The blood–brain barrier and blood–tumour barrier in brain tumours and metastases

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Abstract | For a blood-borne cancer therapeutic agent to be effective, it must cross the blood vessel wall to reach cancer cells in adequate quantities, and it must overcome the resistance conferred by the local microenvironment around cancer cells. The brain microenvironment can thwart the effectiveness of drugs against primary brain tumours as well as brain metastases. In this Review, we highlight the cellular and molecular components of the blood–brain barrier (BBB), a specialized neurovascular unit evolved to maintain brain homeostasis. Tumours are known to compromise the integrity of the BBB, resulting in a vasculature known as the blood–tumour barrier (BTB), which is highly heterogeneous and characterized by numerous distinct features, including non-uniform permeability and active efflux of molecules. We discuss the challenges posed by the BBB and BTB for drug delivery, how multiple cell types dictate BBB function and the role of the BTB in disease progression and treatment. Finally, we highlight emerging molecular, cellular and physical strategies to improve drug delivery across the BBB and BTB and discuss their impact on improving conventional as well as emerging treatments, such as immune checkpoint inhibitors and engineered T cells. A deeper understanding of the BBB and BTB through the application of single-cell sequencing and imaging techniques, and the development of biomarkers of BBB integrity along with systems biology approaches, should enable new personalized treatment strategies for primary brain malignancies and brain metastases.

Pericytes
Mural cells that regulate development, permeability and function of the microcirculation.

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The blood–brain barrier (BBB) regulates homeostasis of the central nervous system (CNS) by forming a tightly regulated neurovascular unit (NVU) that includes endothelial cells (ECs), pericytes and astrocytic endfeet, which together maintain normal brain function[1,2]. However, these same features also hinder the delivery of systemic therapies into brain tumours. The BBB is disrupted during tumour progression and is then referred to as the blood–tumour barrier (BTB). Although the BTB is more permeable than the BBB, its heterogeneous permeability to small and large molecules as well as heterogeneous perfusion contributes to suboptimal drug accumulation in brain tumours[3–6]. As such, the BBB is one of the rate-limiting factors in clinically effective therapy.

In this Review, we discuss how the genotype of brain tumours impacts BBB and BTB (hereafter referred to as BBB/BTB) structure and function, and elaborate on the role of the BBB/BTB in disease progression and treatment. We highlight emerging minimally invasive strategies to improve drug delivery across the BBB/BTB and discuss their potential to improve treatment. Finally, we present emerging insights from systems biology approaches and discuss the impact of the BBB/BTB on immunotherapy, along with recent developments in biomarkers to assess extravasation across the BBB/BTB that should facilitate personalized therapy.

The neurovascular unit

As early as the late 1800s, several studies demonstrated that the CNS vasculature actively maintains CNS homeostasis by tightly regulating molecular and cellular transport across its specialized endothelium[7]. Preclinical and clinical observations in the following decades helped characterize the structure of capillary beds in the neuroparenchyma, which is composed of ECs connected by tight junctions (TJs), surrounded by a specialized basal lamina that is shared with pericytes and astrocytic endfeet, and sparsely interconnected by neuronal endings and microglia[8]. Together, these cells dictate the CNS endothelial structure and define the physical properties of the BBB (FIG. 1). Within these cellular and extracellular networks, specifically regulated transport systems allow efficient efflux of toxic cellular byproducts to move

Astrocytic endfeet
Extensions of astrocytes into the perivascular space, covering the majority of the central nervous system endothelium and regulating development and function of the microcirculation.

back into the circulation while permitting regulated influx of circulating molecules essential for CNS function. Although the CNS is considered immunologically unique, some populations of immune cells can 'loosen' and cross the BBB during neuroinflammation, whereas others can repair damaged nervous tissue[9,10]. Together, the BBB endothelium and neuroparenchymal cells constitute the NVU, which acts as a 'gatekeeper' within the CNS that tightly controls transcellular and paracellular crossing of molecules and cells[10,11] (FIG. 1).

Defects in BBB integrity during development are linked to several neurodegenerative disorders[12]. Investigation of the developmental expression and function of specific transcription factors, TJ proteins, angiogenic factors and growth factors during mouse development revealed that angiogenesis begins at embryonic day 9 (E9) and barriergenesis, formation of the BBB, ends at E15 (REFS[13,14]). The final steps of BBB maturation include a decrease in EC fenestrations and the appearance of TJs followed by a reduction in transcytosis. Although

