in the TGFβ pathway results in gross defects in vasculogenesis and angiogenesis, and often embryonic lethality[108,109]. Knocking out critical factors in TGFβ–ALK5 signalling in ECs[92], neurons[110] or oligodendrocyte precursor cells[111] results in gross BBB dysfunction and hyperproliferative ECs. In adult mice, acutely blocking TGFβ signalling in the retina similarly results in barrier disruption, but also gross vascular defects, including EC cell death, making barrier-specific conclusions difficult[112]. At the same time, leaky BBB states are also often associated with upregulation of extracellular positive regulators of TGFβ signalling, such as thrombospondin[17,67,68,81]. More work needs to be done using acute manipulations of specific TGFβ signalling components to disentangle the role of TGFβ signalling in adult barrier regulation from its importance in development.

Multiple pathways are involved in BBB regulation, and they can share similar molecules and feed back onto one another. Some confusion may be removed by measuring pathway activity directly, for instance with reporter mice (which are available for the WNT[113] and TGFβ[114] pathways), or by staining for known downstream targets, such as lymphoid enhancer-binding factor 1 (LEF1) or phosphorylated SMAD proteins for the WNT pathway or the TGFβ pathway, respectively.

Does neural activity modulate the BBB?

Given the intimate and reciprocal relationship between the nervous system and the vascular system in the brain[28,115], an exciting but incompletely explored question is whether changes in neural activity can modulate BBB function through the mechanisms mentioned in the preceding section. Neural activity has been demonstrated to modulate developmental CNS angiogenesis[116,117], and the neural control of blood flow has been studied for decades (see the subsequent section). But, in a similar way to how NVC serves to efficiently match moment-to-moment energy demand, might neural activity also tune BBB transporter composition to match other demands? Does neural activity modulate general barrier permeability? Below, we highlight recent work examining these questions.

One possible mechanism for BBB modulation in response to neural activity is direct action of neurotransmitters on cells of the NVU. Indeed, glial cells have abundant neurotransmitter receptors. In the developing retina, Müller glia are activated by spillover of neurotransmitters released during spontaneous waves of neural activity[118] that are necessary for refinement of the neural circuit underlying vision[119,120]. Müller glia are also critical to secretion of Norrin, which in turn activates the WNT pathway in retinal ECs, promoting barrier formation[66]. Inhibiting the spontaneous neural activity mediated by retinal cholinergic neurons impairs both angiogenesis and barriergenesis in the deep retinal vascular plexus[121], further demonstrating the influence of neural activity on barrier function.

Acute increases in neural activity in adult animals have also been implicated in changes in BBB function. After being housed in darkness, mice exposed to light show substantial changes in BBB-related gene transcription in the vasculature of the visual cortex[122], including increases in the levels of angiopoietin 2 (ANG2), which can then antagonize ANG1–TIE2 signalling and lead to BBB disruption[123]. Additionally, direct activation of neural activity with transcranial magnetic stimulation led to measurable increases in BBB permeability in rodents and human participants, although in this case leakage may have been principally restricted to perivascular spaces[124]. It will be interesting to examine the relative contributions of paracellular and transcellular permeability in these models as well as the functional consequence of BBB opening therein.

Aberrantly high neural activity has also been correlated with BBB opening. In epilepsy, which features pathologically high amounts of glutamatergic activity, robust BBB opening is observed, consistent with BBB opening in response to direct application of 1 mM glutamate[124]. There is in vivo evidence for active ionotropic glutamate receptors in ECs[125], and in vitro evidence suggests that glutamate can act on ECs directly, inducing paracellular leakage[126]. Transcriptomic data, however, fail to reveal expression of ionotropic receptors in CNS ECs, and evidence for BBB modulation by physiological levels[127] of glutamate is lacking. To what degree does normal physiological neural activity in adults cause general BBB permeability? More work is needed to explore whether specific subsets of neurons can mediate dynamic barrier opening or barrier sealing, or whether different levels of general neural activity are responsible for differential effects on the BBB.

The BBB may also become permeable to specific molecules in response to neural activity. An intriguing example of this is insulin-like growth factor 1 (IGF1), a peptide growth hormone that is essential for brain development and neurogenesis[128–130]. IGF1 is secreted mainly by the liver into the blood and is typically bound to carrier proteins such as IGF-binding protein 3 (IGFBP3). IGF1 acts through IGF1 receptor (IGF1R), which is abundant both in brain ECs and in the parenchyma[4,11]. Increasing neural activity — through exercise[131], exploration of novel environments[132] or whisker stimulation[132] — results in increased brain IGF1 levels. Conversely, inhibiting

neural activity using tetrodotoxin prevents brain IGF1 accumulation[132].

So how does IGF1 transit from the blood to the brain? One possible mechanism is that activity-induced hyperaemia may result in increased blood-borne IGF1 bound to IGFBP3 delivered to active brain areas[133]. Neural activity-induced enzymatic processing of IGFBP3 by matrix metalloproteinase 9 (MMP9)[134,135] allows IGF1 to bind LRP1 (REFS[132,136]) and/or IGF1R[137] (which are expressed at the BBB), and to transcytose into the parenchyma specifically in areas where neural activity is elevated. Studying IGF1 trafficking in the context of endothelium-specific deletion of these proteins may help to resolve the mechanism of transport into active brain areas.

