OPINION

# Engaging neuroscience to advance translational research in brain barrier biology

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Abstract | The delivery of many potentially therapeutic and diagnostic compounds to specific areas of the brain is restricted by brain barriers, of which the most well known are the blood–brain barrier (BBB) and the blood–cerebrospinal fluid (CSF) barrier. Recent studies have shown numerous additional roles of these barriers, including an involvement in neurodevelopment, in the control of cerebral blood flow, and — when barrier integrity is impaired — in the pathology of many common CNS disorders such as Alzheimer's disease, Parkinson's disease and stroke.

There is a commonly held notion that the blood–brain barrier (BBB) is a simple anatomical structure that restricts the traffic of molecules in and out of the CNS but otherwise is not very relevant to neuroscience. This view is flawed however, as brain barrier sciences and neuroscience are inextricably linked in many areas of neurophysiology and neuropathology.

The BBB is one of a number of blood–CNS interfaces, which also include the blood–cerebrospinal fluid (CSF) barrier, the blood–retinal barrier, the blood–nerve barrier and the blood–labyrinth barrier — all of which are important for the physiological functions of the CNS (FIG. 1). Among these interfaces, the BBB occupies by far the largest surface area. A wide range of neurological conditions such as Alzheimer's disease[1–3], Parkinson's disease[4], multiple sclerosis[5,6], trauma[7,8], brain tumours[9,10], stroke[11–14] and epilepsy[15] are associated with perturbations in the normal BBB that contribute to their pathology (TABLE 1). Furthermore, the cells that constitute the BBB play a part in the control of cerebral blood flow[16,17] and neuronal development[18]. Thus, it is important to recognize that the BBB has many other roles and is not simply a control point for molecular trafficking in and out of the brain.

The common wisdom has been that the BBB consists of endothelial cells and is either open or closed depending on the status of tight junction proteins that create a restrictive, fixed barrier. We now know that the BBB is in fact dynamic, with a wide permeability range that is controlled by intra- and intercellular signalling events among endothelial cells, astrocytes and neurons in the BBB (and other cells that are in contact with the BBB), as well as by paracellular changes at the BBB. A further key conceptual advance has been the discovery that the BBB is an integral part of the neurovascular unit (NVU)[19] (FIG. 1a).

The complex regulation of barrier properties is far from understood. Gaining better insight into the physiological and pathophysiological processes that alter intra- and intercellular junction protein distribution and function is important for understanding how the barrier can be fixed when it does not function properly and how it can be manipulated for therapeutic purposes. Suffice it to say, herein lies the opportunity for interdisciplinary approaches to expand our knowledge and improve strategies for treatments of neurological disorders.

Many factors have contributed to the lack of interaction between neuroscientists and brain barrier scientists, including the complexities of each field and the numerous gaps in our understanding of the BBB. This, in turn, has resulted in relatively little emphasis on brain barrier science as an interdisciplinary topic or an educational objective, whereas greater emphasis might facilitate such communication. Recent advances have increasingly demonstrated the common ground between the two fields of study and the urgency for crosstalk. The present article provides a vision for future study that integrates both disciplines, highlighting areas of relevance and convergence (BOX 1).

## Physiology of the NVU

The NVU consists of an endothelial cell monolayer (connected by tight junctions and resting on the basal lamina), integral neighbouring cells (including pericytes and smooth muscle cells) and astrocytic endfeet covering >98% of the vascular wall and occasional neuronal terminals. The astrocytes also extend processes that surround synapses and can thereby link neuronal activity with the oxygen and nutrient supply. Finally, components of the NVU include the circulating blood cells, such as polymorphonuclear (PMN) cells, lymphocytes and monocytes that adhere and roll along the vascular lumen and perform surveillance of neural signalling and cellular activity[20] (FIG. 1a).

The endothelial cells of the NVU are highly polarized, with different integral membrane proteins at the luminal and abluminal surfaces. These include various receptors, enzymes and transporters that support the functions of this cellular barrier within the NVU. For example, the endothelial barrier performs vectorial transport of solutes — including ions, nutrients and drugs — at the blood–brain interface. It also engages in highly specialized interactions with blood cells, through specific luminal receptors, and with elements of the basal lamina and underlying cells (for example, astrocytic endfeet and neuron terminals) at the abluminal