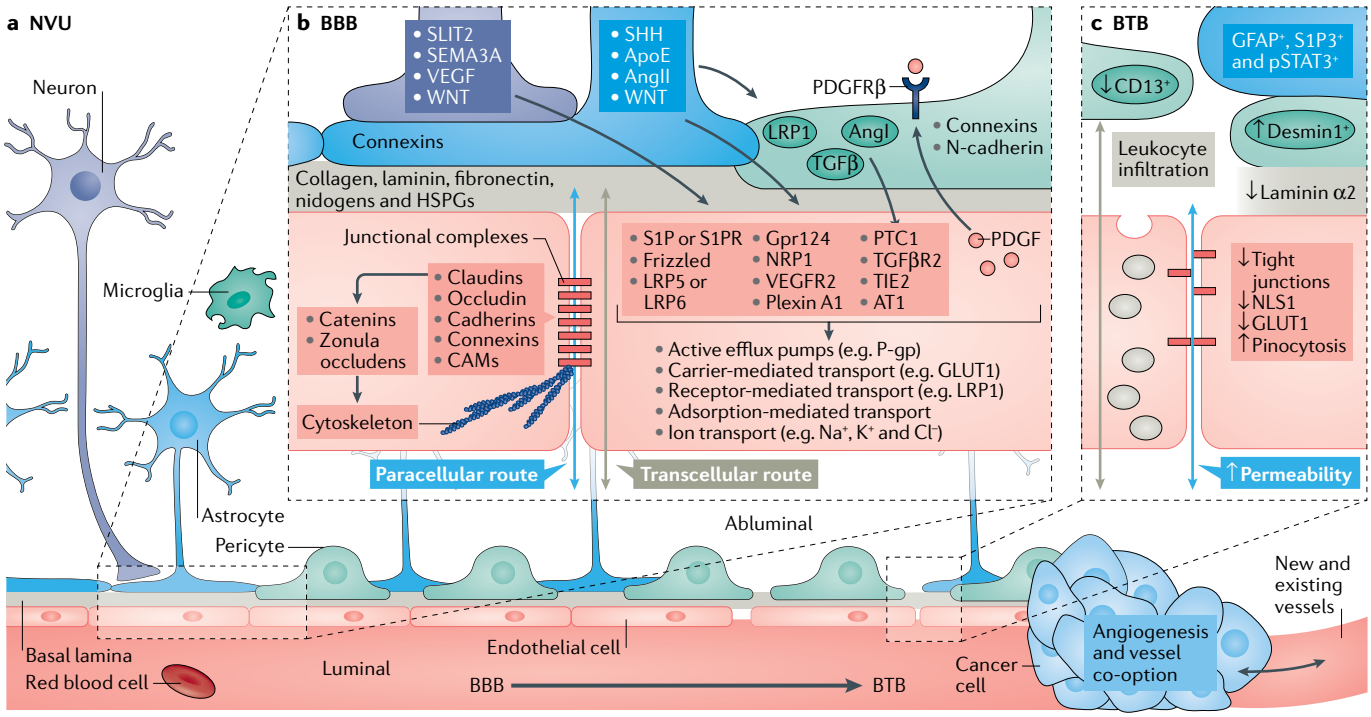

Fig. 1 | **Overview of the neurovascular unit in healthy and tumour-bearing brains. a** | Schematic representation of capillaries in the neurovascular unit (NVU) with the intact blood–brain barrier (BBB, bottom left) and the disrupted blood–tumour barrier (BTB, bottom right) in the neuroparenchyma. BBB development and permeability is dictated by signalling and structural mechanisms that are regulated by multiple cells within the NVU. These mechanisms control both paracellular and transcellular routes and, ultimately, vessel permeability in the central nervous system (CNS). **b** | Schematic representation of notable cellular and molecular components that regulate the development, maturation and function of endothelial cells (ECs) (red) and the NVU (refer to main text for relevant references). Neuronal (purple) and non-neuronal cells regulate the expression of transport and tight junction proteins in ECs, which in turn may 'loosen' or 'tighten' the BBB. Here, we depict examples of key signalling pathways connecting astrocytes (blue), pericytes (green) and neurons to ECs. Together or individually, these pathways will alter transcellular transport by changing the expression of transporters and the paracellular route by disrupting junctional protein complexes. Of note, ECs reciprocally regulate components of the NVU. For example, EC-secreted transforming growth factor-β (TGFβ) can activate cognate receptor on pericytes. During development and maturation, glial cells, pericytes and neurons regulate EC behaviour via multiple ligands and receptors, which in turn activate downstream signalling cascades (for example, Frizzled, G protein-coupled receptor 124 (GPR124), β-catenin, GLI, PI3K, SRC and the p38 MAPK) that dictate expression of junctional and transcytosis proteins and control CNS homeostasis. For example, astrocytes directly modulate NVU demands such as water content in the neuroparenchymal space via the major water channel protein aquaporin 4 (AQP4), regulate immune cell and cancer cell infiltration via specific chemokine and cytokine production, regulate BBB permeability and integrity in part by angiotensin (Angl and AngII), apolipoprotein E (ApoE) and retinoic acid, and regulate pericyte distribution. **c** | In the BTB, NVU integrity and endothelial permeability is compromised due to disruption of the NVU, including displacement of astrocytes (blue) and pericytes (green), neurovascular decoupling, altered pericyte populations and changes in EC tight junctions and transcytosis mechanisms. Additional vascular-related phenotypes such as hypoxia, oedema, angiogenesis and tumour-vessel co-option can influence the NVU in brain tumours. Although BBB features remain present during tumour development, in particular at the cancer–neuroparenchyma edge, the BTB displays increased and heterogeneous permeability. Tumour progression leads to BTB structural changes including neuronal death, astrocyte endfeet displacement (from primary and metastatic cancer cells) and heterogenous pericyte and astrocyte subpopulations, all of which can reduce the barrier functions of the CNS endothelium. Intracellular vesicular transport is represented by grey-coloured vesicles in the schematic. AT1, angiotensin II receptor type 1; CAM, cell adhesion molecule; GLUT1, glucose transporter 1; HSPG, heparan sulfate proteoglycan; LRP, low-density lipoprotein receptor-related protein; NLS1, sodium-dependent lysophosphatidylcholine symporter 1; NRP1, neuropilin receptor 1; PDGF, platelet-derived growth factor; PDGFRβ, platelet-derived growth factor receptor β; P-gp, P-glycoprotein; PTC1, protein patched homologue 1; S1P, sphingosine-1-phosphate; S1PR, sphingosine-1-phosphate receptor; SEMA3A, semaphorin 3A; SHH, sonic hedgehog; SLIT2, Slit homologue 2 protein; TGFβR2, transforming growth factor-β receptor 2; TIE2, tyrosine kinase with Ig and EGF homology domains 2; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.

Microglia
Resident myeloid cells in the central nervous system (CNS) that regulate CNS function and homeostasis.

Astrocytes
Major glial cells in the central nervous system (CNS) that regulate CNS function and homeostasis.

Mural cells
Vascular-associated cells that include pericytes and smooth muscle cells.

Pinocytosis
Small particles or molecules suspended in extracellular fluid that are brought into the cell through cell membrane invagination.

ATP-binding cassette transporters
(ABC transporters). Active transporters that use energy in the form of ATP to transport molecules across the cell membrane against their concentration gradient.

functional at birth, the BBB continues to mature postnatally under the influence of pericytes, astrocytes and neurons[12]. The early onset of the BBB is evident clinically as drug delivery of systemic therapies into the tumour microenvironment of paediatric brain tumours faces similar challenges to those observed in adults[15,16].

Molecular profile

Genetic and transcriptomic studies have confirmed activation of signalling pathways, such as WNT–β-catenin and sonic hedgehog (SHH)-dependent signalling in brain ECs within the BBB[8]. Interestingly, some pathways — including the G protein-coupled receptor (GPCR) GPR124 and WNT–β-catenin axis — also regulate additional features of vascular architecture and function[17–19]. CNS EC signalling pathways can be directly regulated by pericytes and astrocytes (FIG. 1).

Recent single-cell analyses of the NVU have revealed previously unknown properties of ECs in the BBB. Single-cell RNA sequencing (scRNA-seq) of adult mouse NVU cells, including ECs, pericytes and glia, isolated by specific marker expression (for example, claudin 5 (CLDN5) for ECs and platelet-derived growth factor receptor β (PDGFRβ) for pericytes) has provided distinct cell-specific transcriptional profiles[20]. As expected, capillary ECs have high expression of nutrient transporters and TJ proteins (for example, CLDN5) when compared with arterial or venous ECs, whereas other factors such as von Willebrand factor (VWF) and vascular cell adhesion protein 1 (VCAM1) were inversely enriched. Interestingly, subpopulations of PDGFRα-positive perivascular fibroblast-like cells are present between the vessel wall and aquaporin 4-positive astrocytic endfeet throughout the CNS vasculature except for capillaries[20]. A separate single-cell transcriptional profiling study of different areas of the CNS has also confirmed unique molecular signatures of mural cells, fibroblast-like cells and ECs[21]. Another recent transcriptomic and epigenomic analysis compared ECs isolated from the brain, liver, lung and kidney of postnatal mice[22]. Brain ECs particularly expressed transporters including sodium-dependent lysophosphatidylcholine symporter 1 (NLS1; also known as MSFD2A), glucose transporter 1 (GLUT1) and solute carrier organic anion transporter family member 1C1 (SLCO1C1), which transport long-chain fatty acids, glucose and organic anions, respectively, across the BBB. By comparing CNS and non-CNS ECs, these authors observed CNS-specific transcription factor activity including T cell factor/lymphoid enhancer-binding factor (TCF/LEF) and the zinc finger protein ZIC3, which implies a role for canonical WNT signalling. Interestingly, there was heterogeneity among CNS capillary ECs reflecting either a more venous-like or more arterial-like transcriptional signature.

ECs are not the only NVU cells that are organotypic. Although pericytes share ubiquitous characteristics throughout the body, mouse brain pericytes express distinct sets of markers when compared with those isolated from the lung[20]. Current comprehensive single-cell analysis platforms and annotated databases of CNS and non-CNS capillary endothelium are providing organotypic signatures of NVU cells that will directly inform studies evaluating BBB biology and drug delivery across the NVU[23,24]. Performing single-cell analysis of the BTB during primary brain tumour progression and brain metastasis, and comparing it with normal BBB or extracranial primary endothelium in the case of brain metastases, will surely reveal more unique properties of the NVU and yield novel therapeutic strategies.

Reminiscent of the complex synaptic networks within the CNS, the BBB requires a co-dependent cellular structure to maintain normal function. The BBB can be mimicked ex vivo by co-culturing brain ECs, astrocytes and pericytes, allowing for experimental studies in vitro[25]. However, to what extent the full repertoire of BBB features, including the expression of BBB-specific transporters, is reproduced in in vitro models will require further analysis. For example, comparisons of the in vivo and in vitro BBB using scRNA-seq have not yet been published. CNS ECs are connected by TJs enriched in transmembrane cell-surface proteins including claudins, occludins and adhesion molecules[26] (FIG. 1). Brain EC–cell junctions also contain vascular endothelial cadherin (VE-cadherin)-rich adherens junctions and connexin 43 (Cx43) gap junctions[26]. Together, these proteins regulate intracellular signalling, via zonula occludin proteins and β-catenin, and modulate actin cytoskeletal rearrangement to 'tighten' the extracellular space between ECs, thus preventing paracellular transport[27] (FIG. 1).

Functional profile

The BBB is surrounded by the basal lamina, an extracellular matrix (ECM) formed predominantly of glycoproteins that can be proteolytically cleaved to influence BBB function in health and disease[28]. ECs, pericytes and astrocytes contribute to the basal lamina, which provides ligands that activate multiple signalling pathways in the NVU and regulate BBB function. CNS ECs are considered non-fenestrated, as they display a reduced number of pores in their cellular membrane, and have limited intracellular vesicular trafficking and pinocytosis[29,30]. Transcellular movement of cargo across the BBB takes the form of either vesicle-mediated transcytosis, involving receptor-mediated or adsorptive routes, or carrier-mediated transcytosis, which regulates transport of hormones, transferrin and plasma proteins. Passive diffusion across the BBB is largely dependent upon the lipophilicity of small molecules. Small lipophilic molecules such as oxygen and caffeine diffuse through cell membranes. The CNS endothelium displays polarized cellular transporters to dynamically regulate influx and efflux between the neuroparenchyma and blood (FIGS 1,2). Small hydrophilic molecules such as glucose and amino acids cross the BBB via solute carrier (SLC) proteins. Active transport occurs across the BBB endothelium by polarized expression of ATP-binding cassette transporters (ABC transporters) present on the luminal and abluminal sides of vessel walls[31] (FIG. 2). These transporters mediate efflux of xenobiotics and toxins from the endothelium away from the neuroparenchymal space, effectively clearing these agents into the luminal compartment[32]. Most anti-neoplastic low molecular weight drugs are substrates for ABC proteins. For example, several targeted therapies for gliomas and