Collectively, these studies suggest that neural activity can affect barrier permeability. In normal physiology, these effects are likely to be finely tuned and spatiotemporally restricted to avoid disease. It will be exciting to unravel the contexts in which neural activity can influence the BBB and whether specific classes of activity have differential effects on BBB function. To investigate these mechanisms, future studies will benefit from cutting-edge pharmacological and optogenetic tools to acutely and specifically manipulate neural activity and study the impact on barrier function.

Does BBB permeability modulate neuronal functions?

The blood contains many molecules that can act on cells in the brain parenchyma and be detrimental to neurons if they pass through the BBB. Unsurprisingly, then, breakdown of BBB integrity has often been found to correlate with neural dysfunction and behavioural phenotypes[138,139]. Direct experimental evidence is available, for example, in recent work demonstrating that infusion of albumin into the parenchyma results in neural hyperexcitability[90,140]. However, barrier function also controls nutrient and waste exchange with the parenchyma, so decreases in permeability may also have considerable effects on neural activity.

For instance, recent work in mice has shown that EC-specific ablation of *Slc7a5* (which encodes a transporter of neutral amino acids) results in an altered metabolic profile in the brain[141]. These mice display autism spectrum disorder (ASD)-like behaviour (including reduced social interactions), which was rescued by intraventricular injection of leucine and isoleucine, thus bypassing the BBB[141]. Importantly, there are known mutations in the human *SLC7A5* gene that are associated with ASD[141]. Likewise, reductions in the levels of GLUT1 can have pathological effects on neural function and are associated with Alzheimer disease. Haploinsufficiency of the gene that encodes GLUT1, *Slc2a1*, at the BBB also results in abnormal TJs and BBB leakage[142], which may exacerbate possible reductions in energy availability with additional leakage of molecules such as fibrinogen.

Similarly, MFSD2A functions to transport phospholipids from the outer leaflet to the inner leaflet of the plasma membrane in brain ECs, including omega-3 fatty acids such as docosahexaenoic acid, which is critical for brain development. Enrichment of these unsaturated phospholipids changes the plasma-membrane lipid composition, which in turn inhibits the formation of caveolae and thus suppresses non-specific fluid transcytosis across the BBB[25,143]. Known human mutations in *Mfsd2a* are associated with microcephaly, and in mice and zebrafish, loss of MFSD2A results in microcephaly, cognitive impairment and BBB breakdown[25,143–146]. Furthermore, EC-specific knockouts of *Mfsd2a* also result in microcephaly, highlighting the importance of MFSD2A at the BBB in regulating the specific transport of nutrients crucial for brain function during development[146]. Epistasis experiments show that double knockout of *Cav1* and *Mfsd2a* rescues the BBB leakage but not the microcephaly[26], suggesting the gross neural pathology observed in *Mfsd2a*-knockout animals may be due to impairment of the nutrient transport that is crucial for neuronal survival during early development, and that BBB leakage resulting from *Mfsd2a* knockout is not due to neuronal defects. It will be interesting to acutely knockout *Mfsd2a* in adult animals when neuronal survival is no longer critically dependent on transport of these essential fatty acids.

Aside from nutrient exchange, changes in BBB function can result in permeability to signalling molecules that influence neural function. Emerging evidence has demonstrated that variation in BBB permeability between individuals can affect their susceptibility to neurological and psychiatric diseases. For example, the resilience to social stress among wild-type laboratory mice correlates positively with levels of various BBB-related proteins, including claudin 5 (REF.[147]). In stress-susceptible mice, social stress led to the BBB becoming more permeable in the nucleus accumbens and hippocampus. Notably, transient reduction of claudin 5 levels via short-hairpin RNA in stressed mice exacerbated depressive-like behaviour, suggesting that the BBB dysfunction is directly causative for the neuronal phenotype[147].

Stress-susceptible mice also showed substantially higher levels of the neuropoietic family member interleukin-6 (IL-6) than did resilient mice[148], raising the possibility that BBB disruption allows leakage of this chemokine into the parenchyma, where it alters neuronal function and thus behaviour. Indeed, direct infusion of IL-6 into the nucleus accumbens resulted in increased stress susceptibility[147], whereas *Il6*-knockout mice are more resilient to stress-related depression[149]. As mice globally lacking claudin 5 still show restricted paracellular leakage of molecules the size of IL-6 (REF.[50]), it is not clear whether TJ disruption is what causes the observed leakage in these animals.

It has also recently been demonstrated in mice that maternal inflammation triggered by injection of the immune stimulant polyinosinic:polycytidylic acid results in increased neural activity specifically in the primary somatosensory cortex of the pups, and ASD-like behaviour in these animals via increased IL-17 signalling[150]. Notably, this phenotype was observed only if the injection was performed at E12.5, before the BBB has fully matured, but not at E15.5, after functional BBB formation[23,25]. This suggests that the timing of maternal inflammation relative to functional BBB formation in the fetus might contribute to the offspring's susceptibility to neurological and psychiatric disorders.