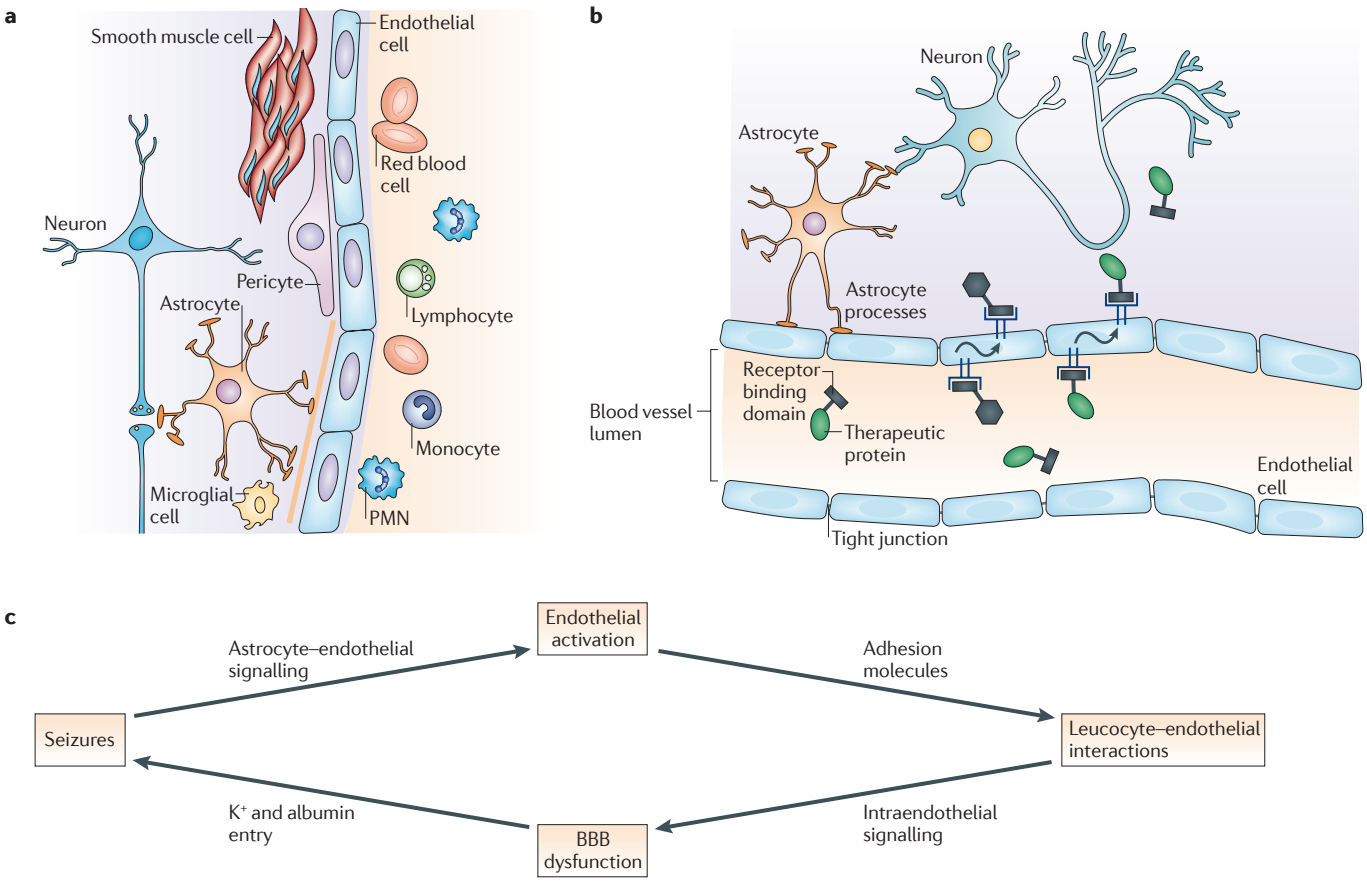


Figure 1 | **The extended neurovascular unit. a** | The blood–brain barrier (BBB) is an essential part of the neurovascular unit (NVU). A classical view of the NVU incorporates neurons, glial cells such as astrocytes and microglial cells closely juxtaposed with vascular endothelial cells, pericytes and smooth muscle cells. Blood cells, particularly polymorphonuclear (PMN) cells, lymphocytes and monocytes, also interact with the BBB endothelium and are therefore an integral part of this unit. The interactions between these cellular components and inter- and intracellular signalling regulate NVU function to maintain homeostasis, or to respond to inflammation and disease. **b** | Receptor-mediated transcytosis of proteins at the BBB. Transcytosis is a receptor-mediated transport mechanism by which proteins that are targeted to the CNS bind extracellular receptors in vascular lumen, are transported across the BBB endothelial cells, and are released in brain parenchyma. The presence of specific receptors (for example, the insulin receptor) on the surface of BBB endothelial cells has allowed targeting and transport of some therapeutic proteins to the CNS[44,128]. **c** | Pathological signalling in the extended NVU. The proposed sequence order is based on data available from the epilepsy field[144] and requires further exploration in the context of other brain diseases, including stroke and Alzheimer's disease. The cycle starts with altered expression of vascular cell adhesion molecules and interactions of leucocytes with the endothelium, initiating intraendothelial signals that alter BBB function and lead to neural tissue dysfunction as a consequence of $K^+$ and albumin entry into the brain interstitium. Astrocytes detect the altered neuronal activity and transmit signals back to the BBB, thereby facilitating interactions with leucocytes and turning the sequence into a vicious circle that maintains and exacerbates the pathological state. The activated endothelium may, as an integral part of the extended NVU, disturb neuron–astrocyte interactions, thereby adding an additional layer of pathological signalling to the process. Astrocytes emerge from this cascade as a primary target for interventions that aim to interrupt the proposed cycle.

surface, through specific abluminal plasma membrane proteins. Furthermore, astrocytes and pericytes possess their own complement of transporters, channels, receptors and signalling mechanisms with which they coordinate the role of the NVU in supporting nervous system function.

***The tripartite synapse and the NVU.*** A major notion that has emerged from neuroscience over the past few years is the concept of the 'tripartite synapse', which has compelled neuroscientists to consider the influence of glia in synaptic function[21,22]. In the cerebral microcirculation, tripartite synapses composed of pre- and postsynaptic endings, together with their related glia, are structurally and functionally related to the brain's capillary bed, and together form the NVU (FIG. 1a). The role of the NVU interface in the context of the tripartite synapse is just beginning to be understood. The cellular components include the endothelium (forming the barrier proper at the capillary level), astrocytic endfeet, pericytes and circulating immune cells, which are adjoined at some distance by nerve endings and vascular smooth muscle cells found at the arterial level[19,23] (FIG. 1a,b). It should be noted that immune cells indeed sneak into the NVU and are defined below as part of the extended NVU.

The NVU, together with the basal lamina and extracellular matrix components, engages in complex signalling processes (fast and slow; active and trophic). Documented examples include the propagation of $Ca^{2+}$ waves through the NVU[24], neuro–metabolic coupling[25], neuro–haemodynamic coupling, neuro–angiogenic coupling, and neuro–trophic coupling[26,27] and cell adhesion-based signalling networks[28]. These NVU signalling processes are also linked to membrane transport[29–33], regulation of cellular permeability (specific or

Table 1 | **Diseases that affect, or that are affected by, the blood–brain barrier (BBB)**

| Disease or process | BBB proteins and mechanisms affected | Refs |
|---|---|---|
| ***Neurodegenerative diseases*** | | |
| Alzheimer's disease | RAGE products — influx of amyloid-β | 163 |
| | LRP1 multi-ligand lipoprotein receptor — efflux of amyloid-β | 163 |
| | P-glycoprotein (also known as ABCB1) is reduced at the BBB and seems to play a crucial part in clearing amyloid-β from the brain | 60,164 165 |
| | Changes in ABCG2 are related to cerebral amyloid angiopathy and control BBB transfer of amyloid-β | 166 |
| Parkinson's disease | Polymorphism in P-glycoprotein drug transporter *MDR1* gene association and *ABCB1* gene encoding the P-glycoprotein | 135,141 |
| ***Cerebrovascular diseases*** | | |
| tPA- and reperfusion-induced haemorrhage | MMPs released by neutrophils and possibly endothelial cells degrade tight junction proteins and basement membrane — increased risk of haemorrhage | 167–170 |
| VEGF-mediated BBB breakdown | Occludin and claudin 5 — downregulation of mRNA and protein | 171 |
| Familial cerebral cavernous malformations | CCM1 (also known as KRIT1), CCM2 or CCM3 (also known as PDCD10) localized in endothelial cells and perhaps astrocyte endfeet — venous malformations with bleeding | 172,173 |
| Ischaemic brain oedema | BBB breakdown due to MMP9 release by neutrophils —degradation of occludin, claudins, junctional adhesion molecule (JAM) family proteins and basement membrane; SUR1 (also known as ATP-binding cassette subfamily C member 8)-regulated non-selective cation channel NC(Ca-ATP) mediates ischaemic cerebral oedema | 174–177 |
| Acute mountain sickness and high-altitude cerebral oedema | Vasogenic oedema | 120 |
| ***Epilepsy and seizures*** | | |
| Epilepsy and exercise-induced dystonia | GLUT1 mutations in brain endothelial cells | 136,137, 139 |
| Resistance to pharmacotherapy in some patients with epilepsy | Multidrug efflux pumps from the ABC superfamily (for example, P-glycoprotein) at the BBB | 79,178 |
| Alexander's disease — large brain, seizures and retardation | GFAP mutations — BBB abnormalities | 179 |
| Leukoencephalopathy with epilepsy | ClC2 is a broadly expressed plasma membrane chloride channel — epilepsy, white matter degeneration and retinal degeneration in mice | 143 |
| ***Infections*** | | |
| HIV entry into brain | HIV activation of STAT and RHO kinase downregulate claudin 5, ZO1 and ZO2 in endothelial cells, which may increase HIV entry | 180–182 |
| Susceptibility to certain types of brain infections (for example, malaria and CNS listeria moncytogenes) | P-glycoprotein (also known as MDR1A andABCB1) deficiency at BBB — susceptibility to cerebral malaria; *opc* gene in *Meningococcus* produces protein that binds HBMECs via α5β1 integrin receptors on fibronectin | 183–185 |
| CNS infections in general | Pathogens hijack BBB cellular machinery to enter the brain | 185 |
| NeuroAIDS in HIV | BBB efflux systems keep out antivirals from thebrain, fostering neuroAIDS | 186 |
| Malaria | Effects protein expression and permeability of human endothelial cells selectively in the brain | 125 |
| ***Neuroinflammation and brain tumours*** | | |
| White blood cell oxidative stress causes adhesion to endothelium and transmigration across BBB | White blood cell proteins (such as selectins, VLA4, CD44 and α4β7 integrin) and brain endothelial proteins (such as selectin ligands, ICAM1, VCAM1 and CD44) mediate migration across BBB. | 32,187, 188 |
| $CD8^+$ cytotoxic T cell-mediated BBB breakdown and oedema | Perforin release degrades tight junction proteins | 189 |
| Brain oedema — tumour, inflammation and others | Aquaporins (astrocyte endfeet) | 190,191 |
| Brain oedema — role of steroids. Prednisone and dexamethasone decrease BBB leakage in acute multiple sclerosis plaques, tumours and other pathologies | Steroids act on glucocorticoid response elements on promoters of tight junction genes (occludin, claudins and cadherin) to increase tight junction proteins and increase BBB tightness | 192–194 |