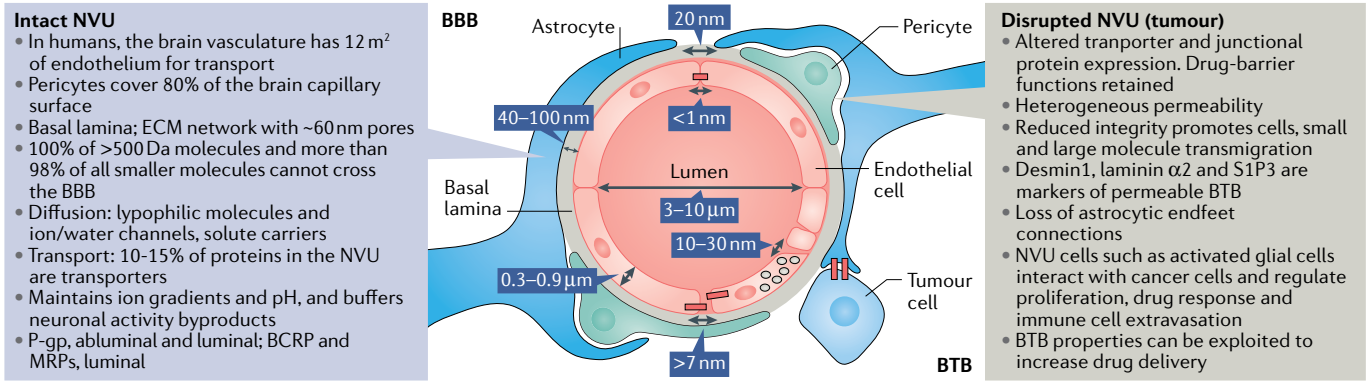

Fig. 2 | **Physical and chemical properties of the BBB.** The blood–brain barrier (BBB) structure displays unique physical properties that tightly regulate molecular and cellular flow in the neuroparenchyma. The brain barrier's importance is evident from its functional conservation across organisms, from fruit flies to humans[1]. As the BBB develops and matures, endothelial cell fenestrations decrease, and the subsequent appearance of tight junctions is followed by a reduction in transcytosis. Centre panel: cross-section of a central nervous system (CNS) capillary depicting estimated distances and spaces within the BBB, which circulating drugs are required to overcome to permeate the brain parenchyma. Left panel: in the non-diseased brain, the neurovascular unit (NVU) includes an intact BBB that displays multiple characteristics that limit drug permeability into the CNS. Right panel: during tumour progression, stroma–cancer cell interactions in the brain tumour microenvironment dictate vessel permeability and cancer cell proliferation. The blood–tumour barrier (BTB) characteristics listed here contribute to the heterogeneous permeability observed in the disrupted NVU. BCRP, breast cancer resistance protein; ECM, extracellular matrix; MRP, multidrug resistance protein; P-gp, P-glycoprotein; S1P3, sphingosine 1-phosphate 3.

brain metastases have affinity for the multidrug-resistant (MDR) ABC transporters, P-glycoprotein (P-gp; also known as ABCB1), breast cancer resistance protein (BCRP; also known as ABCG2) and multidrug resistance proteins (MRPs), which are all expressed in the BBB[33]. Furthermore, some transporters are also expressed in other NVU cells including astrocytes, microglia and neurons[34]. The above transport mechanisms form a delicate balance between influx and efflux routes that is essential for neuroparenchymal homeostasis. However, this homeostatic mechanism will also directly affect any therapeutic delivery.

Pericytes at the abluminal side of the endothelium are key regulators of vascular function throughout the body and control vessel function, vessel remodelling and neuroinflammation during ageing[35–38]. In addition to communicating with astrocytes within the NVU (FIG. 1), pericytes support BBB maintenance in the postnatal brain. Pericyte-deficient mutant mice showed increased BBB permeability to low and high molecular weight tracers[36,38]. Pericytes can directly regulate the expression of transporters. NLS1, which regulates transport of docosahexaenoic acid (DHA, an omega-3 fatty acid) across the BBB, is necessary for neuronal function and neuroprotection[39]. Loss of NLS1 expression in ECs leads to increased transcytosis and a leaky BBB. Pericytes wrap the CNS endothelium and together they produce a basal lamina that attracts astrocytic endfeet during development[38,40]. Astrocytes are the most abundant cell type in the brain, are metabolic sensors and play an integral role in BBB development and function[41]. Astrocytes appose their endfeet and cover the majority of the abluminal surface area of the BBB, and regulate signalling pathways that maintain junctional complexes and produce additional basal lamina. By connecting via gap junctions and TJs, astrocytes can form an additional barrier in the CNS called the glia limitans[42,43]. Interestingly, astrocytic endfeet removed by laser ablation repopulated blood vessels in 50% of cases[44]. Despite studies suggesting a key role for astrocytes in BBB function, this study showed limited BBB disruption after removing astrocytic input in the adult mouse brain. Together, astrocytes and pericytes play a plethora of direct and indirect roles in regulating BBB integrity during development, adulthood and disease progression.

BBB function is dynamic and can be influenced by local and circulating factors. Microglia are the most abundant innate immune cells in the neuroparenchyma and contribute to early CNS vasculogenesis[45,46]. Microglia activation can cause BBB disruption or repair and, during inflammation, microglia and peripheral immune cells such as leukocytes can increase BBB permeability through interleukin-1β (IL-1β) secretion or through the adhesion proteins intercellular adhesion molecule 1 (ICAM1), VCAM1 or E-selectin[47]. The BBB can also be innervated by synaptic endings. GABA-ergic, cholinergic, noradrenergic and serotonergic neurons can directly contact the CNS endothelium to regulate blood flow, neurovascular coupling and BBB permeability[48,49]. In sum, BBB development is modulated by intracranial cues, which control the underlying molecular pathways. In addition, the maintenance of BBB integrity is likely regulated on multiple fronts ranging from direct cellular contact to long-range factors including signalling proteins and molecules, cells and even exosomes, which may help extravasation into the neuroparenchyma[50].

BBB heterogeneity in brain tumours

Owing to the confined space within the brain, tumour growth can impair blood flow even in peritumoral regions by compressing vessels[51]. Furthermore, as brain tumour lesions expand, the NVU gains different properties in the tumour core when compared with the periphery of the tumour and the neuroparenchyma, with the latter harbouring an intact BBB[52]. During primary brain tumour progression and the development of brain metastasis, the tumour vasculature becomes increasingly heterogeneous. An expanding neoplastic

lesion causes local and distal changes that can directly compromise neuronal viability and vascular function[51] (FIGS 1,2). As the vasculature dramatically changes during tumour expansion, nutritional demand of proliferating cancer cells requires co-opting existing vessels and/or creating new ones via angiogenesis. In addition, tumours can increase the blood vessel supply via other mechanisms: postnatal vasculogenesis, intussusception, vascular mimicry and transdifferentiation[53,54].

Tumour vessels are tortuous and the architecture can differ between tumours of the brain and other tumour types[4,55]. Vascular dysfunction during tumour progression, in part mediated by deregulated expression of angiogenic factors such as vascular endothelial growth factor (VEGF), leads to hypoxia and an acidic microenvironment that fuels tumour progression in part through hypoxia-inducible factor 1α (HIF1α)-induced transcriptional programmes[52,56,57]. Blocking VEGF signalling transiently prunes the immature and leaky vessels of brain tumours in mice and actively remodels the remaining vasculature so that it more closely resembles the normal vasculature. We have shown the survival benefit of vascular normalization in patients with newly diagnosed as well as recurrent glioblastoma receiving antiangiogenic agents[58,59]. However, an adverse consequence of anti-angiogenic therapy, at least at high doses, is that the resulting hypoxia can increase invasiveness of cancer cells[60]. Furthermore, VEGF itself can regulate BBB permeability; therefore, anti-angiogenics at high doses might decrease BTB permeability, which could potentially affect delivery of other therapeutics[61,62]. Realizing an optimal balance between these vascular phenotypes remains a challenge for anti-angiogenic therapies of primary brain tumours and brain metastases.

The BTB

BBB disruption is evident from the higher drug accumulation within brain tumours as compared with the unaffected brain and, indirectly, by the detection of brain tumour markers in the circulation, such as circulating tumour cells from gliomas. The BTB is generally considered 'leakier' than the BBB (BOX 1). Imaging techniques including magnetic resonance imaging (MRI) and positron emission tomography (PET) have confirmed disruption of the BTB, in particular with high-grade brain tumours such as glioblastoma[6]. Despite these observations, it is clear that there can be an intact BBB in progressive glioblastomas displaying a range of efflux transporters that prevent the entry of anticancer agents[6,63]. The BTB is characterized by aberrant pericyte distribution and loss of astrocytic endfeet and neuronal connections[64]. Furthermore, invading glioma cells can physically displace astrocytic endfeet and disrupt BBB integrity[65]. T cell subpopulations and peripheral monocytes are detected in brain tumours, indicating permeability of the NVU to circulating immune cells[66]. Moreover, junctional proteins decrease in BTB ECs and the intratumoural vasculature never fully re-establishes a normal BBB in brain metastases[67]. Despite being characterized as a disrupted NVU, the BTB retains critical aspects of the BBB including expression of active efflux transporters in ECs and tumour cells[63].