Polyinosinic:polycytidylic acid
Synthetic mimic of double-stranded RNA mimicking the effect of viral infection on the immune system.

The blood carries many neuroactive molecules, including growth factors and cytokines, that normally cannot permeate the BBB. At the same time, the BBB specifically facilitates passage of nutrients into, and waste products out of, the parenchyma. Changes in BBB permeability, therefore, stand to have considerable effects on neural function, as described above. Moreover, the nature of this effect will be critically dependent on the spatiotemporal dynamics of BBB permeability.

Future directions

It has long been appreciated that maintenance of CNS homeostasis is highly dependent on the tight restriction of the entry of molecules from the circulation. However, although generally increased barrier permeability correlates with various pathologies, the healthy BBB is not a monolithic passive barrier. Rather, transit of various molecules through the BBB occurs in specific regions of the brain and during certain periods of development. It will be interesting to learn the functional importance of these modulations of barrier function. For instance, EC TJs are functional throughout CNS angiogenesis, but transcellular leakage is repressed only later; what role then, if any, does the transcellular leakage have during this period? And more generally, are vascular proliferation and BBB function necessarily antagonistic processes?

Besides the examples discussed here, there are several other emerging lines of evidence suggesting modulation of BBB permeability throughout life. For example, sleep and circadian mechanisms seem to regulate barrier permeability, at least in invertebrate models[151,152], and it will be very interesting to see whether and how these findings apply to mammals. However, when BBB permeability correlates with neural activity, it can be unclear whether BBB opening causally changes neural activity or vice versa, or whether there is just a correlation. Furthermore, BBB permeability and blood flow can feed back onto one another, particularly in ageing. The development of sensitive real-time assays of BBB permeability[153] to accompany real-time imaging and stimulation of neural activity may help shed light on this relationship.

NVC

The ability of neural activity to increase local cerebral blood flow — that is, NVC — has been recognized for more than a century[154]. In vivo studies have demonstrated that NVC is rapid, with changes in blood flow occurring less than a second after neural activity[155–157], and that the vessel responses can occur hundreds of microns to millimetres from the centre of neural activation[155,156]. Despite decades of investigation, the mechanisms underlying NVC and the extent of spatiotemporal correlation between dynamics of neural activity and vascular responses have remained poorly understood. Recent advances have provided insights into the complexity of NVC and its involvement of coordinated crosstalk among neurons, astrocytes, mural cells and ECs[155,158,159]. Here we highlight the studies that have informed our understanding of the cellular and molecular mechanisms underlying NVC as well as some experimental caveats (BOX 3).

What executes NVC?

In the brain's vascular network, pial arteries descend into the brain parenchyma, narrow and branch into penetrating arterioles and eventually branch into a dense network

Box 3 | **Experimental caveats of studying NVC**

Although the mechanisms underlying neurovascular coupling (NVC) has been intensely investigated, there have been conflicting results, which may be attributed to four experimental caveats. Here we contextualize these caveats by highlighting studies investigating the role of astrocytic calcium in NVC.

Ex vivo versus in vivo

Many studies use ex vivo preparations, including acute brain slices and isolated vessels, to study NVC[238]. However, such preparations do not account for many of the physiological dimensions of NVC, as they lack the vascular tone associated with the blood flow and pressure in an intact brain, neural and vascular connections are severed and tissue is immersed in an artificial composition of nutrients for viability. Cumulatively, these conditions can result in haemodynamic responses different from those observed in vivo, including delaying NVC kinetics from hundreds of milliseconds in vivo to minutes ex vivo. For example, calcium signalling via ITPR2 was shown to be required for arteriolar dilation on glutamate stimulation in brain slices[239], but in vivo imaging in *Itpr2*-null mice found that, following sensory stimulation, arteriolar dilation persisted, even though increases in astrocytic calcium were abolished[192–194]. Thus, *Itpr2* is dispensable for NVC in vivo.

Anaesthesia versus awake

As some experimental manipulations in studies of NVC in vivo are invasive, anaesthetics are often used. The mechanisms of many anaesthetics are poorly understood but they can affect neural[240], astrocytic[241] and vascular[242,243] functions, and different anaesthetics have different effects on NVC[244–246]. Furthermore, anaesthetized and awake subjects display different NVC responses[246–248]. Sensory-evoked NVC responses in awake mice are larger and faster than those in anaesthetized mice[249]. Anaesthesia may explain the confounding kinetics of astrocytic calcium release and vasodilation, as increases in astrocytic calcium preceded vasodilation in anaesthetized mice, whereas the reverse was true in awake mice[157,187]. Thus, anaesthesia could substantially confound interpretations of NVC studies, and future studies should confirm findings in awake subjects.

Artificial versus natural stimuli

Many studies used artificial stimuli to evoke NVC, including calcium uncaging, implanted electrode stimulation and pharmacological agents[187,238]. However, it is imperative to assess whether these artificial stimuli are reflective of normal physiology, especially when their use produces conflicting conclusions. For example, in anaesthetized mice, light-mediated calcium uncaging in astrocytes induces robust vasodilation[187], whereas increasing astrocytic calcium through the use of Gq-associated designer receptors exclusively activated by designer drugs (Gq-DREADDs)[250] did not evoke vasodilation[192]. Moreover, vasodilation evoked by a physiological stimulus preceded increases in astrocytic calcium in anaesthetized mice[192] and awake mice[157]. Although artificial stimuli are invaluable and necessary to obtain a mechanistic understanding of NVC, cross-examinations with natural stimuli are needed too.