Table 1 (cont.) | **Diseases that affect, or that are affected by, the blood–brain barrier (BBB)**

| Disease or process | BBB proteins and mechanisms affected | Refs |
|---|---|---|
| BBB breakdown in multiple sclerosis: monocyte–endothelial interactions induce tPA in endothelial cells | tPA induction of ERK1 and ERK2 in endothelial cells mediates monocyte transmigration across BBB and control breakdown of occludin | 195 |
| BBB breakdown in multiple sclerosis: role of IL-17 and IL-22 | Helper T lymphocytes ($T_H17$ cells) release IL-17 and IL-22 that act on receptors on brain endothelial cells that results in degradation of tight junction proteins and opening of the BBB | 196,197 |
| Prevent leukocyte trafficking across the BBB — decreased multiple sclerosis relapses | Monoclonal antibody to α4 integrin (Natalizumab) — adhesion molecule on leukocytes necessary to attach to and cross the BBB in EAE | 198–200 |
| Inflammatory pain — cytokines mediate BBB breakdown | Downregulation of occludin and claudin 5 | 201 |
| ***Metabolic and psychiatric diseases*** | | |
| Brain oedema — associated with diabetic ketoacidosis and cerebral ischaemia | Na-K-Cl cotransporter, and Na–H exchanger at BBB | 202–207 |
| Adrenoleukodystrophy — abnormal white matter in brain with a wide range of neurological findings; retinal degeneration | ABCD1 gene mutation — ATP binding cassette disorder; ABC superfamily | 140,142 |
| Obesity — leptin released from adipose tissue and binds to leptin receptor to modulate food intake | Deficient BBB transporter protein function — reduced leptin transport across the BBB | 208–210 |
| Imerslund–Gräsbeck syndrome familial vitamin B12 malabsorption — dementia and white matter abnormalities | Amnionless mutations — possible vitamin B12 transport into brain | 211 |
| Canavan's disease — large brain, seizures, retardation, white matter degeneration and other signs | Mutations in aspartoacetylase lead to accumulation of *N*-acetylaspartate | 212 |
| Mucopolysaccharidosis | Loss of the GUSB transporter with maturation underlies difficulty in treatment | 213 |
| Depression | Polymorphisms in the drug transporter gene ABCB1 predict antidepressant treatment response in depression | 214 |
| Hepatic encephalopathy | Affects potassium homeostasis in astrocytes, produces swelling and disrupts control of extracellular potassium | 215–217 |

ABCB1, ATP-binding cassette, subfamily B, member 1; ABCG2, ABC transporter G family member 2; CCM1, mitochondrial group I intron splicing factor CCM1; CIC2, chloride channel protein 2; EAE, experimental autoimmune encephalomyelitis; ERK1, mitogen-activated protein kinase 3 (also known as extracellular signal-regulated kinase 1); GFAP, glial fibrillary acidic protein; GLUT1, solute carrier family 2, facilitated glucose transporter member 1; GUSB, beta-glucuronidase; HBMEC, human brain microvascular endothelial cell; ICAM1, intercellular adhesion molecule 1; IL-17, interleukin-17; LRP1, low-density lipoprotein receptor-related protein 1; MDR1, multidrug resistance protein 1; MMP, matrix metalloproteinase; *opc*, class 5 outer membrane protein; RAGE, advanced glycosylation end product-specific receptor; STAT, signal transducer and activator of transcription protein family; SUR1, sulfonylurea receptor 1; VCAM1, vascular cell adhesion protein 1; VEGF, vascular endothelial growth factor; VLA4, integrin alpha 4; ZO1, tight junction protein ZO1.

selective regulation, or through paracellular pathways)[32,34,35] and intracellular metabolic cascades[25,36,37].

***Dynamic regulation of brain barrier permeability by the NVU.*** Cells of the NVU form a complex and fine-tuned transport machine that balances the influx of nutrients and the efflux of wastes, toxins and drugs to maintain CNS homeostasis[38]. Numerous factors regulate the barrier permeability of the NVU, including modulation of membrane transporters and transcytotic vesicles, and modulation of transcellular permeability[34] (FIG. 1b).

The importance of investigating NVU transport proteins is underscored by the recent finding that 10–15% of all proteins in the NVU are transporters[39]. In 2003 it was estimated that only about 50% of brain barrier transporter proteins had been identified[39,40]. Since then, several new transporters have been detected and localized in the brain endothelium and the choroid plexus (which forms the blood–CSF barrier). The identity and cellular location of multiple — generally efflux — transporters that are present in the NVU and that function possibly in drug transport are shown in FIG. 2. New neuroscience discoveries include structural and mechanistic insights into coupled transporters (for example, GABA and norepinephrine transporters (sodium- and chloride-dependent GABA transporter (GAT) and sodium-dependent noradrenaline transporter (NET) family proteins), excitatory amino acid transporters (EAATs)[41] and ATP-binding cassette (ABC) transporters[42]. Improved understanding of the physiological and biophysical mechanisms underlying transport function[43–46] should be applicable to both general brain function and dysfunction in disease.

Ion transporter proteins in cells of the NVU play an important part in maintaining fluid balance in the brain, and our understanding of their role in water and electrolyte movement among cells of the NVU has recently been expanded. These studies largely focused on whole brain and/or hypoxia in neurons and astrocytes, and led to the discoveries of a family of $HCO_3^-$ transporters, the electrogenic sodium bicarbonate cotransporter (NBCe) and electroneutral sodium bicarbonate cotransporter 3 (NBCn) families, and electroneutral sodium-driven chloride/bicarbonate exchanger 1 (NDCBE)[47–49]. Expression levels of these transporters vary with brain region and cell type, with prominent expression of both NBC and NDCBE transporters reported for neurons and choroid plexus, and little or no expression in astrocytes. Little is known about expression and function of the NBC and NDCBE transporters in cells of the cerebrovasculature.

Recent studies have provided important new insights regarding the role of aquaporins in astrocytic endfeet[50] and the control of water distribution within the brain[51–55], and have shown that abnormal fluid dynamics

Box 1 | **A meeting of minds**

In an effort to bring the two fields of neuroscience and brain barrier science closer, an international panel of experts was assembled in March 2009 to discuss current areas of overlapping interests in which the expertise and observations of one group might advance the research progress of the other. Leaders in the fields of neuroscience and brain barrier science identified five topics as central to advancing the treatment of CNS disorders; these included molecular physiology of the brain and brain barriers; intercellular communication within the neurovascular unit (NVU); transport biology in the brain and brain barriers; neurodevelopment and the brain barriers; and imaging the structure, function and dynamics of the brain and brain barriers.

Within each topic, four main questions were addressed: what are the key scientific opportunities in the neuroscience field that may be applied to the brain barriers field and vice versa? What is the status of the science in the topic, including key scientific advances made in the respective fields over the past 4 years, and are they relevant to the other field? What are the barriers to progress in the topic? What are the highest-priority recommendations for developing and advancing knowledge in the topic, including the key resources and approaches needed?