BBB/BTB structural integrity is heterogeneous between metastatic lesions and between tumour types (BOX 1). For example, characteristics of BBB ECs such as fenestration differ between four molecular subtypes of medulloblastoma, thus directly impacting transcytosis of drugs across the BTB and therapeutic efficacy; the WNT subtype of medulloblastoma has a fenestrated vasculature, allowing higher accumulation of chemotherapeutic agents in the neuroparenchyma, whereas the SHH medulloblastoma subtype has an intact BBB[15]. The extent of BBB function and characteristics can vary among brain metastases from different subtypes of breast cancer[68]. Human epidermal growth factor receptor 2 (HER2, also known as ERBB2)-positive breast cancer brain metastases generally express more GLUT1 and BCRP than other subtypes. Although vessels are considered abnormal, the BTB may retain expression of efflux pumps such as BCRP.

In addition to genetic phenotypes of brain tumours, brain stroma signalling cascades that are important during barriergenesis can also influence progression of primary brain tumours. The Norrin–Frizzled 4–β-catenin signalling cascade (WNT pathway) can control tumour burden when activated in ECs[69,70]. Norrin–Frizzled 4 suppresses neoplastic progression of medulloblastoma by maintaining an antitumour stroma[61]. WNT signalling through the endothelial GPCR GPR124 regulates TJs and microvascular haemorrhage during glioblastoma progression in mice to prevent BBB dysfunction and oedema[70]. Although it is still unclear how molecular subtypes of gliomas might differentially dictate BTB function, emerging data are providing new insights. For example, oligodendrocyte transcription factor 2 (OLIG2)-positive precursor-like glioma cells co-opt blood vessels by activating WNT–β-catenin signalling[17], which could influence BTB integrity.

BTB heterogeneity. Elegant preclinical studies have shown that despite being leakier on average, the BTB also displays heterogeneous permeability (BOX 1). For example, low molecular weight compounds are unevenly distributed within the tumour lesion when injected systemically in mouse models of brain metastasis[71,72]. In preclinical models, the lipophilic low molecular weight inhibitor lapatinib displays increased heterogeneous distribution in HER2-positive breast cancer brain metastasis lesions when compared with the surrounding unaffected brain[73]. Molecular and cellular components of the NVU are disrupted in brain metastases. Pericyte subpopulations are altered in mouse and human brain metastasis, where desmin-positive pericytes largely populate the drug-permeable BTB when compared with unaffected vessel areas[71]. Reactive astrocytes lose connections to the ECs, reducing the expression of the lipid transporter NLS1 in ECs, which directly disrupts transport of DHA into the brain tumour microenvironment[74]. Furthermore, the sphingosine 1-phosphate receptor 3 (S1PR3) that is expressed in reactive astrocytes and brain metastatic cancer cells loosens the BTB via astrocytic IL-6 and CC-chemokine ligand 2 (CCL2) secretion[75]. EC S1PR1 signalling can also regulate BBB integrity within the mature CNS[76].

In glioblastoma, the BTB also displays abnormal barrier features such as reduced TJs, non-uniform pericyte vessel coverage and stem cell-derived pericytes, which disrupt vascular integrity during tumour progression[64,77]. Of particular note, glioma stem cells, which are considered refractory to therapies, reside in perivascular, hypoxic and invasive niches that maintain 'stemness' and also display abnormal barrier integrity[78]. Leakier and dysfunctional vessels cause water and metabolic waste retention in the neuroparenchymal space, increasing the interstitial and intracranial fluid pressure, often requiring glucocorticoid administration to reduce oedema in malignant brain tumours[79]. Interestingly, targeting glioma stem cell-derived pericytes in a preclinical model of glioma disrupts the BTB and enhances drug delivery. Furthermore, pericyte coverage is inversely correlated with the prognosis of patients with glioblastoma treated with chemotherapy[80]. Thus, the structural and

Box 1 | **Examples of BBB/BTB heterogeneity in preclinical and clinical studies**

Preclinical mouse models

Glioma

- Delivery can vary greatly across a primary brain tumour lesion such that the centre of the tumour will demonstrate higher leakiness when compared with the peritumoral region and the surrounding brain microenvironment. In the intracranial GBM8401 glioma model, the distribution of liposomes containing doxorubicin is higher within the tumour when compared with the surrounding brain tissue; however, focused ultrasound further increases drug accumulation indicating that the blood–tumour barrier (BTB) is a compromised blood–brain barrier (BBB)[197]. Delivery of the targeted therapy dasatanib is heterogeneous within the tumour lesion and higher than the corresponding normal brain tissue in an oncogene-induced spontaneous model of malignant glioma[198].
- The orthotopic GL261 mouse glioblastoma model was studied using contrast-enhanced magnetic resonance imaging (MRI), Evans blue staining and histopathology. GL261 tumours displayed increased BBB disruption as they progressed, whereas the Hs683 human oligodendroglioma model had less disruption[199,200]. The U251 xenograft model has an intense rim on contrast-enhanced MRI representing vasogenic oedema (disrupted BBB and vasculature)[201].

Medulloblastoma

All subtypes, with the exception of the WNT subtype, have an intact functional BBB in preclinical models and patients[15]. This could explain why the WNT subgroup has the best prognosis owing to improved drug delivery.

Brain metastasis

- In the intracardiac injection (ICD) brain metastasis model using breast cancer cell lines 231-BR-Her2 and 4T1-BR5, BTB permeability is highly heterogeneous[72]. The 231-BR brain metastasis model displays areas of higher permeability due to sphingosine 1-phosphate receptor 3 (S1PR3)-positive astrocyte-dependent disruption of BTB integrity[75].
- In the ICD brain metastasis model using breast cancer cell lines SUM190-BR3 and JIMT-1-BR3, the BBB is more permeable than that with the 231-BR-Her2 cell line[202].
- In an intracranial injection brain metastasis model using the human breast cancer cell line BT474 (oestrogen receptor-positive, human epidermal growth factor receptor 2 (*HER2*)-amplified), the BBB is permeable to chemotherapy and antibodies but BBB/BTB disruption significantly increases delivery[128]. This preclinical model recapitulates clinically observed subpar central nervous system (CNS) delivery of targeted therapies such as lapatinib when compared with the site where the primary tumour arose[203]. Brain metastases formed from BT474 cells injected intracranially, intravenously or intracardially display some differences in the delivery of therapeutic nanoparticles. The intracranial model system is 'leakier' as more drug uptake and inhibition is observed[204].
- In the ICD brain metastasis model using the melanoma cell line A2058, there are regions with intact BBB[112,205].
- Intracranial brain metastasis models using melanoma cells (MDA-MB-435 and A2058) display a vessel co-option phenotype in the brain, whereas those using lung carcinoma cells (PC14-PE6 and HTB177) harbour more angiogenesis[82].
- In the intracranial brain metastasis model using the ALK-mutated lung adenocarcinoma cell line H3122 EML4-ALK^{L1196M}, brain lesions respond to brain-penetrant ALK inhibitor (PF-06463922)[206]. These observations highlight the benefit of designing systemic therapies that bypass barriers of the neurovascular unit (NVU). Designing brain-permeable drugs is essential when targeting non-permeable micrometastases and macrometastases, for example, those observed in the ICD model of melanoma brain metastasis[112].
- Patient-derived xenograft models of brain metastases (from the breast, lung, melanoma, neuroendocrine, prostate) display reduced sodium-dependent lysophosphatidylcholine symporter 1 (NLS1) expression when compared with normal brain. Breast cancer brain metastases display heterogeneously disrupted BBB[74].

Clinical data

Glioma

- Glioblastomas (high-grade gliomas) display heterogeneous permeability to circulating drugs. Contrast-enhanced MRI of high-grade gliomas suggests there are areas of vasogenic oedema[5,6]. Together with positron emission tomography (PET) imaging and MRI, and analysis of surgically removed tissues, it has been shown that some regions of gliomas also display an intact BBB.
- Contrast-enhanced MRI and immunohistochemistry (IHC) analyses (GFAP, AQ4, CD31) suggest that there are regions of intact BBB in low-grade and high-grade gliomas[207].

Medulloblastoma

Contrast-enhanced MRI has revealed that the BBB/BTB is tumour subtype specific with high variability within the subgroups and heterogeneity across the tumour[208,209]. These observations highlight that genetic and molecular phenotypes may influence barrier function and dictate drug delivery.