Pharmacology versus genetics

Studies have used pharmacology to examine the molecular mechanisms underlying NVC. Although researchers can use pharmacology for acute perturbations, it often lacks molecular and cellular specificity. Furthermore, owing to the BBB, delivery and bioavailability of drugs are challenging in vivo. These caveats could explain why conclusions obtained with use of pharmacology conflict with some results obtained with use of genetics. For example, astrocytes were suggested to synthesize prostaglandins via cyclooxygenase 1 (COX1) to trigger vasodilation following increases in intracellular calcium, because antagonizing COX1 using SC-560 impaired vasodilation following a stimulus[187]. However, sensory-evoked NVC was reported to be unaffected in anaesthetized *Cox1*-null mice[191]. Instead, genetically ablating or pharmacologically antagonizing COX2 results in impaired sensory-evoked NVC[174,180]. Although SC-560 inhibits COX1 less potently than it does COX2 (REF. [251]), the studies that used this compound may have used a sufficiently high concentration to also block COX2. Brain RNA sequencing studies found that the gene encoding COX1 is expressed at low levels in astrocytes but is robustly expressed in interneurons and microglia[4,169], which could confound the alleged contribution of astrocytes in NVC[187]. Although pharmacology is invaluable for understanding NVC mechanisms, future studies should verify findings using genetics.

of capillaries[160]. Arteries and arterioles constitute only a small fraction of the entire brain vasculature and can, themselves, be far from active neurons. Nevertheless, they are the only vascular segments ensheathed by contractile SMCs, which canonically have the ability to regulate cerebral blood flow[156,161–163]. There is also evidence that pericytes can be contractile and control blood flow by regulating capillary diameter[164–166]. Notably, compared with aged wild-type controls, aged, moderately pericyte-deficient mice exhibit reduced capillary vasomotion in response to stimulus without reductions in neural activity. These mice also display BBB leakage and increased cerebral hypoxia[165]. Similar blood-flow deficits are seen in moderately pericyte-deficient mice before these animals display other, more overt pathologies[167]. However, the contribution to NVC of pericyte-mediated regulation of capillary diameter has been controversial, and recent studies have challenged this model, reporting that capillaries fail to dilate on neural activity[162,163].

Part of this controversy may stem from inconsistent criteria used to distinguish SMCs from pericytes, and arterioles from capillaries. Classically, these criteria have been morphological, including vessel size, branch order in the vascular tree or shape of the mural cells covering the vessel[162,168]. Unfortunately, all these distinctions become imprecise at precapillary arterioles[162]. A molecular definition of capillaries and arterioles (and therefore pericytes and aSMCs) may alleviate some confusion.

Studies using unbiased single-cell RNA sequencing have already adopted the presence and absence of smooth muscle actin (SMA, encoded by *Acta2*) expression to cluster brain SMCs and pericytes, respectively[4,169]. Notably, prior work shows cells with canonical SMC morphology display positive staining for SMA, whereas canonical pericytes do not[168]. Furthermore, transcriptomic studies have also identified other potentially unique molecular markers to further define the various mural cells, such as *Tagln* and *Myh11* for SMCs and *Abcc9* and *Kcnj8* for pericytes (TABLE 1). Going forward, these may be preferable to NG2 or PDGFRβ, which have been used to identify pericytes but are also expressed by SMCs[164,165,170]. TABLE 1 also highlights two reagents, Hydrazide 633 (REFS[156,161]) and NeuroTrace 525 (REF.[163]), that have been serendipitously shown to label large arteries and pericytes, respectively. Importantly, they can be used in live animals, presenting an attractive in vivo avenue to accurate identification of arteries and capillaries.

Using molecular criteria to define mural cells, Hill et al. examined the precapillary arteriolar vessels and showed that although some morphologically hybrid mural cells express SMA, the adjacent downstream mural cells can lack SMA despite being on the same vascular branch[162,163]. Furthermore, SMA$^+$ mural cells are also found on vessels with diameters as small as 3 µm and on fourth-order vascular branches downstream of pial arteries[162,163] — morphological criteria that Hall et al. used to classify pericytes and capillaries[164]. Using in vivo two-photon imaging of sensory-evoked neural activity, Hill et al. found that, regardless of morphological criteria such as cell shape, vessel diameter or vessel branch order, only SMA$^+$ mural cells dilate, whereas SMA$^-$ mural cells failed to display vasomotion[162].

As described in the section 'The BBB', intersectional genetic strategies can be used to specifically target pericytes[78]. Using such an approach, Nikolakopoulou et al. showed that specific ablation of ~60% of pericytes results in decreased baseline cerebral blood flow. In follow-up work, Kisler et al. found that these mice with acute pericyte reduction have reduced capillary vasomotion but not reduced arteriole vasomotion[166]. The extent of the role of pericytes in acute vasomotion, the sensing of neural activity, signal propagation and the fine-tuning of vascular tone should be further investigated in consistent, molecularly defined studies.