For each of the five key topics, a panel of experts, co-chaired by renowned neuroscientists and brain barrier scientists, drafted reports answering the four primary questions as well as addressing the key question, 'What is the single most important issue that would advance research in each topic area?' The draft reports were discussed among approximately 150 neuroscientists and brain barrier scientists at the 2009 Annual Blood–Brain Barrier Consortium Meeting in Oregon, USA (see Supplementary information S1,S2 (boxes)). The co-chairs and working groups incorporated into their final reports the discussion and input from the combined group of scientists (see Supplementary information S3 (box) for the final reports from each of the five topics).

or aquaporin malfunctions may have pathological consequences[56]. Several aquaporins are found in the brain including aquaporin 1 (AQP1), AQP3, AQP4, AQP5, AQP8 and AQP9, with AQP1, AQP4 and AQP9 most heavily studied. Whereas AQP9 is found in the astrocyte cell body, AQP4 is abundant in perivascular astrocyte endfeet and also where the astrocyte is in close apposition to neurons. Choroid plexus exhibits AQP1 and AQP4, and endothelial cells of the NVU appear to have minor amounts of AQP4 at best. Pathways by which water moves through the endothelial cells of the NVU are largely understudied. A relatively recent finding is that that $CO_2$ and $NH_3$ conductances are regulated by AQP1 and AQP4 (REF. 57). This has created a paradigm shift in the way that we think about how metabolically relevant gases move through the NVU — that is, that diffusion of the gases across plasma membrane is not by simple diffusion but rather, by facilitated diffusion via the aquaporins.

It has long been accepted that the NVU functions as a selective barrier to various substances passing between blood and brain, but these new discoveries have led to a more developed understanding of the NVU, which recognizes the NVU as a functionally complex blood–brain interface with multiple, interacting roles.

## The NVU as a barrier to xenobiotics

One of the most important roles of the NVU is to limit xenobiotics, including CNS drugs, from entering the brain. This barrier function is mainly achieved by two components in the brain capillary endothelium — ATP-driven membrane transporters known as 'efflux transporters' and tight junctions that 'seal' spaces between endothelial cells.

***Signalling pathways that regulate efflux transporters.*** Transporter-mediated export of xenobiotics can affect the pharmacokinetics and pharmacodynamics of a large number of therapeutics, and poses a challenge for the ability to deliver drugs into the CNS. Direct transporter inhibition has been pursued as one strategy, but it leaves little control over the extent and duration of the inhibition. Accordingly, transporter inhibitors are currently not in clinical use. Recent efforts have therefore focused on targeting the intracellular signalling pathways and molecular switches that control efflux transporter regulation, for several reasons. First, modulating these pathways and switches would allow fine-tuning of transporter activity so that transporters can be turned off for controlled periods, thus providing a time window to deliver drugs[58]. Second, such strategies could be used to upregulate expression and activity of efflux transporters in the NVU to minimize brain side effects associated with the treatment of a disease in the periphery (for example, 'chemobrain' in cancer patients)[59]. Third, efflux transporters are affected by — and likely contribute to — disease pathology of CNS disorders that are accompanied by inflammation, oxidative stress and neurotransmitter release, and that include cancer, epilepsy and Alzheimer's disease[60–62]. Thus, understanding the signalling pathways that regulate efflux transporter expression and activity is likely to be useful for improving CNS drug delivery, protecting the brain during systemic treatment and preventing pathogenesis or slowing the progression of several CNS diseases. For example, three major pathways have been identified that regulate P-glycoprotein (Pgp), a major efflux transporter at the BBB that limits brain penetration of therapeutic drugs[63]. One pathway is triggered by the inflammatory mediator tumour necrosis factor α (TNFα), which signals through tumour necrosis factor receptor superfamily member 1A (TNFR1), resulting in release of endothelin 1 (ET1). This in turn signals through the endothelin B receptor (ETBR), resulting in signalling through nitric oxide synthase and protein kinase Cβ (PKCβ) to alter Pgp expression and function[58,64–66]. Indeed, activating PKCβ reduced Pgp activity and enhanced delivery of small molecule therapeutics into the brain[58].

A second pathway involves the neurotransmitter glutamate, which signals through the NMDA receptor, cyclooxygenase 2 (COX2) and the prostaglandin E2 receptor EP1 to upregulate Pgp expression and activity[67–70]. Inhibiting this pathway prevents seizure-induced Pgp upregulation and improves brain penetration of anti-epileptic drugs and reduces epileptic seizures[71].

The third pathway involves activation of xenobiotic-sensing nuclear receptors, such as the aryl hydrocarbon receptor (AHR), the glucocorticoid receptor, the pregnane xenobiotic receptor (PXR) and the constitutive androstane receptor (CAR)[72–78], to regulate transporter expression. In a recent study, for example, activation of PXR has been used to restore brain endothelial Pgp in an Alzheimer's disease mouse model, which resulted in enhanced amyloid-β clearance from the brain[60]. In addition, several of the signalling pathways have common elements (for example, TNFα, nuclear factor κB (NF-κB) and COX2) that may be potential therapeutic targets.

Thus, findings from studies using physiological and pathophysiological modulators, pharmacologic inhibitors and activators of efflux transporters may be useful for improving the delivery of drugs into the brain, protecting the brain from harmful xenobiotics and alleviating CNS disorders[79,80]. In addition to studying brain barrier transporters, understanding the molecular regulation of tight junctions may provide therapeutic opportunities in diseases in which endothelial barrier integrity is disrupted[81].

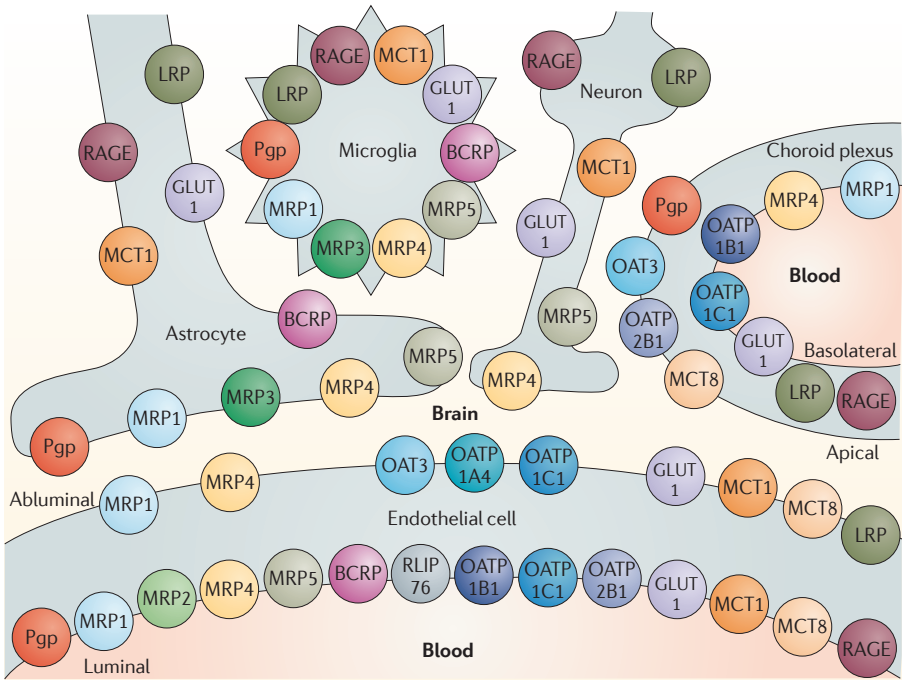


Figure 2 | **Primary transporters in the neurovascular unit.** The spatial and cellular relationships of the transporters are shown. Only proteins detected at the protein level are depicted. For a more complete listing of carrier-mediated transport systems at the blood–brain interface see Ohtsuki and Terasaki[38]. BCRP, breast cancer resistance protein (also known as ABC transporter G family member 2); GLUT, solute carrier family 2, facilitated glucose transporter member; LRP, low-density lipoprotein receptor-related protein family member; MCT, monocarboxylic acid transporter family member; MRP, multidrug resistance-associated protein family member; OAT, organic anion transporter family member; OATP, organic anion transporter polypeptide family member; Pgp, P-glycoprotein; RAGE, advanced glycosylation end product-specific receptor; RLIP76, Ral-binding protein 1. Figure is modified, with permission, from REF. 161 © (2011) Bentham Science.

