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A neurocentric perspective on glioma invasion

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Abstract

Malignant gliomas are devastating tumours that frequently kill patients within 1 year of diagnosis. The major obstacle to a cure is diffuse invasion, which enables tumours to escape complete surgical resection and chemo- and radiation therapy. Gliomas use the same tortuous extracellular routes of migration that are travelled by immature neurons and stem cells, frequently using blood vessels as guides. They repurpose ion channels to dynamically adjust their cell volume to accommodate to narrow spaces and breach the blood-brain barrier through disruption of astrocytic endfeet, which envelop blood vessels. The unique biology of glioma invasion provides hitherto unexplored brain-specific therapeutic targets for this devastating disease.

Every year, more than 22,000 Americans are diagnosed with a malignant glioma. Current therapy for these primary brain cancers is inadequate, and approximately 95% of patients succumb to the disease within 5 years of diagnosis¹. Treatment involves a three-pronged approach, which consists of maximal tolerable surgical resection followed by radiation and chemotherapy. Together, these typically add only months of additional survival. Major reasons for treatment failures include the challenge of delivering sufficient dosages of chemotherapeutics across the blood–brain barrier (BBB) and the diffuse invasion of tumour cells into the surrounding brain, which shelters them from surgery and radiation. Early radical surgical interventions attempted to remove the entire affected brain hemisphere only to witness recurrence from cells that had crossed into the other hemisphere². Even now, in the era of modern microsurgical techniques, tumour recurrence is the norm, typically occurring within 1–2 cm of the original tumour border³. In light of the overall poor outcome from current therapies, a better understanding of glioma invasion is crucial for the future development of more effective interventions to contain this rapidly progressing disease. In this article, we review recent research, which offers hope that new strategies are emerging.

The past decade has witnessed important progress in our understanding of the genetic changes that characterize gliomas. Research from a consortium of scientists, The Cancer Genome Atlas Research Network, suggests that malignant gliomas comprise a genetically heterogeneous disease⁴, with core defects primarily in three signalling axes: the tyrosine kinase receptor pathway, the anti-apoptotic retinoblastoma pathway and the cell cycle

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regulatory (p53) axes. Not surprisingly, recent attention has focused on harnessing these molecular insights to tailor treatment to individual patients (for reviews, see REFS 5,6).

Another major advance has come from studies on the cells-of-origin from which gliomas derive. Gliomas may arise from adult neural stem cells or multipotent neural progenitor cells that persist in proliferative niches in the human CNS, namely the subventricular zone (SVZ) and the subgranular zone⁷ (for excellent recent reviews on this topic, see REFS 8,9). However, recent evidence indicates that the SVZ may be non-contributory in adults¹⁰. Gliomas may also arise from more differentiated lineages within the brain, including NG2 (neuron-gial antigen 2; also known as chondroitin sulphate proteoglycan 4 (CSPG4))-positive oligodendrocyte precursor cells^{11,12}, astrocytes and even mature neurons¹³.

Despite their genetic differences, and possibly divergent cells-of-origin, all malignant gliomas share one conserved feature: aggressive invasiveness. Strikingly, unlike other high-grade solid cancers, malignant gliomas do not rely on intravascular or lymphatic metastasis to spread; instead, glioma cells actively migrate through the tortuous extracellular spaces of the brain, which leads to the formation of distant satellite tumours. Hence, they behave much more like non-malignant brain cells during embryonic development, or adult stem cells in the mature brain, which similarly migrate along extracellular routes, often exploiting the brain vasculature or, after injury, nerve bundles as guides (BOX 1). Other shared features — such as their responsiveness to neurotransmitters and neuropeptides, and their interactions with the extracellular matrix (ECM) and neighbouring cells — suggest that gliomas retain much of their neurobiological root. We review these brain-specific biological traits in greater detail here.

Gliomas rarely metastasize outside the brain

Malignant cancers spread in two phases. The first requires metastasis to another organ and typically occurs through haematogenous and lymphatic routes. The second phase involves local intra-organ invasion whereby cells infiltrate an organ to form a new tumour. Gliomas are exceedingly adept at infiltrating organs, but only 0.4–2% metastasize outside the brain^{14–16}. This is in stark contrast to other solid cancers, including small-cell lung carcinoma, mammary ductal carcinoma, prostate cancer and colorectal cancers, which characteristically metastasize beyond the original organ. Several hypotheses have sought to explain the dearth of extracranial metastasis. First, although glioma cells associate with blood vessels, they may be unable to breach the basement membrane and enter the vasculature¹⁷. Second, the extraneural tissues may not contain the right milieu of growth factors that are capable of supporting glioma growth. Third, individuals with gliomas may not survive long enough for extracranial metastasis to become apparent. In support of this hypothesis, average postoperative survival time in patients with extracranial metastasis is 16–24 months¹⁸, which is longer than average. Although any or all of these explanations may be valid, gliomas are nevertheless exceedingly adept at intra-organ invasion, as are the neural precursor cells from which they are derived.