Finally, although the field has generally used mural cells to categorize the various vascular segments, it has comparatively ignored ECs even though ECs undergo distinct genetic programmes for arterial–venous and capillary differentiation, which influences the differential mural cell recruitment to arteries, capillaries and veins[171]. Consequently, ECs from different vascular segments also display different functions and transcriptomes.

Molecular and cellular mechanisms

NVC begins with increased neural activation that eventually induces vasodilation and increases blood flow. Although the signals that induce NVC have been investigated, broad questions remain unresolved. Can any activated neuron induce vasodilation, or are there specific subtypes of neurons that mediate NVC? Is NVC regulated by general or specific neural signals? Do neurons directly signal to SMCs, or do they signal to other cell types that then communicate to SMCs? Here we review and discuss the evidence that helps to address these questions.

Which neurons induce NVC? Given that activation of excitatory neurons in the cortex elicits an increase in net neural activity, many studies have investigated the contribution of excitatory neurons to NVC. Optogenetics and chemogenetics have enabled specific activation of excitatory neurons, confirming that their activation causes increased local blood flow. For example, activating channelrhodopsin expressed in excitatory neurons specifically (for example, in *Camk2*$^+$ or *Emx1*$^+$ neurons) increases local blood flow in anaesthetized rodents[172–174].

Inhibitory neurons tend to closely associate with blood vessels[175]. Specific activation of inhibitory neurons in the cortex (by targeting *Vgat*$^+$ neurons) using optogenetics also increased blood flow in anaesthetized and awake mice, despite causing a net decrease in neural activity[176,177]. Similarly, chemogenetic stimulation of parvalbumin-expressing neurons in the dentate gyrus leads to hyperaemia[133].

These findings highlight two interesting observations: that activation of both excitatory and inhibitory neurons can induce NVC and that NVC can occur despite a net decrease in overall neural activity. However, it remains undetermined whether all or specific subtypes of excitatory and inhibitory neurons can induce NVC[178]. Single-cell RNA sequencing has revealed that there are

at least 56 glutamatergic and 61 GABAergic subtypes of neurons in the mouse cortex[169]. Thus, future studies should determine whether specific subtypes of neurons and circuits differentially contribute to NVC.

What are the neural signals that induce NVC? By understanding the specific cellular players that mediate NVC, we can pinpoint the molecules released by neurons that can directly or indirectly communicate to arterioles to induce NVC. This will address whether NVC is induced by common signals released by all neurons or by specific neurotransmitters, neuropeptides and neuromodulators that are unique to specific subtypes of neurons. In support of the former hypothesis, studies have reported that signals released by all activated neurons, such as K^+, can induce vasodilation both ex vivo and in vivo[179]. Given that all neurons release K^+ during action potentials, these findings suggest that any neuron firing action potentials can elicit vasodilation. Although it is possible that increased extracellular K^+ elevates the excitability of specific neurons to then release other, vasoactive factors, increased extracellular K^+ was found to be sufficient to directly dilate isolated brain arterioles ex vivo[179].

By contrast, many studies have reported that specific neural signals induce NVC. Excitatory neurons express cyclooxygenase 2 (COX2) to generate prostaglandin E_2 (PGE_2), which putatively binds to EP2 receptors (encoded by *Ptger2*) and EP4 receptors (encoded by *Ptger4*) on SMCs, causing them to relax[174]. Consistent with this, single-cell RNA sequencing studies demonstrate that COX2 is expressed in excitatory neurons[4,169],