*Role of tight junctions in NVU function.* In the brain capillary endothelium, tight junctions that seal the spaces between neighbouring endothelial cells represent a passive barrier that restricts paracellular diffusion of water-soluble solutes, including drugs, from blood to brain. The role of tight junctions and their key constituent proteins, claudins and occludins, in the regulation of barrier function is beginning to be elucidated. However, it is not yet known how these and other proteins interact to create the highly effective and precisely regulated tight junction. For example, although genetic ablation of claudin 5 has shown that this tight junction protein is necessary to limit movement of small molecules into the brain[82], other studies have shown that claudin 5 is expressed in all endothelial cells, not just those in the NVU. Conversely, occludin is brain endothelial cell-specific but is not required for barrier function[83]. Thus, the molecular basis for the tightness of the cerebrovascular endothelium compared to endothelia in most other tissues remains unknown.

## NVU and the control of cerebral blood flow

Communication between neurons and glial cells — especially astrocytes[84] — in response to electrical and synaptic activity can influence cerebral blood flow. This occurs under conditions of physiological levels of neuronal activity[85], strong or pathological stimuli[86] and spontaneous activation[87]. Astrocytic calcium signals propagate to astrocytic endfoot extensions that are in contact with blood vessels and also extend to neighbouring endfeet[88], thereby triggering the release of vasoactive messengers[89–91] and altering local cerebral blood flow. Neuroimaging techniques such as functional MRI use these changes in cerebral blood flow as an indicator of CNS activity.

A key finding is that astrocytic endfeet release vasodilatory as well as vasoconstrictive messengers and this depends on the availability of oxygen: if oxygen availability is low, vasodilators prevail, whereas if oxygen availability is high, vasoconstrictors predominate[92]. Vasoactive messengers released by astrocytes include arachidonic acid, prostaglandin members of the epoxyeicosatrienoic acid family and nitric oxide as vasodilators, and ET and OH-eicosatetraenoic acid products as vasoconstrictors[17]. As astrocytes make extensive contacts with smooth muscle cells at the arteriolar level[93], vasotropic actions of astrocytic calcium signals may also affect smooth muscle cells directly or indirectly via brain endothelial cells (which in turn secrete vasoactive substances such as nitric oxide)[89].

The observation that calcium can act as a signal in astrocytic endfeet located at the blood–brain interface suggests that these signals may influence endothelial cells at distances of less than 0.1 μm and thus, could cause dynamic alterations in BBB function[25]. Clearly, the astrocyte is a major communication link between the multiple parts of the NVU.

## Neurodevelopment of the brain barriers

Research on vascular and neuronal development has been converging over the past decade — for example, in the study of angiogenesis in fetal brain. There are at least two major reasons for this: first, there is evidence for shared molecules and coordinated cellular mechanisms during the development of these systems[94,95]; and second, there is evidence that neurogenesis and angiogenesis are co-regulated in embryonic and adult brains[94,96,97].

The CNS vasculature develops by angioblastic invasion of the head region that occurs in early phases of embryogenesis, and this vasculogenic process establishes the extracerebral vascular plexus that eventually covers the entire surface of the neural tube[98–100]. After the primary vascular plexus is formed, further vascularization of the CNS is exclusively achieved by angiogenesis from the perineural vascular complex. Driven by metabolic demands of the expanding neuroectoderm, capillary sprouts invade from the extracerebral vascular plexus toward the periventricular zone[101]. Once formed, the nascent brain vasculature is further stabilized by the recruitment of mural cells and the formation of the extracellular matrix, and is fine-tuned by microenvironmental cues from the neighbouring cells[102,103]. Through this process of maturation all the components of the brain vascular network acquire the phenotype that allows them to form a fully differentiated NVU.

It has been known for decades that there is a functional NVU well before the middle of the 150-day gestation of sheep[104–106], and the existence of tight junctions during brain development has also been noted in various other species, including humans, as summarized elsewhere[107]. Over the past 5 years,

unequivocal evidence has been published of both structural and functional barriers in the developing brain. In fact, studies using small molecular weight markers have shown that functionally effective tight junctions are present as soon as blood vessels begin to penetrate the early CNS parenchyma and as soon as epithelial cells of the choroid plexuses begin to differentiate[108,109].

These tight junctions provide the basis for selectivity of barrier interfaces. Efflux transporters (for example, Pgp, breast cancer resistance protein (also known as ABC transporter G family member 2) and multidrug resistance proteins), which can reduce the accumulation of drugs and toxins in the brain, are expressed in cerebral endothelial and choroid plexus epithelial cells early in development[110–112]. A recent study reported that pericytes are required for BBB integrity during embryogenesis[113]. Specifically, the data indicated that pericyte–endothelial cell interactions regulate some properties of the BBB during development, and disruption of these interactions may lead to BBB dysfunction and thus, to neuroinflammation as part of the response to CNS injury and disease[113].

The functional role of the brain barriers during development is to provide the brain with a specialized internal environment. As shown in FIG. 3, one major barrier difference is that the neuroependyma lining the cerebral ventricles constitutes a barrier during early development but not at later times, when it has become the adult ependyma. The molecular properties[114] and specific functions of the brain barriers alter as the brain matures, to reflect its changing role, influenced by the surrounding neural environment and its intrinsic developmentally regulated properties. In addition, the vasculature interacts with the neural environment — this includes shared molecular processes that influence the growth and maturation of the brain at specific stages of its development[94]. Several key studies have identified important CNS parenchymal cell-derived molecular signals, including angiotensinogen and Wnt, that seem to regulate the formation and function of the cerebrovasculature and thereby the NVU[18,27,94,104,115].

In addition to being essential for angiogenesis, Wnt and β-catenin signalling seems to be essential for expression of cerebral endothelial cell-specific transporters such as solute carrier family 2, facilitated glucose transporter member 1 (SLC2A1; also known as GLUT1), high affinity cationic amino acid transporter 1 (SLC7A1; also known as CAT1) and large neutral amino acids transporter small subunit 1 (SLC7A5; also known as LAT1), but not tight junction molecule, including occludin and tight junction protein ZO1 or pan-endothelial molecules including platelet endothelial cell adhesion molecule (PECAM) and vascular endothelial cadherin[27]. The finding that Wnt regulates CNS-specific angiogenesis and induces specific NVU properties, such as gene expression and restricted permeability[27,94], suggests that CNS angiogenesis and brain barrier formation are linked by Wnt regulation and mutual interactions. The similarity of some immune and neural molecular mechanisms during development might also have implications for vascular development of CNS barriers, but this has so far remained unexplored. Combining vascular and neuronal developmental approaches to tackle questions that relate to brain barrier development promises to unravel the poorly understood mechanisms of barrier development, as has recently been reviewed[94]. The blood–CSF barrier seems to be especially important during development as the choroid plexuses are functional, possess protein specific transport mechanisms and restrict paracellular passage at a time in development when the brain parenchyma has low levels of vascularization[108,116,117].