Brain metastasis

- BBB function is tumour subtype specific: triple-negative or basal-type tumours often disrupt the BBB, whereas HER2-positive tumours tend to preserve the BBB[68]. HER2-positive breast cancer brain metastases express high levels of glucose transporter 1 (GLUT1) and breast cancer resistance protein (BCRP) compared with brain metastases derived from other breast cancer subtypes.
- Desmin-positive pericytes are present in specimens of human brain metastases. This phenotype correlates with areas of high permeability in preclinical models of breast cancer brain metastasis[71].
- Lapatinib concentration in breast cancer brain metastases ranges from 1.0 to 6.5 μM (REF.[210]). PET studies show higher levels of lapatinib in tumour lesion than normal brain[211] and higher trastuzumab accumulation in breast cancer brain metastases[212]. Although tumour lesions maintain BBB features, the BTB 'leakiness' may allow more drug accumulation compared with unaffected brain parenchyma. Brain tumour permeability to targeting antibodies such as trastuzumab is evident in human and mouse preclinical orthotopic models of brain metastasis[89,203,213,214].
- Dynamic contrast-enhanced MRI has also been used to assess barrier integrity disruption in patients with brain metastases and revealed heterogeneity[215].

functional heterogeneity of the BBB/BTB in the brain tumour microenvironment must be considered for developing effective systemic therapies.

Although primary brain tumours arise from CNS cells, metastatic cells must first cross the intact BBB. Elegant studies have demonstrated that specific circulating neoplastic cells express cell-surface markers that allow their extravasation into the brain parenchyma by facilitating transcellular transport[81]. Real-time imaging of a mouse brain metastasis model showed early extravasation and proximity to microvessels as a necessary element for colonization[82]. Metastatic cells that breach the BBB can disrupt CLDN5 TJs, and this can be reduced by blocking expression of angiopoietin 2 in ECs[83]. Once arrested within the CNS capillary bed, brain-tropic metastatic cells that successfully colonize the neuroparenchyma express cell-surface proteins, including ligands and proteases, which mediate extravasation across BBB and survival in the brain tumour microenvironment (FIG. 3). For example, expression of the 2,6-sialyltransferase ST6GALNAC5 on circulating metastatic breast cancer cells facilitates paracellular crossing of the BBB[84]. Brain metastatic cells can also express proteases such as cathepsin S to degrade the junctional adhesion molecule JAM2 (REF.[85]). Multiple steps during brain tumour progression, including the brain metastatic cascade and tumour expansion in the neuroparenchyma, alter BBB integrity, permeability and structural composition[67]. Although the BBB is regarded as the main barrier to successful therapy of brain tumours, the local microenvironment must be considered to improve patient survival[86,87]. Indeed, preclinical and clinical findings demonstrate that cancer cells that colonize the brain microenvironment display enhanced oncogenic and pro-survival signalling that can counteract the efficacy of targeted therapies and chemotherapies[88–90].

Drug delivery across the BBB/BTB

Although essential for normal CNS function, the BBB represents a formidable barrier during therapeutic intervention. However, emerging insights into BBB/BTB structure and function have yielded novel strategies to overcome this barrier and deliver drugs to brain tumours, including those infiltrating the peritumoral regions. All the facets described above are directly or indirectly responsible for poor drug delivery within brain tumours. Circulating drugs are subject to multiple barriers posed by the NVU: reduced paracellular transport of hydrophilic molecules, reduced transcytosis and regulated polarized efflux transporters that prevent neuroparenchyma access to lipophilic synthetic molecules[91]. With the BBB intact, hydrophobic molecules with molecular weight <500 Da (smaller than 1 nm) diffuse transcellularly into the neuroparenchyma, whereas small hydrophilic compounds can enter the brain via the paracellular route[92]. However, many circulating pharmacological compounds have affinity for the multidrug resistance ABC transporters, as discussed above. As a result, ABC transporters are often responsible for decreasing the uptake rate of potential drugs crossing the BBB, as well as enhancing the barrier properties of the BTB[53]. The most common ABC transporters are P-gp, responsible for the poor penetration of large (>500 Da) hydrophobic drugs in the brain, the BCRP, conferring resistance to non-chemotherapeutic drugs and xenobiotics, and several members of the MDR ABC transporter family[93–97].

Multiple strategies are being developed or optimized to bypass or hijack the cellular and molecular barriers of the BBB (FIG. 3). The various approaches to improve drug delivery across the BBB/BTB can be categorized as invasive or non-invasive, with minimally invasive included in the latter category. Although invasive approaches, which involve direct access to the disease site, have not lived up to their full potential, they are currently undergoing extensive refinements and optimization with promising preclinical findings (BOX 2). In addition to refining invasive methods, many minimally invasive (and non-invasive) approaches are currently in preclinical or early-stage clinical evaluation with encouraging findings (TABLE 1). Below, we will discuss these approaches.

Molecular approaches

Hijacking the endogenous influx transport. Knowledge of the proteins expressed on ECs that line the BBB/BTB has been exploited for receptor-mediated BBB/BTB transcytosis (that is, a transcellular route)[98]. In this case, a targeting ligand (for example, a monoclonal antibody) binds to its receptor to trigger endocytosis, using the vesicular trafficking machinery to transport to the abluminal surface. A drug can be molecularly linked to this ligand, thus enabling transport across the BBB/BTB. Some well-characterized receptors that have been used for this approach include transferrin, insulin and insulin-like growth factor 1 receptors[99,100]. Although receptor-mediated transport is a very promising strategy for the delivery of macromolecular pharmaceuticals (up to 80 nm in diameter; see below for a discussion of nanoparticles)[99] in the treatment of brain tumours, the widespread expression of these receptors in other tissues, the small dissociation rate and potential toxicity need to be carefully considered. Other targeted approaches take advantage of the endogenously expressed BBB/BTB receptor low-density lipoprotein receptor-related protein 1 (LRP1). LRP1-targeted peptide–chemotherapy conjugates, consisting of three paclitaxel molecules covalently linked to angiopep-2, a peptide designed to utilize the LRP1 transport system, increased delivery (>50-fold) into brain metastases and improved median survival in preclinical and clinical studies[101,102].

Another approach is to employ the SLC proteins on the endothelial surface to cross the BBB/BTB. Examples include the glucose transporter GLUT1 that is responsible for the passive transport of glucose through the cell membrane and LAT1 that transports neutral amino acids bidirectionally between the blood and the brain[93]. Interestingly, overexpression of GLUT1 is correlated with poor survival in most solid tumours, including glioblastoma[103], supporting further exploration of GLUT1 targeting to improve penetration across the BBB/BTB and enhance cancer cell uptake. Likewise, LAT1 could be targeted as its overexpression has been correlated with the malignant phenotype and proliferation of gliomas, although its function might be sensitive to hypoxia[104].