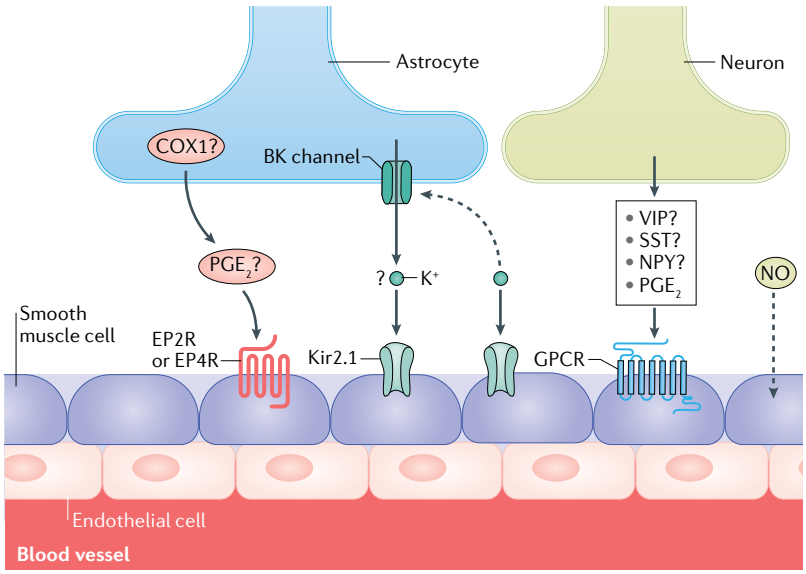

Fig. 3 | **The direct pathway to elicit neurovascular coupling.** Brain cells, including excitatory neurons, interneurons and astrocytes, can release vasoactive compounds directly onto the smooth muscle cells located near the surface of the brain. Potential neural signals, which may include prostaglandin E_2 (PGE_2), K^+ ions, vasoactive intestinal peptide (VIP), somatostatin (SST) and nitric oxide (NO), may act on or travel through putative receptors or channels on smooth muscle cells, including the prostaglandin EP2 and EP4 receptors (EP2R and EP4R), the inward-rectifier potassium ion channel 2.1 (Kir2.1) and G protein-coupled receptors (GPCRs). A question mark indicates some confounding evidence, as described in the text. Dotted arrows indicate hypothesized modes of action. COX1, cyclooxygenase 1; NPY, neuropeptide Y.

and *Cox2*-knockout mice have impaired NVC in response to sensory-evoked neural activity[180].

In addition, specific neurotransmitters, neuropeptides and neuromodulators, including vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), somatostatin (SST) and nitric oxide synthase 1 (NOS1), each of which defines various subtypes of inhibitory interneurons, have been implicated in modulating NVC ex vivo. Notably, however, in vivo evidence is still largely lacking. Bath application of VIP increases vasodilation in ex vivo brain slices, whereas NPY causes vasoconstriction[181–183]. However, *Vip*-knockout mice have not been studied to assess for defects in NVC. Similarly, although NPY has been implicated in vasoconstriction[177], *Npy*-knockout mice have yet to be assessed for NVC deficits. Finally, there are three NOS isoforms: NOS1 (also known as neuronal NOS), NOS2 (also known as inducible NOS) and NOS3 (also known as endothelial NOS). Although all three enzymes use L-arginine to synthesize nitric oxide (NO), a potent vasodilator, the expression of these isoforms varies across tissues and organs.

Nos1 is expressed by multiple populations of neurons throughout the cortex[169,184]. Surprisingly, early work analysing global *Nos1*-knockout mice found they have normal sensory-evoked NVC in the barrel cortex as assessed under anaesthesia by laser Doppler flowmetry[185]. By contrast, subsequent work in the same genetic system found impaired sensory-evoked NVC in the cerebellum[186]. Additionally, acute short interfering RNA-mediated knockdown of *Nos1* in the dentate gyrus results in substantially reduced NVC in awake mice[133]. Inducible, conditional deletion of *Nos1*, or additional genetic ablation of other NOS isoforms, could resolve potential compensation issues and demonstrate the role of NOS1 in NVC.

Do neurons signal directly to SMCs or indirectly via other cell types? Do excitatory and inhibitory neurons release vasodilatory signals directly onto SMCs (FIG. 3), or do these neurons first release signals to other cell types, which then release vasoactive cues to SMCs? In support of the former scenario, excitatory pyramidal neurons express COX2 to metabolize arachidonic acid to PGE_2, which is released onto SMCs to target EP2 and EP4 receptors to promote relaxation[174]. However, both EP2 and EP4 receptors are undetectable in SMCs by single-cell RNA sequencing[4,169] but are expressed in GABAergic interneurons[4,169]. Thus, excitatory neurons might release PGE_2, which binds to EP2 receptors or EP4 receptors on interneurons, which then release vasoactive cues onto SMCs.

Analogously, certain subtypes of interneurons have been reported to secrete vasoactive compounds directly onto SMCs[159,183]. In particular, the role of VIP in NVC in vivo remains unclear. Specifically, the receptors for VIP — VPAC1 and VPAC2 — are expressed at only low levels in SMCs and are instead found mainly in excitatory neurons[4,169]. Cell type-specific deletion of these receptors in SMCs will help elucidate the role of VIP signalling in NVC in vivo.

A recent study found that interneurons release NPY to enhance the vasocontraction phase of NVC in vivo[177].

Pharmacological antagonism of NPY type 1 receptor (NPY1R; encoded by *Npy1r*) attenuated the magnitude of the contraction[177]. Although single-cell RNA sequencing demonstrates that *Npy1r* is expressed by SMCs, it is also expressed by excitatory and inhibitory neurons[4,169]. Thus, pharmacological approaches may antagonize NPY1R molecules on neurons and indirectly impair NVC. A cell type-specific deletion of *Npy1r* would determine the role of NPY signalling in SMCs.