## Glossary

**Abluminal**
Facing the neural cells or brain.

**Basal lamina**
A thin, continuous layer of extracellular matrix surrounding the brain endothelial cells and pericytes.

**Blood–cerebrospinal fluid (CSF) barrier**
The blood–CSF barrier is at the choroid plexus epithelial cells, which are joined together by tight junctions. The capillaries in the choroid plexus differ from those of the blood–brain barrier in that there is free movement of molecules between endothelial cells via fenestrations and intercellular gaps.

**Blood–labyrinth barrier**
The cochlea is a structure of the inner ear involved in sound transduction and is vascularized by a dense set of capillaries that are essential for delivering the nutrients and ions necessary for producing the fluids (endolymph and perilymph) present in the cochlea. These capillaries are lined with endothelial cells that are joined by tight junctions and physiologically form the blood–labyrinth barrier that is essential for sensitive auditory function.

**Blood–nerve barrier**
The endothelial lining of blood vessels in peripheral nerves is formed by continuous, non-fenestrated endothelia in which individual cells are linked by tight junctions, rendering them impermeable to intravascular macromolecules. This blood–nerve barrier, and a similar mechanism in the innermost perineurial sheath, isolate the endoneurial interstitium, in much the same way as the blood–brain barrier. Other factors, such as the absence of lymphatics, are also analogous to the central nervous system.

**Blood–retinal barrier**
The blood–retinal barrier has two components: the retinal vascular endothelium and the retinal pigment epithelium. The retinal vascular endothelium is non-fenestrated and has anatomical properties similar to those of cerebral vascular endothelium. The retinal pigment epithelium consists of a layer of epithelial cells, joined by tight junctions, that forms a barrier between the neuroretina and the choroid.

**Ependyma**
A thin cellular layer lining the ventricular system of the brain. The cells of the ependyma are called ependymal cells and are a type of glia. They are linked by gap junctions, which do not provide an impediment to diffusion of molecules, even against large proteins between cerebrospinal fluid and brain interstitial fluid.

**Luminal**
Facing the capillary lumen.

**Neuro–angiogenic coupling**
The coupling of the development of neurons (neurogenesis) with new blood vessel formation (angiogenesis and vasculogenesis).

**Neuroependyma**
(Also known as neuroepithelium or ventricular zone.) A deep pseudostratified layer of cells lining the embryonic ventricular system that proliferate into radial glial cells and neurons in the embryo, and into glial cells later in development. The cells of the neuroependyma are linked by strap junctions, which limit intercellular movement of molecules — particularly proteins — from cerebrospinal fluid to brain interstitial space in the embryo. By adulthood these cells have transformed to the layer of thin generally non-dividing ependymal cells lining the ventricular system of the mature brain.

**Neuro–haemodynamic coupling**
The coupling of neuronal firing and synaptic activity with haemodynamic changes (for example, blood volume and blood flow).

**Neuro–metabolic coupling**
The coupling of neural activity, an energy consuming process, with the energy producing metabolic processes to maintain cellular homeostasis.

**Neuro–trophic coupling**
The coupling of neuronal production of activity-dependent signals such as growth factors (for example, brain-derived neurotrophic factor (BDNF)) with control of neurogenesis.

**Paracellular**
Paracellular is used here to refer to the transfer of substances between cells of an endothelium or epithelium. It is in contrast to 'transcellular transport', in which the substances are transported through the cell.

**Tripartite synapse**
A tripartite (three-part) synapse consists of a presynapse, a postsynapse and a glial cell functioning as a single unit.

**Xenobiotic-sensing nuclear receptor**
A xenobiotic-activated transcription factor that controls the expression of proteins involved in xenobiotic metabolism and efflux transport.