Where do glioma cells migrate?

The pathways through which glioma cells migrate can be roughly divided into two compartments: the perivascular space and the brain parenchyma. These spaces differ with regard to inherent mechanical and physical constraints. The perivascular space is fluid-filled, continuous with the subarachnoid space and surrounds all blood vessels, including penetrating arteries, arterioles and veins. The parenchyma contains neuronal and glial cell bodies and their processes, so extracellular spaces in the parenchyma are narrow and tortuous, and provide considerably greater physical resistance than the perivascular space. These compartments provide constitutive trails for glioma cell migration¹⁹.

Extracellular space and the importance of the ECM

Both the perivascular space and the interstitial spaces of the parenchyma contain a combination of ECM molecules, some of which constitute basement membranes, which separate functional domains. On the whole, the brain lacks the stiff fibrillar collagen matrix that is typical of other tissues, explaining its gelatinous consistency¹⁹. The interstitial spaces in the parenchyma are filled with a matrix mainly composed of proteoglycans (lectican family; also known as the hyalactan family) and their binding partners, hyaluronan and tenascins²⁰. These water-binding ECM molecules are primarily produced by astrocytes and oligodendrocytes and form a gellike filling throughout the extracellular brain spaces. Many of these molecules — for example, neurocan and brevican (lectins), and phosphocan (a CSPG) — are brain-specific and particularly suited to support cell migration²¹. Tenascins form a family of CSPGs that bind to other ECM components and to cell surface receptors, most notably integrins. Interestingly, gliomas lay down their own pro-migratory ECM proteins and secrete brevican and tenascins to increase their invasiveness^{21–23}. Tenascin C, which plays an important part in embryonic cell migration, is also produced by invading gliomas²⁴. In addition, both tenascin C²³ and tenascin W localize to blood vessels that are occupied by gliomas and are believed to stimulate vessel sprouting or angiogenesis^{25,26}.

Basement membranes are specialized and highly organized self-aggregating sheets composed of ECM molecules that are 50–100 nm thick and surround blood vessels in all tissues. In the brain, parts of the vascular tree, including the meninges, large vessels and postcapillary venules, are encircled by two basement membranes, whereas only one composite basement membrane surrounds capillaries^{27,28}. Basement membranes are rich in fibronectin and vitronectin, molecules that increase glioma cell motility^{29–31}. The basement membrane functions as a physical and biochemical barrier that separates mesoderm-derived epithelial cells from ectoderm-derived neurons and glia. It is also an important component of the BBB, serving as a second physical barrier, and in addition signals from the basement membrane induce tight junction formation, which is necessary to maintain the BBB. Disruption of the basement membrane in pathological states has been linked to dysfunction of the BBB³².

Invading glioma cells interact with the ECM

Cell movement is an orchestrated biological process that requires a coordinated sequence of adhesion of the leading edge of a migrating cell, anchoring to the ECM, and last, detachment

of the trailing end³³. Actin–myosin molecular motors provide the main contractile force, and myosin II is specifically important in glioma invasion through narrow spaces³⁴. Cell attachment is mediated by cell–cell and cell–matrix receptors, such as integrins, cadherins and neural cell adhesion molecules, whereas detachment requires the activity of proteases that also degrade ECM components, such as matrix metalloproteinases (MMPs)^{35–37}.

Integrins are transmembrane receptors involved in cell–cell and cell–matrix interactions and enable cells to sense their environment and adjust their behaviour to environmental cues³⁵. Integrins are heterodimers composed of one of 18 α - and one of 8 β -subunits, and the combination determines substrate specificity and signalling modality. Upregulation of the $\beta 1$ subunit is associated with increased glioma invasion^{37,38}, whereas $\alpha v\beta 3$ and $\alpha v\beta 5$ support tumour-induced angiogenesis^{37,39}. The integrin $\alpha v\beta 5$ can be selectively blocked with a synthetic peptide, cilengitide, which demonstrated modest anti-tumour activity in a Phase II trial⁴⁰ but failed to increase overall survival in a large randomized Phase III trial.