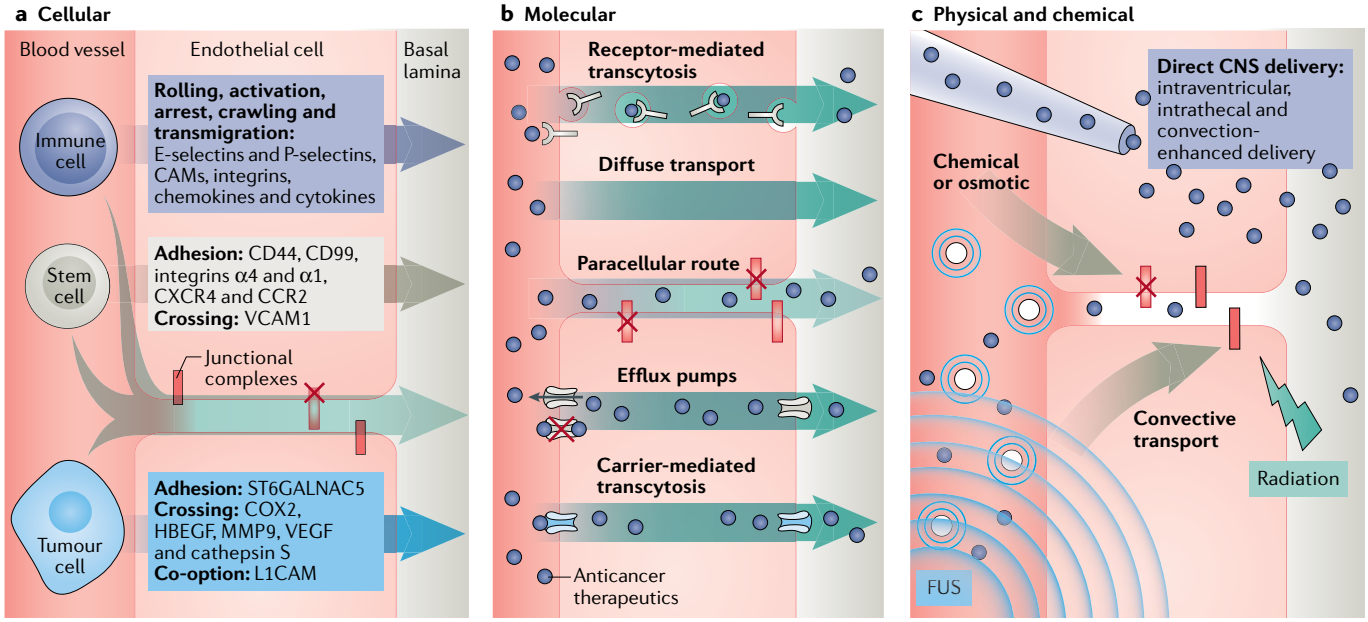

Fig. 3 | **Improving drug delivery through the BBB/BTB.** Schematic presentation of key molecular, cellular and physical mechanisms and systems to overcome the blood–brain barrier and blood–tumour barrier (BBB/BTB). **a** | The BBB prevents cellular extravasation into the neuroparenchyma unless compromised by circulating cells equipped with necessary 'brain-tropic' molecular components[197], including soluble and membrane-bound proteins, to disturb the BBB integrity. Immune cell extravasation into the central nervous system (CNS) occurs by the following steps: rolling, activation, arrest, crawling, transmigration. The transcellular route is preferred when the BBB is intact, whereas the paracellular route is preferred when there is reduced tight junction integrity (red X = disrupted junction) and formation of intercellular gaps. Transmigration across the BBB is mediated by actin-containing protrusive structures and occurs on the timescale of minutes. Stem cells that have been engineered to contain anticancer cargo localize to sites of neuroinflammation and display coordinated rolling and adhesion behaviour, and transcellular and paracellular transmigration. In contrast to immune cells, mesenchymal stem cell (MSC) transmigration does not involve substantial lateral crawling. Stem cells migrate by the paracellular or transcellular route through discrete gaps or pores in the CNS endothelium. Stem cell transmigration is mediated by membrane blebbing and occurs on the timescale of hours. In contrast, circulating metastatic cancer cells that accumulate in the CNS capillary bed must express specific proteins in order to adhere and breach the BBB. Metastatic cells express proteases that disrupt junctional complexes. Although preclinical studies show that this process may occur within days, the time course of symptomatic brain metastasis in patients varies greatly (from months to years after metastatic dissemination of primary tumour). **b** | Several molecular strategies are employed to hijack or bypass barriers posed by the neurovascular unit (NVU) (described in main text and TABLE 1). Anticancer therapeutics (purple circles) can be designed with low affinity to efflux pumps or may hijack carriers or receptor-mediated transcytosis mechanisms. Alternatively, inhibitors of efflux pump or junctional complexes can be used to limit the clearance of drugs from the tumours. **c** | Direct delivery into the neuroparenchyma and physical disruption of the BBB/BTB. Specifically, focused ultrasound (FUS, indicated by the waves) with microbubbles (concentric small blue circles), radiation, osmotic, direct and convective-mediated delivery of therapeutics into the CNS is depicted. CAM, cell adhesion molecule; CCR2, CC-chemokine receptor 2; COX2, cyclooxygenase 2; CXCR4, CXC-chemokine receptor 4; HBEGF, proheparin-binding EGF-like growth factor; L1CAM, L1 cell adhesion molecule; ST6GALNAC5, a 2,6-sialyltransferase; MMP9, matrix metalloproteinase 9; VCAM1, vascular cell adhesion protein 1; VEGF, vascular endothelial growth factor.

Overcoming the efflux pumps. Efflux pumps contribute to poor brain-to-blood drug ratios by actively pumping out pharmacological compounds with sufficient binding affinity. Efflux transporters including P-gp and BCRP have affinity for several FDA (US Food and Drug Administration)-approved drugs, including chemotherapeutics and various targeted therapeutics. They are often overexpressed in tumour vessels when compared with unaffected brain and keep drug levels well below the required effective dose in the brain tumour microenvironment[105]. ABC transporters (for example, P-gp and BCRP) are expressed in the BBB/BTB as well as in tumour cells of mouse and human brain tumours, which may exclude drugs from the cellular cytoplasm[106–108].

To overcome efflux transporter-mediated drug reduction in the brain and reach sufficient bioactive concentrations in the brain tumour microenvironment, preclinical studies have focused on the co-administration of drugs with transporter inhibitors (for example, inhibitors of ABC transporters). This approach has shown significant enhancement in the brain concentrations of concurrently administered chemotherapeutic agents and targeted therapies (1.5-fold for temozolomide (TMZ)[105], 5-fold for the poly(ADP ribose) polymerase (PARP) inhibitor ABT-888 (REF.[106]) and 40-fold for the mutant BRAF inhibitor vemurafenib[109]). Although the effectiveness of these strategies in the clinics has been questioned in the past[110] (this was most likely due to methods used at that time), the increasingly recognized role of the efflux transporters in BBB and brain tumours combined with current knowledge[31,111] suggests that more potent inhibitors with high specificity that concurrently target multiple pathways can lead to increased penetration of substrate drugs across the BBB/BTB[31]. In addition to blocking ABC transporters, structural refinement of molecularly targeted agents such as kinase inhibitors (for example, PI3K and mTOR inhibitors), with reduced affinity to P-gp and BCRP, is a promising alternative approach[33,112]. This approach may

Diffusive transport
The net movement of molecules from a region of higher concentration to a region of lower concentration.

Convective transport
Mass transport mediated by bulk fluid flow that is driven by a pressure gradient.

not only improve penetration across the BBB but also lead to improved cancer cell uptake[106,113].

Cellular approaches

Harnessing the homing ability of certain stem cells is another exciting option for enabling therapeutic delivery across the BBB/BTB. These cells can disseminate in solid tumours, thus enabling site-specific delivery, and potentially circumvent the short half-lives that many chemotherapeutic agents exhibit by stably expressing or releasing various anticancer agents[114].

A wide range of stem cell-based systems has been tested against brain malignancies; however, the high tumour tropism and ability to cross the BBB/BTB of neural stem cells (NSCs) and mesenchymal stem cells (MSCs) makes them the preferred carriers of therapeutics against primary brain tumours and brain metastases[115,116]. How stem cells migrate across the BBB is a controversial topic, but there are many reported similarities to immune cell infiltration (FIG. 3), including rolling on and adhesion to the endothelium and transmigration across the BBB. However, the specific molecular mechanisms might be different[117,118]. Importantly, stem cells can migrate along metastatic or invasive tumour borders, even when implanted intracranially at distant sites from the tumour, providing a promising platform to target brain micrometastases[119]. Although the reasons for the high tumour tropism of NSCs and MSCs are not yet fully elucidated, it is assumed that the chemoattractants and cytokines released by the tumour microenvironment play a central role[114].

Current approaches for cell-mediated drug delivery involve the genetic modification of the carrier cell to secrete anticancer proteins, antiangiogenic factors or immunosupportive factors, such as IL-12 (REF.[114]). The NSC-mediated expression of an enzyme that converts a separately administered non-toxic pro-drug into a cytotoxic drug via the bystander effect is another approach that was also found to be well tolerated in a recent phase I trial in glioblastoma[120]. MSCs can shield oncolytic viruses from the host immune system to maintain long-term reservoirs of the therapeutic virus in the tumour microenvironment[121]. Nanoparticles can also be loaded into stem cells via several mechanisms including passive and/or caveolin-assisted or clathrin-assisted uptake[122]. When these cells reach the tumour, unloading takes place via exocytosis (passively or in response to external stimuli) or by membrane disruption due to stem cell death[123].

Looking forward, analysis of stem cell migration kinetics and their short-term and long-term fate[124], along with further understanding of the signalling pathways mediating stem cell migration and their interaction with cancer cells, may not only offer the means of increasing the migrating pool of stem cells but also further potentiate this promising therapeutic strategy.

Physical approaches: focused ultrasound

The combination of low-intensity focused ultrasound (FUS) pulses and circulating microbubbles — lipid, albumin or polymer-shelled gas pockets (1–10 μm) that scatter sound and vibrate in response to ultrasound waves — provides a physical method to transiently disrupt the BBB/BTB (for 6–24 h) and increase the permeability of the BBB/BTB[125–127]. Although the exact mechanism of action of FUS-mediated BBB/BTB disruption remains unknown, it appears that the forces exerted by the microbubbles in the brain vessels while they vibrate in response to ultrasound pressure waves (the microbubbles expand during peak negative pressures and contract during peak positive pressures) can change vessel permeability. Recent studies indicate that FUS and microbubbles can increase the BBB/BTB permeability to small molecules by 2–4-fold in tumours in rats and mice[128,129], and experiments in the brains of healthy animals have shown that molecules with a diameter around 10 nm can readily extravasate[130,131]. Cellular and molecular evidence suggests that microbubble oscillation in the vessels can promote paracellular transport via transient reorganization of the TJs and facilitate transcellular passage through caveolae-dependent vesicular transport[125]. Although it is not well understood how the microbubble oscillation promotes the above changes, it appears that the microbubble dose and FUS exposure settings can be optimized to modulate transvascular transport, while limiting micro-haemorrhages and other adverse effects[132,133]. In addition to increasing BBB/BTB permeability[125], FUS and microbubble-mediated BBB/BTB disruption induces a shift from diffusive transport to convective transport in the tumour interstitial space[128]. Due to their very low diffusivity, the transport of large molecules is primarily mediated by convection[134], supporting the use of this technology with larger therapeutic agents, including antibodies (3–9 nm) and nanoparticles (10–60 nm)[125].