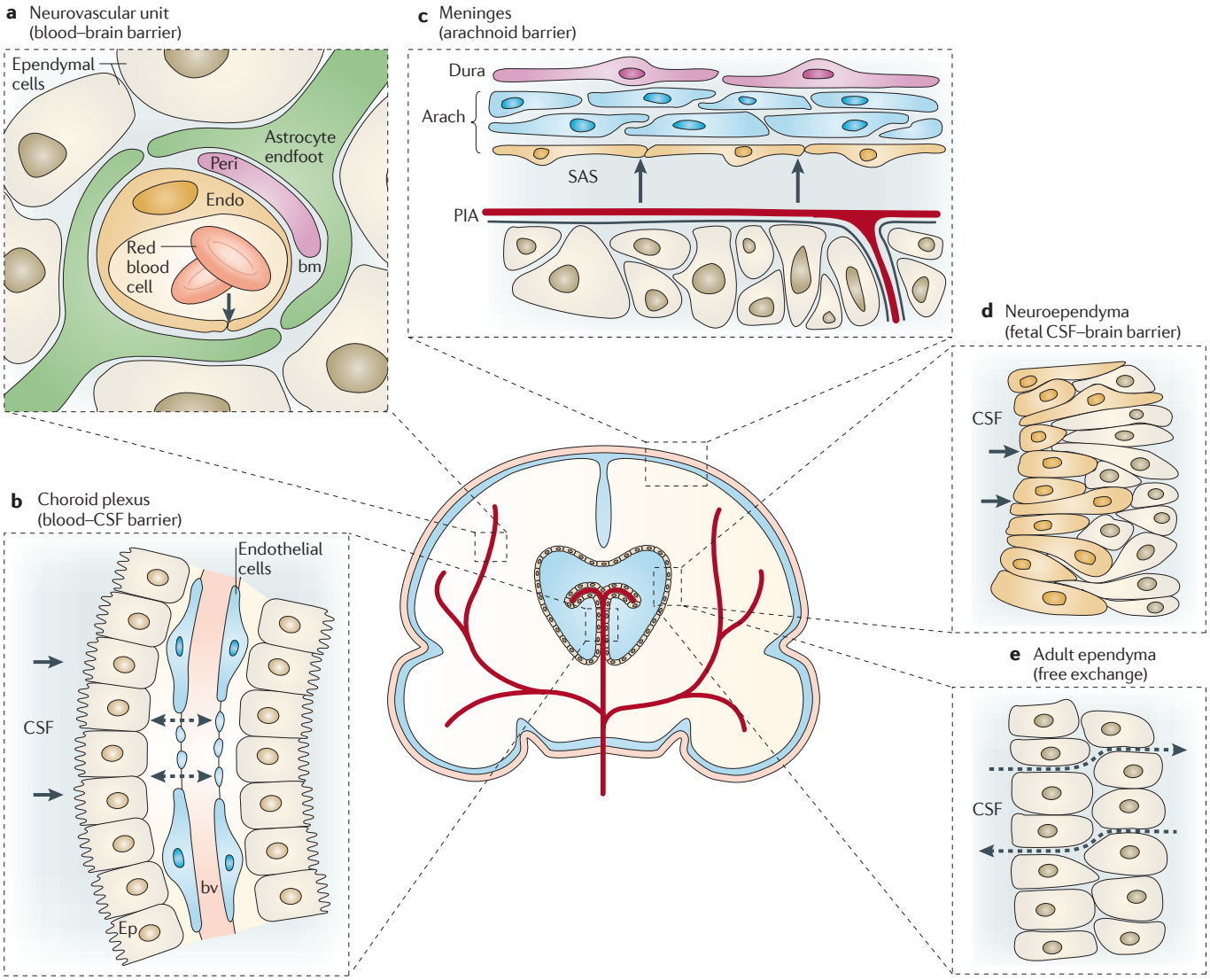


Figure 3 | **Barrier interfaces. a** | Endothelial cells (Endo) in the neurovascular unit have luminal tight junctions (shown by the arrow) that form the physical barrier of the interendothelial cleft. Outside the endothelial cell is a basement membrane (bm) which also surrounds the pericytes (Peri). Around all of these structures are the astrocytic endfeet processes from nearby astrocytes. **b** | The endothelial cells of choroid plexus blood vessels are fenestrated and form a non-restrictive barrier (shown by dashed arrows) between the cerebrospinal fluid (CSF) and blood vessel (bv). The epithelial cells (Ep) have apical tight junctions (shown by arrows) that restrict intercellular passage of molecules. **c** | In the meninges, the blood vessels of the dura are fenestrated and provide little barrier function (not shown). However, the outer cells of the arachnoid membrane (Arach) have tight junctions (shown by arrows) and this cell layer forms the physical barrier between the CSF-filled subarachnoid space (SAS) and overlying structures. The blood vessels between the arachnoid membrane and the pial surface (PIA) have tight junctions (not shown). **d** | In early development the neuroependymal cells are connected to each other by strap junctions (shown by arrows) that are believed to form the physical barrier restricting the passage of larger molecules, such as proteins, but not smaller molecules, such as sucrose. **e** | The mature adult ventricular ependyma does not restrict the exchange of molecules (shown by dotted arrows). The neurovascular unit (**a**), blood–CSF barrier (**b**) and arachnoid barrier (**c**) are common between developing and adult brain, whereas fetal neuroependyma (**d**) differs from adult ependyma (**e**). Figure is reproduced, with permission, from REF. 162 © (2008) Cell Press.

## The NVU in disease

The NVU is usually considered in the context of its role in preventing CNS access of drugs and proteins with neurotherapeutic potential. The NVU is also affected in many CNS conditions and plays a part in their pathology. Brain abscess, trauma, multiple sclerosis, diabetic ketoacidosis and stroke can all alter brain endothelial cell function and NVU function, with consequent oedema that may be life threatening. NVU abnormalities themselves can result in distinct disease entities (see TABLE 1). These disorders range from acute mountain sickness causing vasogenic oedema[118–120] and hepatic encephalopathy causing astrocyte swelling in the NVU[121,122], to malaria impacting the cerebral endothelium[123–125].

The complexity of the cellular interactions in the NVU offers numerous potential targets for treatment. For example, one of the newest treatments for multiple sclerosis is a monoclonal antibody that binds the α4 integrin receptor found on leukocytes and that prevents adhesion of the leukocytes to brain endothelial cells[126]. This reduces the effects of the leukocytes that lead to

the demyelination and brain injury seen in acute multiple sclerosis plaques. In addition, molecules found on the luminal side of brain endothelial cells are now recognized as receptors or ligands for proteins associated with various infectious microorganisms that have a predisposition to invade the brain, for example, malaria (see TABLE 1). Furthermore, the large number of transporters and receptors on the luminal and abluminal membranes of brain endothelial cells and astrocyte endfeet provide numerous potential therapeutic targets for treatment of CNS diseases[127]. One example is co-opting insulin receptors for transport of drugs across the NVU[128], a possibility that is now being examined[129,130].

Cerebral vascular abnormalities have been reported in brains from patients with Alzheimer's disease and Parkinson's disease[131], and human genetics studies have linked vascular phenotypes to amyloid precursor protein (APP)[132]. Specifically, a particular point mutation associated with hereditary cerebral haemorrhage with amyloidosis of the Dutch type (HCHWAD) leads to cerebral amyloid angiopathy (CAA) in both humans and transgenic mouse models[133]. CAA induces intracranial haemorrhages, with cognitive decline and seizures, which may also contribute to the pathology and symptoms of Alzheimer's disease. Patients who are amyloid-β A4 protein (APOE4)-positive, who carry the most common late-onset genetic risk factor for Alzheimer's disease, also have a higher rate of CAA[134]. Recent advances have also described an influx and efflux mechanism for amyloid-β, via advanced glycosylation end product-specific receptor (RAGE), low-density lipoprotein receptor-related protein (LRP1) and, more recently, Pgp[20,60]. These membrane proteins might be targets for modulating movement of amyloid-β out of the brain of patients with Alzheimer's disease to slow or reverse plaque formation. Although barrier-specific genes have not been implicated in directly inducing CNS disease, there are genes associated with barrier function that have been linked to disease[135–143].

Recent data from the epilepsy field indicate a prominent role of interactions of leucocytes, in particular PMN cells, with brain endothelium in the initiation of seizure activity[144]. This is a key observation that not only points to the importance of the BBB in initiating pathology but also stresses the need to consider the blood cells (leucocytes in this case) as members of the family of NVU cells. We therefore propose to use the term 'extended NVU' (FIG. 1a) to underscore the importance of blood cells and inflammatory signals as key players in disturbed NVU and barrier function. We further anticipate that astrocytes may play an active part to maintain disturbed NVU function, by feeding back pathological signals from the disturbed NVU to the BBB (FIG. 1c). Indeed, astrocytes as well as microglial cells are physiological sensors of brain function and pathology[145]. The work by Appel and colleagues[146], in which neuroprotective signals may cause microglia to become protective in axonal injury models, in Parkinson's disease and in amyotrophic lateral sclerosis (in contrast to the majority of the literature, in which microglia are damaging) further emphasizes the need for greater communication between neuroscientists and brain barrier scientists.

## Imaging brain barrier function

At the microscopic level, new imaging techniques, including confocal and time-lapse microscopy, which allow simultaneous tagging and visualization of multiple molecular targets, molecular imaging of brain cells *in vitro* and brain tissues *ex vivo*, have made tremendous advances in recent years[147]. Although imaging techniques at the atomic level and 'label-less' techniques such as Raman spectroscopy[148] have much improved resolution, they can only be used to detect one or a few molecular species simultaneously. With imaging mass spectroscopy (IMS), tissue sections can be directly analysed for the spatial distribution of multiple molecular markers[149]. IMS is a powerful method for *ex vivo* imaging biomarkers that define particular regions, or following 'biomarker' responses to disease, pharmacological treatment, electrical stimulation and so on[149].

Further, the field of bioimaging relying on confocal, multiphoton and spinning disk confocal microscopy has been enhanced through the use of fluorescent murine transgenic reporter systems, mostly using green fluorescent protein (GFP) as a reporter. These techniques have made great contributions to *in vivo* tracking of exogenously added cells (that is, tumour cells, immune cells and progenitor cells) and have gained popularity as proxy reporters for endogenous genes (that is, transgenic mice)[150–152]. These approaches have greatly enhanced our understanding of the trafficking of inflammatory cells across the BBB in models of ischaemic brain injury and autoimmune demyelination, among others[153]. In addition, the recent introduction of optogenetic approaches[154] will allow investigators to examine discrete neuronal signalling events in the context of the NVU, providing a degree of *in vivo* analytical power previously achievable only using *in vitro* systems. As brain vasculature is functionally implicated in many brain diseases (TABLE 1), molecular changes in the NVU could be exploited as imageable biomarkers for early diagnosis or monitoring of the disease using targeted molecular imaging agents[155]; the added advantage of such biomarkers is their accessibility from the systemic circulation.

At a macroscopic level, analysis of drug delivery to the CNS will be advanced by imaging technologies. For example, studies in animals using positron emission tomography (PET) indicate that it is possible to assess endothelial Pgp function, and its role in the uptake and binding of drugs in the intact CNS, by using suitable Pgp modulators that are labelled with a positron emitting isotopes[156] (FIG. 4). In fMRI, signal intensity changes are detected as changes in local blood flow and oxygenation, presumably linked to changes in neural activity. The opportunity exists to apply this technology (as well as other methods for imaging cerebral perfusion, for example, dynamic magnetic resonance) with nanoparticle-based brain mapping methods to advance our understanding of neuro–glio–vascular coupling and BBB pathophysiology[157]. As no single method can cover the several orders of magnitude in temporal and spatial resolutions and at the same time capture cellular and vascular events, one of the key opportunities to be harnessed in the future is a combination and integration of data and knowledge obtained through multimodal imaging techniques, such as MRI and PET.