Many studies have reported that neurons first signal to astrocytes, which then signal to SMCs to mediate NVC. Excitatory pyramidal neurons release glutamate, which binds to metabotropic glutamate receptor 1 (mGluR1) and mGluR5 on astrocytes. This leads to the opening of inositol trisphosphate receptors on the endoplasmic reticulum to elevate intracellular calcium levels in astrocytes, activating COX1 to metabolize arachidonic acid to PGE_2 (REF.[187]) and opening BK channels (encoded by *Kcnma1*) to release K^+ — which hyperpolarizes SMCs[188], triggering NVC. However, the role of these astrocytic molecular players in triggering NVC has been controversial. First, mGluR1 and mGluR5 were claimed to be essential for NVC, as blocking them impairs NVC as assessed by laser Doppler flowmetry in vivo[189]. However, a later study demonstrated that astrocytes in adult rodents lack these receptors[190]. Next, the requirement for COX1 in NVC has been questioned, as COX1-null mice have normal NVC as assessed by laser Doppler flowmetry[191]. Furthermore, the role of astrocytic calcium in NVC has also been controversial. *Itpr2* encodes the predominant inositol trisphosphate receptor in astrocytes, and three studies showed that although *Itpr2*-null mice have abolished calcium release in astrocytes, their arteriolar dilation on sensory-evoked neural activity remains intact in vivo[192–194]. Last, although astrocytes express the BK channel, its role in NVC is unclear, as knocking out *Kcnma1* does not impair NVC on sensory-evoked neural activity[195].

Moreover, there are also temporal discrepancies between the increase in astrocytic calcium and arteriolar dilation. Although many studies found that the increase in astrocytic calcium precedes arteriolar dilation[187,189], a recent study showed that the onset of astrocytic calcium release occurs after, and is triggered by, arteriolar dilation[157]. Collectively, these studies do not provide clear conclusions about the role of astrocytes in NVC. However, it is still possible that astrocytes may signal to SMCs via other mechanisms independently of calcium and the molecular players discussed here to mediate NVC.

How is NVC sensed? Previous NVC models postulated that neurons and astrocytes release vasodilatory factors onto the SMCs of penetrating arterioles, causing them to relax and dilate[196]. However, changes in neural activity occur deep in the brain parenchyma within capillary beds, whereas SMCs surround only upstream arterioles, up to 200 µm away. It is unlikely that locally generated factors, such as NO, can diffuse over this long distance in a few hundred milliseconds to elicit a vasodilatory response in such a short time frame[155]. Moreover, such a diffusive mechanism would stand to lose the spatial specificity of vasodilation. Thus, the previous models did not account for the spatiotemporal realities of NVC dictated by the anatomy of the vascular network.

Recent evidence suggests ECs play a pivotal role in NVC[197], with capillaries in deeper cortical layers sensing neural activity (FIG. 4). Capillary ECs are ideal for sensing neural activity because they are deep in the brain and close to all neurons[1]. One study found that brain capillary ECs express the potassium channel Kir2.1 and therefore

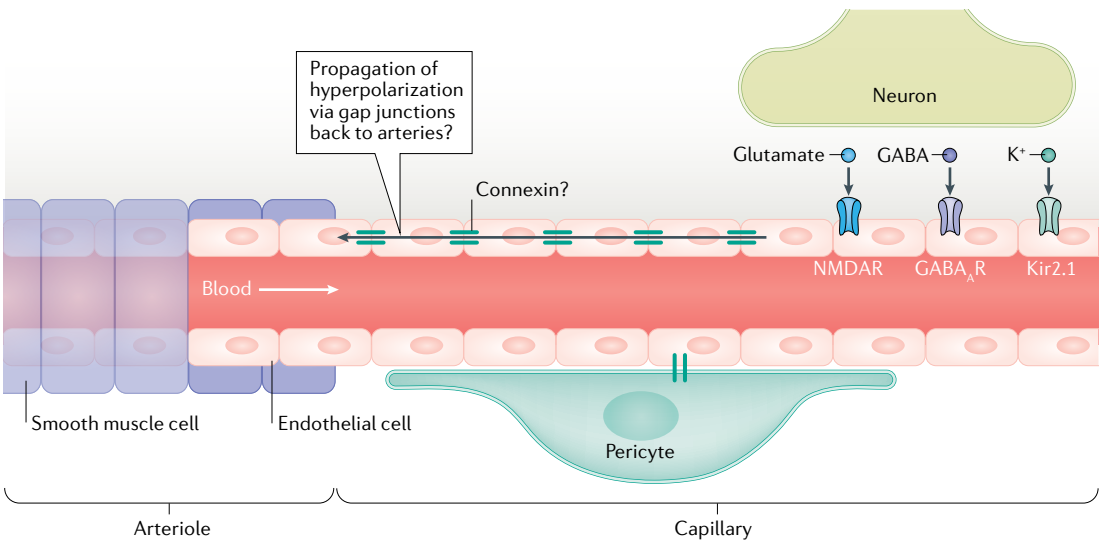

Fig. 4 | **The endothelium-dependent model of neurovascular coupling.** Most brain cells are juxtaposed to capillaries and can release neural signals, including glutamate, GABA and K^+, to capillary endothelial cells, which express the corresponding neurotransmitter receptor and ion channels such as NMDA receptors (NMDARs) and GABA type A receptors ($GABA_AR$s) as well as the inward-rectifier potassium ion channel 2.1 (Kir2.1). The occurrence of neural activity may be signalled via a retrograde propagation of hyperpolarization through putative gap junctions between capillary endothelial cells to upstream arteries, to dilate and increase blood flow. A question mark indicates some confounding evidence, as described in the text.

can sense increases in extracellular potassium generated during neural activity; indeed, mice lacking Kir2.1 in ECs showed attenuated NVC[179]. Other studies have also reported that brain ECs express neurotransmitter receptors, including those for glutamate[125] and GABA[198]. Conditional genetic ablation of the *Grin1*-encoded subunit of the NMDA receptor in ECs attenuates sensory-evoked NVC[199]. Although conditional genetic ablation of GABA type A receptors ($GABA_A$ receptors) in ECs impairs brain vascularization and interneuron migration and leads to behavioural defects, NVC effects have not been examined[200]. Nevertheless, collectively, these studies support the notion that capillary ECs are the ideal sensors of neural activity.