Of interest, this minimally invasive method to disrupt the BBB/BTB can be repeated numerous times over a long period without any apparent functional deficit,

Box 2 | **Invasive approaches to improve drug delivery to brain tumours**

Convection-enhanced delivery by direct injection
Direct intraparenchymal infusion of a drug via a cannula is used to bypass the blood–brain barrier (BBB) and promote bulk convective flow of the drug in the interstitial space. Although several phase I–III clinical trials have been carried out in patients with glioblastoma, the improvement in survival remains modest[216]. Optimization of the catheters and their placement, the administration protocol (for example, infusion rate and so on) and the physicochemical properties of the drug, along with methods to track it, may improve drug penetration and uptake[217,218].

Intrathecal and intraventricular injection
The drug is administered to the lumbar subarachnoid space or ventricular system, which contains cerebrospinal fluid (CSF), and diffuses to the brain through the brain–CSF barrier. Intrathecal chemotherapy is currently the main treatment for patients with neoplastic meningitis[219]. Intrathecal or intraventricular administration delivers much higher doses to the leptomeningeal space than systemically administered chemotherapies[219], but drug penetration to the brain parenchyma is limited[220].

Implantation of wafers, gels and microchips
Macromolecules incorporated in biodegradable polymers are released by a combination of diffusion and polymer degradation. Microchips can provide even more control over the release. Biodegradable discs or wafers that contain the chemotherapy drug carmustine led to modest improvement in survival in patients with high-grade glioma[221] but were also associated with high complication rates[222]. Current, preclinical investigations with injectable gel-like lipid capsules and microchips have shown promising results[223–225]. Evaluation of these methods in large animal models (for example, dogs with spontaneous gliomas) to demonstrate their improved ability to deliver drugs at larger distances, similar to those found in humans, is needed.

Table 1 | **Emerging minimally invasive approaches to improve transport across the BBB/BTB**

Method	Route	Compound	Clinical trial	Main findings and comments	Refs
Molecular					
Receptor mediated	Transcellular (transcytosis)	LRP1-targeting peptide bound to paclitaxel	Phase II	Similar toxicity to paclitaxel alone, increased delivery and doubled the median survival in patients with breast cancer brain metastasis	101,102
Lipid soluble	Transcellular (diffusion)	Enzastaurin — a synthetic bisindolylmaleimide that targets/inhibits both the PKC and the PI3K–AKT pathways	Phase III	Well tolerated but did not have superior efficacy compared with chemotherapeutic agent lomustine in patients with recurrent glioblastoma	226,227
Evade active efflux	Transcellular	None	NA	None	
Tight junction pathways	Paracellular (diffusion)	None	NA	None	
Biological					
Cellular	Paracellular and transcellular (diapedesis)	NSCs expressing cytosine deaminase plus 5-fluorocytosine prodrug	Phase I	Therapy was well tolerated in trials against glioblastoma; intracerebral microdialysis revealed that NSCs produced 5-fluoruracil in the brain in a dose-dependent manner	120
Viral	Paracellular (diffusion) and transcellular (receptor-mediated endocytosis: infect and spread)	Adenovirus-mediated gene therapy	Phase III	Marginal improvement in overall survival in patients with newly diagnosed glioblastoma with more treatment-related adverse events, including hemiparesis and aphasia, as compared with the control group	228
Physical					
Focused ultrasound with microbubbles	Paracellular and transcellular (diffusion and convection)	Carboplatin	Phase II	Demonstrated safety and provided preliminary evidence of its efficacy in patients with glioblastoma	138
Radiation	Paracellular and transcellular	None	NA	Inconsistent results; more rigorous assessment on the direct versus indirect effects (for example, inflammation) of radiation on blood–brain barrier is needed	154–156
Nanoparticles	Paracellular (diffusion) and transcellular (transcytosis)	Liposomal doxorubicin plus radiotherapy and temozolomide	Phase II	Well tolerated but did not add any significant clinical benefit in patients with glioblastoma	161–163

BBB, blood–brain barrier; BTB, blood–tumour barrier; LRP1, lipoprotein receptor-related protein 1; NA, not available; NSC, neural stem cell; PKC, protein kianse C.

delivering agents to white matter structures in Rhesus macaques[135]. Whereas FUS is a focal therapy, intraoperative MRI of the tumours combined with fast electronic beam steering can be used to target and treat a broad region of the brain[135,136]. Extensive preclinical research has shown that the method of FUS with microbubbles can lead to a more than 4-fold increase in the delivery and penetration of a range of intravenously administered anticancer agents in brain tumours[125]. This improvement in the delivery of anticancer agents has also led to a significant increase in the median survival time (3-fold) in multiple orthotopic murine tumour models, including glioma and breast cancer brain metastasis[128]. Numerous phase I clinical trials have confirmed the preclinical findings of increased BBB permeability by FUS in combination with microbubbles, demonstrated its safety and provided preliminary evidence for its efficacy[137,138].

Identifying optimum drug combinations to use with FUS and microbubbles is a critical next step for evaluating the therapeutic efficacy of this method. Effective integration of quantitative imaging, molecular analysis, minimally invasive assays and physiologically accurate mathematical modelling should help realize this goal[128]. Moreover, methods for imaging the cerebrovascular microbubble dynamics — a highly non-linear and, to some extent, stochastic physical process — through the skull along with methods to control them (that is, promote stable oscillation) are important for safe and effective BBB/BTB disruption[139–142].

Other strategies

Manipulate EC signalling pathways to increase BBB porosity. Intravenously injected angubindin 1, a protein which binds to the tricellular TJ protein lipolysis-stimulated lipoprotein receptor (LSR)[143], can increase the paracellular transport between brain microvascular ECs[144]. This increased transport across the BBB enabled the transient delivery of antisense oligonucleotides into the mouse brain that silenced a target RNA without any overt adverse effects[144]. Another approach includes the use of RNA interference (RNAi) to reduce the expression levels of TJ proteins and transiently modulate BBB permeability[145]. Although these methods have so far only been explored in the context of neurological disorders, they may provide a novel approach for targeting brain tumours and metastases. Moreover, exploiting

Fast electronic beam steering
Changing the direction of the ultrasound focus or pattern by changing the relative phases of the radiofrequency signals driving the transducer elements. Multiphase array transducers that are composed of hundreds of elements are able to perform electronic beam steering within a few microseconds.

the role of VEGF receptors to destabilize the TJs is an alternative method to increase BBB/BTB vessel permeability[61]. In addition to manipulating TJ pathways, modulating WNT–β-catenin signalling to induce fenestrations in ECs, as observed in the WNT subtype of medulloblastoma, could also provide a viable strategy to increase BBB/BTB leakiness[146].

Radiation. In addition to killing cancer cells, radiation is known to induce functional and structural changes in the tumour microenvironment[147]. Recent investigations in glioma-bearing rats have demonstrated increased BBB/BTB permeability[148–150], increased levels of an MRI contrast agent[151] and increased nanoparticle delivery after radiation[152] (dose >5 Gy; typical radiotherapy doses range from 30–50 Gy at 5–25 dose fractionation)[153], implicating radiation in changes of BBB/BTB permeability[151,152]. These findings are corroborated by recent clinical trials showing increased levels of gefitinib in cerebrospinal fluid in a dose-dependent manner in patients with brain metastasis[154]. Although these findings have opened up the possibility that radiation therapy could also improve therapeutic delivery across the BBB/BTB, other studies have failed to reproduce these findings[155]. Further research is needed to resolve whether enhancement in therapeutic delivery indeed can occur with radiotherapy, and if so, whether it is driven by direct effects of radiation on the vasculature or by other effects of radiation on the microenvironment, such as radiation-induced (neuro) inflammation[156] or other mechanisms.