## Barriers to progress

The most important barrier to progress in our understanding of the role of brain barriers in brain functioning is the lack of communication between neuroscientists and brain barrier scientists. This lack of communication contributes to the omission of the brain barrier sciences in interdisciplinary education programs, thus perpetuating the gap between the fields. If the relationships among neurons, astrocytes and cells of the NVU are to be fully appreciated, it is essential for researchers in both fields to expand their knowledge of the cellular and molecular mechanisms at play in all cells of the NVU, not just those of the endothelial cell (currently studied by brain barrier scientists) or neurons and astrocytes (currently studied

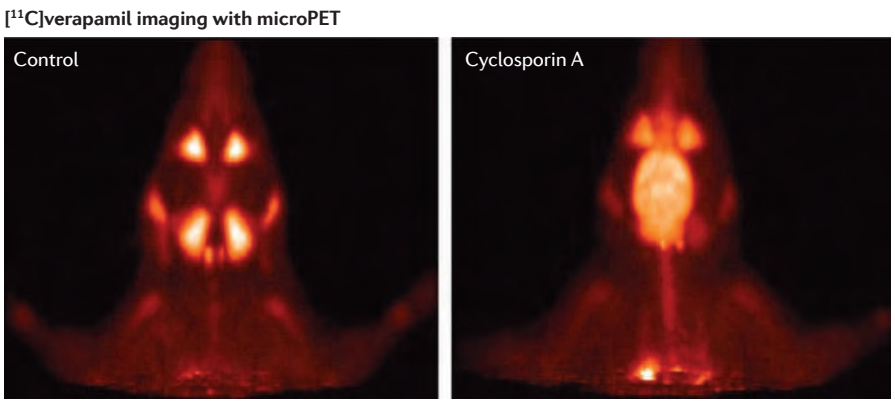


Figure 4 | **MicroPET images of the head biodistribution of a calcium channel blocker.** Micro positron emission tomography (PET) images of the head of a Wistar rat, showing the biodistribution of the calcium channel blocker [$^{11}$C]verapamil, injected systemically, either alone (control) or after pre-treatment of the animal with the P-glycoprotein (Pgp) inhibitor cyclosporin A. [$^{11}$C]verapamil, a substrate for the blood–brain barrier efflux transporter Pgp, gains access to the brain only after Pgp inhibition by cyclosporin A. Images courtesy of P. Elsinga, University Medical Center Groningen, The Netherlands.

by neuroscientists). This broader approach should include, for example, attention to all classes of membrane proteins involved in transport across the barriers and among cells of the NVU — such as ion transporters and channels, nutrient transporters, drug transporters — and to proteins that are involved in transport mechanisms (including receptor-mediated endocytosis) as well as the receptors and transduction pathways signalling to these proteins.

Because the endothelial cells are thin and tightly embedded within the brain parenchyma, they are not easily isolated for routine biochemical, molecular or cellular analysis. This has posed substantial technical difficulties that have delayed progress in the study of blood–brain interfaces. This interface is a highly interactive structure, in which endothelial cells engage with multiple neighbouring cells including pericytes, astrocytes, neurons and blood cells such as leukocytes. Brain endothelial cells are very flat cells, with a thickness of less than 0.5 μm outside the nuclear region, comparable in size to dendritic spines. The major technical difficulty here is the fact that the numerous cellular partners at the barrier interface interact with each other in a very thin compartment. As such, it is very inaccessible. In addition, barrier function can only be studied *in vivo* because blood cells and blood flow are now considered as essential elements for its normal function. The presence of an intact circulation is a technical difficulty for microscopic imaging studies because of the presence of mechanical vascular pulsations. We propose to apply highly specialized microscope imaging techniques based on two-photon excitation that have been employed to study dendritic functions and dynamics for investigating the functional interactions at the BBB interface.

Finally, there are misconceptions that need to be overcome, particularly with regard to the status of the NVU during development. A perception persists for some researchers in the field of brain barrier physiology, and therefore in the wider area of neurobiology, that the barrier systems are immature in the developing brain in both their structure and function. This misunderstanding of barrier function during neural development represents an impediment to a full understanding of the biological processes involved in barrier development and the contribution of barrier functions to neural development. The emphasis that is currently placed on understanding the role of the NVU in neuronal development, and recent evidence that vascular and neuronal development have common mechanisms, make a fruitful merger of fields possible. Similarly, there are misconceptions that the blood–brain interface is either open or closed in brain tumours[158], that is, opened as in systemic tissues or closed as in normal brain. In truth, cerebral microvessels coursing through most malignant brain tumours have intermediate paracellular permeability so that some drugs and proteins can move from blood into tumour[158]. However, recent evidence has shown that enhanced delivery of antitumour agents by further opening the blood–brain interface may improve survival in malignant brain tumour patients[159]. One key obstacle is the lack of efficacious yet non-invasive methods. Modelling animal models of the BBB in human diseases such as stroke is difficult since our clinical knowledge in patients over time is so limited. Improved models may open up new avenues to define opportunities and time windows for therapeutic interventions.

## Conclusions and future directions

In order to further the science in both fields, it is important that understanding what constitutes the functional blood–CNS interface under various physiological and pathophysiological conditions is paramount for developing appropriate therapies to address different disease states. New and improved animal models, including transgenic rodents, will be beneficial in achieving this aim. The use of zebrafish as an easily accessible comparative model for mechanistic *in vivo* studies should be further explored[160]. Investigations on the contribution of blood cells and inflammatory signals as part of the NVU are needed. Although blood cells and inflammatory signals are generally not considered part of the NVU proper, they must be considered part of the extended NVU and are important mediators of CNS pathophysiology and need further investigation. Furthermore, animal models will be useful with application of advanced microscopy tools for real-time and spatial resolution of cellular interactions, signalling events and metabolism.

Transporters, receptors and their signalling pathways in the NVU are important targets for improving CNS drug delivery and brain protection, and in preventing CNS disease. Advancing knowledge of transporter function, expression, localization and regulation in the brain vasculature and CNS tissues will surely aid progress.

Further consideration of the role of the NVU in research into nervous system development will likely continue to lend insight into both developmental neuroscience and the brain barrier sciences. There is also a need for improved animal models that are appropriate for studies investigating the links between barrier versus neural development. Furthermore, neuroscientists should consider the possible contributions of the NVU to the interpretation of their neuroscience data, including analysis of genetically engineered mouse models, drug efficacy studies and so forth. Acceptance of the recent evidence in support of barrier function in the developing brain, along with the advent of an increased research focus on the development of brain barriers will help to overcome impediments to progress in these fields.

The simultaneous and remarkable advancements in neuroscience and brain

barriers research over the past decade have followed relatively independent tracks. Because of the mutual interests in understanding the mechanisms underlying neural function and disease and in delivering therapeutics through the blood–brain interface, it is now becoming clear that these dual tracks must become one. A careful analysis of common interests, as reviewed here, indicates that many underlying biological principles and technical approaches in neuroscience apply to the brain barriers and vice versa. Further progress in both fields will be advanced by continued and greater crosstalk and collaboration among the respective scientists and clinicians in the brain barrier and neuroscience fields.

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### Competing interests statement
The authors declare no competing financial interests.

### FURTHER INFORMATION
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