How is NVC propagated? In addition to acting as sensors of neural activity, ECs have been proposed to 'retrogradely' propagate this information up the vascular tree to dilate upstream arterioles, via electrical coupling[156,179,197]. As the spread of electrical signals across ECs is fast and can traverse long distances, this model fits the spatiotemporal constraints inherent to NVC. Indeed, peripheral ECs are electrically coupled via gap junctions and can rapidly conduct signals between ECs. These waves of ions, including potassium and calcium ions, trigger EC release of vasodilatory factors onto SMCs[201]. A similar mechanism may exist in the brain vasculature, as micropipette application of 6–10 mM potassium to capillaries generated robust hyperpolarization in ECs that was transmitted retrogradely to penetrating arterioles at an estimated speed of 2 mm per second[179]. Many have speculated that, like peripheral ECs, the brain ECs are also coupled by gap junctions, allowing the spread of electrical hyperpolarization up the vascular tree from capillaries to arteries. However, the evidence for gap junction coupling in brain ECs has been scarce. Most vascular gap junction studies have been performed in the peripheral vasculature[202–206] but very few studies have examined the role of gap junctions in the brain vasculature[207], especially in the context of NVC in vivo. Furthermore, studies examining the role of gap junctions in the cerebral vasomotion mostly used putative gap junction blockers[208,209], which have been repeatedly demonstrated to have non-gap-junction-related effects[210,211]. These effects include blocking ion channels such as $GABA_A$ receptors[212] and, more pertinently, endothelial small- and intermediate-conductance calcium-activated K^+ channels (IK and SK channels), which are implicated in NVC[213]. Thus, the role of gap junctions and the identities of the connexins that form them in NVC remain unknown. Given the advent of new genetic and imaging tools, it is finally possible to study the effect of gap junctions on NVC using cell type-specific deletion of their various connexin components.

How does the endothelium communicate to SMCs to mediate NVC? Once the electrical conductance travels from the capillary endothelium to the arterial endothelium, how do arterial ECs then communicate to the underlying SMCs to mediate NVC? A recent study demonstrated that the arterial ECs actively mediate NVC through cell type-specific mechanisms[214]. Unlike capillary endothelium, the arterial endothelium lacks MFSD2A, and arterial ECs have abundant caveolae, which relay signals to SMCs to mediate arterial dilation[214]. Indeed, arterial endothelium-specific ablation of caveolae impairs vasodilation during NVC. Furthermore, the caveola-mediated pathway is independent of the NOS3-mediated pathway, as ablation of both caveolae and NOS3 completely abolished NVC, whereas single ablation of *Cav1* or *Nos3* resulted in partial impairment, demonstrating that a caveola-mediated pathway in the arterial endothelium is a major contributor to NVC[214]. Future work should investigate the mechanism by which caveolae facilitate signalling to SMCs to promote dilation.

Future directions

NVC is a complex process involving the coordination of multiple cells and feedback cycles. The cellular players and molecular determinants involved in NVC have been intensely investigated. As highlighted in this Review, many NVC studies have resulted in incomplete conclusions. We believe that with the development of novel technologies to investigate molecular and cellular mechanisms in vivo, the field should revisit the old dogmas of NVC. Additionally, new genetic tools[78,214] for perturbing NVC will allow for precise exploration of the effects of impaired NVC on neuronal function. This will lead to substantial breakthroughs in our understanding of the sequence of events underlying the mechanisms of NVC. Insights gleaned from future mechanistic NVC studies could also facilitate the development of novel therapeutics to enhance cerebral blood flow in disease, as well as better interpretation of the BOLD signal that is crucial for human brain-imaging studies.

Conclusions

The work discussed in this Review demonstrates the tight interplay between neural activity and neural vasculature. As many diseases of the nervous system and ageing are associated with dysfunction of both the BBB and NVC, a clearer mechanistic understanding of this interplay in health and disease will be crucial for the development of new therapies.

The complexity of the NVU has historically made studying the physiology of the BBB and NVC daunting. The NVU has considerable heterogeneity and zonation, and is composed of several highly related but functionally distinct cell types. Fortunately, recent technical advances have helped push past these hurdles and stand to clarify mechanistic questions in BBB regulation and NVC. For instance, single-cell sequencing and multiplexed fluorescent in situ hybridization have helped to distinguish which cell types express molecules important for BBB function and NVC. This, in combination with cell type-specific gain-of-function and loss-of-function genetics, is a powerful tool for elucidating mechanisms. Additionally, the increasingly large optogenetic, chemogenetic and biosensor toolkits provide a means of acutely controlling and detecting activity in live animals. These approaches stand to answer many long-standing questions in the BBB and NVC fields.

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