Nanoparticles. Several passive and active strategies (as discussed earlier and in FIG. 3) based on different nanoparticle formulations have been proposed for improving drug delivery across the BBB/BTB[157–159]. Unfortunately, nanoparticle accumulation in the brain and brain tumours is very low[160], potentially explaining their limited therapeutic efficacy in clinical studies of high-grade gliomas[161–163]. Although several improvements in nanoparticle formulation for enhancing drug delivery in brain tumours have been proposed, most efforts either led to marginal improvements or were limited by the capabilities of the nanoparticle technology employed[157–159]. Although new and multifunctional nanoparticles are constantly being developed[164], a transition from formulation-driven designs to tumour microenvironment-based development that takes into account BBB/BTB heterogeneity and tumour biology may provide an alternative path towards identifying optimum nanoparticle designs[165–167]. Such approaches would first establish the rules for design of BBB/BTB-penetrable nanoparticles, such as identifying the optimum nanoparticle size[168] and shape[165], and should then fuel technological developments that are more targeted to the brain tumour microenvironment. Tracking the nanoparticles and their payload is also important for gaining insights into nanoparticle penetration and cargo delivery to the brain tumour microenvironment, and further support the refinement of nanoparticle designs for maximum drug delivery to brain tumours[167]. In concert with improved nanoparticle designs and understanding of the mechanism for drug delivery, remodelling of the tumour microenvironment to promote nanoparticle accumulation[169], potentially combined with triggered-release mechanisms and other strategies employed in extracranial malignancies[170], may further improve the cargo delivery and penetration by supporting higher concentration gradients from the blood to the tumour core (that is, diffusive transport).

Future directions

BBB and immunotherapy

Although the BBB plays a key role in limiting antigen presentation and immune cell infiltration, both of which are immunological hallmarks of brain tumours and especially glioblastomas[52,171,172], recent investigations have revealed potential strategies to alleviate its immunoprotective function. In this Review we focus only on the BBB/BTB, so for an extended review on brain cancer immunotherapy we direct the reader to a companion review in this issue by Sampson et al.[173].

The compromised BBB may facilitate the presentation of tumour-associated antigens, as implied by a recent clinical trial showing improved responses when immune checkpoint inhibitors were administered before surgery in patients with glioblastoma[174]. Consequently, the physical approaches discussed above, such as FUS, might enhance antigen presentation as well as enable immune checkpoint inhibitor penetration across the BBB/BTB and increase immune cell infiltration to further sensitize brain tumours and micrometastases to immunotherapies[175,176]. Although these approaches seem promising, the relative contribution of reprogramming immune cells within the brain tumours versus systemically needs further exploration[52]. For example, the penetration of anti-PD1 immune checkpoint antibodies through the BBB/BTB in medulloblastomas in mice did not seem necessary for an effective antitumour response[177]. Nevertheless, there is a clear need for strategies that can deliver immunotherapeutic agents into tumours that have high immune cell infiltration (that is, $CD8^+$ T cells) but no antitumour immunity and/or can improve T cell infiltration into the brain tumour microenvironment. Approaches that will limit or avoid the use of corticosteroids to control oedema[178], which causes immunosuppression and reduces BBB/BTB permeability[179,180], will also be crucial for effective immunotherapy of brain malignancies[181].

Brain tumours and metastases also pose a substantial challenge for adoptively transferred T cell therapy and chimeric antigen receptor (CAR) T cell therapy[182,183]. T cell infiltration from the circulation into the brain involves several stages, including adhesion to the endothelium and transmigration across the BBB/BTB (FIG. 3). However, in brain tumours, such as glioblastoma, the ECs express little or no cell adhesion molecules[52]. Recent studies indicate that administration of CAR T cells in the circulation elicited inferior antitumour responses when compared with intracranial tumour infusions and local intracranial delivery[184], further supporting the rate-limiting role of the BBB/BTB in T cell therapy. Hence, strategies that enhance the ability of T cells to penetrate the BBB/BTB[185] and/or change the BBB/BTB phenotype to become more amenable

to immune cell infiltration could lead to enhanced tumour control.

Biomarkers of BBB integrity

Assessment and quantification of BBB/BTB heterogeneity pre and post therapy to identify appropriate treatment windows has emerged as a clinical priority[186]. Several methods and biomarkers to assess extravasation across or disruption of the BBB have been proposed (summarized in TABLE 2). Contrast-enhanced MRI is the gold standard method for assessing BBB dysfunction. Most notably, K^{trans} — a bulk transport parameter obtained using dynamic contrast-enhanced (DCE) MRI that is dependent on both capillary permeability and perfusion — provides a semi-quantitative assessment of BBB permeability. This parameter has been used to quantify changes in BBB/BTB permeability after anti-angiogenic therapy (drop in K^{trans})[187] and characterize the effectiveness of targeted BBB/BTB disruption methods (increase in K^{trans})[129]. PET is another modality for delineating brain tumours and assessing the extent of BBB disruption. Whereas the most widely used PET tracers are those that penetrate the BBB (for example, ^{18}F-fluorodeoxyglucose (FDG), *O*-(2-^{18}F-fluoroethyl)-L-tyrosine (^{18}F-FET)), ^{18}F-3′-deoxy-3′-fluorothymidine (^{18}F-FLT) might be of interest for assessing BBB integrity as FLT uptake is dependent on BBB integrity[188].

Systems biology and the BBB

Although still in an early phase, systems biology approaches may provide a quantitative framework to meaningfully combine the increasing amount of data and information gathered during cancer diagnosis and lead to the development of rational strategies to overcome the BBB/BTB. Most notably, physiologically based pharmacokinetics modelling expanded to include new knowledge in cancer biology, including mechanics, blood vessel function and tissue oxygenation, can be used to identify effective new treatment regimens and drug combinations along with vascular remodelling strategies for effective delivery of nanomedicines[169]. Mathematical models combined with quantitative imaging can also be used to analyse drug transport across the BBB/BTB after modulating the paracellular and transcellular routes (for example, using FUS and microbubbles) to capture agent-specific transport parameters at the cellular level and optimize therapeutic interventions[128]. Moreover, by incorporating human-derived in vitro (that is, human cell lines) and ex vivo data on drug interaction with the efflux and uptake transporters and drug metabolism, model analysis can suggest in which patients or for which drugs the BBB/BTB may not pose major transport obstacles[189]. Direct assessment of drug concentrations via microdialysis methods or biopsy, during and after drug administration, respectively, may further increase the precision of these methods[190,191]. Likewise, data from The Cancer Genome Atlas may also be used to develop computational approaches to identify rate-limiting factors for effective antitumour immunity, including the type and abundance of immune cell populations that can cross the BBB/BTB, in order to inform effective cancer vaccine and immune checkpoint inhibitor therapies[192]. Incorporating data from in vivo measurements, including single cell profiles of the evolving NVU during primary brain tumour progression and metastasis, is a critical next step to improve these potentially personalized approaches.

Conclusion and perspectives

The BBB/BTB harbours considerable structural and functional heterogeneity within the microenvironment of the same lesion and across different cancer subtypes. Emerging insight into cellular, molecular and tumour subtype-specific features of the BBB/BTB has led to improved understanding of its role during tumour progression and treatment. These findings highlight the need to optimize and define tumour-specific therapeutic windows to disrupt CNS barriers and increase drug efficacy in the CNS with minimal side effects. Combining the strategies to improve penetration of anticancer agents across the BBB/BTB with biomarkers of BBB integrity may allow better delivery of therapeutics across the BBB/BTB and improved treatment outcomes.

Table 2 | **Biomarkers of BBB/BTB permeability**

Method	Agent or molecule	Measurement of BBB permeability	Clinical approval	Refs
Intravital microscopy	Fluorescent molecules	• Direct quantification of BBB/BTB permeability • Direct measurement of the extravasation of fluorescently labelled drugs and cells	No	128,229
MRI (contrast enhanced)	Gadolinium or magnetic nanoparticles	• Semi-quantitative assessment of BBB permeability (K^{trans}) • Indirect assessment of the extravasation of drugs that have similar permeability with gadolinium or magnetic nanoparticles • Overall limited correlation with the extravasation of drugs with different physiochemical properties from those of the agents	Yes	129,187
PET	^{18}F	• Relative assessment of BBB permeability (extravasation of imaging tracer) • Direct measurement of radiolabelled drug and cell accumulation in the brain	Yes	230–233

BBB, blood–brain barrier; BTB, blood–tumour barrier; K^{trans}, bulk transport parameter obtained using dynamic contrast-enhanced MRI that is dependent on both capillary permeability and perfusion; MRI, magnetic resonance imaging; PET, positron emission tomography.

Notes added in proof

Intriguingly, the recent discovery of bona fide functional synapses between gliomas and neurons that are associated with tumour proliferation and growth[193,194] suggests that it is possible for brain tumours to hijack CNS pathways to control, and not necessarily disrupt, the NVU in order to support their metabolic demands and fuel tumour growth. Interestingly, another finding elegantly demonstrates that breast cancer cells that colonize the brain can replace astrocytes in tripartite synaptic complexes by forming pseudo-tripartite synapses with neurons[195].

Another study published after this Review was accepted demonstrated that tumour-derived extracellular vesicles can breach the intact BBB in vivo by decreasing the expression of the late endosomal marker RAB7 in brain ECs and thereby increasing transcytosis[196].

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