Although the ECM molecules of the basement membrane are important for attachment of cell processes, the dense matrix that fills the extracellular space can be an obstacle for migrating glioma cells. To overcome this, glioma cells express a large number of secreted proteases, including the MMPs membrane type MMP1 (also known as MMP14), MMP2 and MMP9, the serine protease uPA and cell surface proteases, including ADAMs (disintegrin and metalloproteinases; also known as adamalysins). In addition, they recruit microglia, astrocytes and endothelial cells to also secrete proteases. The combined activity of these proteases remodels the ECM to favour tumour invasion while also regulating the activity of growth factors and chemokines that increase glioma proliferation and migration^{21,37}. The use of MMP inhibitors to halt glioma invasion seems to be a promising therapeutic strategy, but clinical trials carried out thus far have not achieved clinical success.

Glioma cells migrate along the vasculature

As already described by Scherer (BOX 2), gliomas frequently populate and migrate along existing brain structures, including nerve tracts, blood vessels and the meninges. Whether they accidentally reach these structures or purposefully seek these out is important to understand. Our knowledge regarding this question is limited, in part, because it is difficult to dynamically assess cell migration within these structures. However, several biochemical and histological studies that use a combination of *in vivo* and *ex vivo* preparations are beginning to shed light on this question.

Blood vessels in particular are a critical substratum for glioma cell migration. *In situ* studies of human glioma cells demonstrate that when injected into the brain, the vast majority (>85%) of glioma cells move into contact with a blood vessel⁴¹ (FIG. 1). The recruitment of glioma cells to blood vessels occurs through bradykinin, which acts as a chemotactic signalling peptide⁴¹. Bradykinin is produced in vascular endothelial cells by enzymatic cleavage of high-molecular-weight kininogen by kallikrein. Binding of bradykinin to bradykinin 2 receptors (B2Rs) activates these G protein-coupled receptors (GPCRs) and causes inositol-1,4,5-trisphosphate receptor 3 (IP3R3)-dependent increases in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in glioma cells⁴². The resulting cyclic changes in $[\text{Ca}^{2+}]_i$ activate downstream ion channels that support cell shape and volume changes that are

necessary for cell invasion. Pharmacological inhibition or genetic elimination of B2Rs on gliomas impairs their homing onto blood vessels⁴¹. As a result, preclinical studies are evaluating the use of the bradykinin receptor inhibitor icatibant (Firazyr; Shire) as a treatment option. In the United States, icatibant was approved by the US Food and Drug Administration (FDA) in 2011 for the treatment of acute attacks of hereditary angioedema, an autosomal dominant disease that is characterized by unrestrained bradykinin activity. In rodent models of glioma, icatibant significantly reduced the percentage of glioma cells associated with blood vessels from 77% to 19%, which resulted in blunted glioma cell migration through the cerebral parenchyma and smaller tumour volumes⁴¹. Although bradykinin is probably not the only chemoattractant that guides glioma cells to blood vessels, it does seem to be the predominant molecule (responsible for approximately 75% of glioma cells on blood vessels). These data suggest that preventing the association of glioma cells with the vasculature could be an important therapeutic strategy to curtail glioma dispersion. Further clinical evaluation of this FDA-approved drug for the treatment of gliomas is warranted.

Located along larger brain vessels, the Virchow–Robin space is continuous with the subarachnoid space and is separated from the parenchyma by the basement membrane created by astrocytes along their endfeet. The cerebrospinal fluid (CSF) contained in this compartment comprises the glymphatic system⁴³ in which the CSF is emptied into collecting veins along the meninges, leading to the clearance of parenchymal deposits such as amyloid. Hence, it stands to reason that for cells entering this space, convection towards the brain surface and into the subarachnoid space could influence cellular movement. The perivascular space provides little physical resistance to glioma migration. It thus serves as an accessible route for glioma invasion. Whether cells take advantage of the convective forces of the CSF or instead move through guided migration along the basement membrane remains to be seen.

How do glioma cells migrate?

Whether glioma cells migrate along the basement membrane of blood vessels, intraparenchymally or follow white matter tracts, they are challenged by the brain's limited extracellular space. Measurements *in vivo* in the murine neocortex suggest that the width of the undisturbed extracellular space in the brain is 38–64 nm⁴⁴, which is vastly smaller than the size of migrating glioma cells. In light of these spatial constraints, it is remarkable that cells can move at all.

Hydrodynamic mode of cell invasion

Time-lapse imaging studies conducted *in vivo* and *ex vivo* show that invading glioma cells undergo remarkable shape and volume changes^{34,45,46}. Using quantitative, three-dimensional imaging *in vivo*, these studies demonstrate that enhanced green fluorescent protein-expressing human gliomas implanted into mice show periodic changes in cellular volume (as much as ~33%) as cells cycle between extension and retraction of processes⁴⁶. Interestingly, volume changes of similar magnitude occurred as cells migrated across Transwell barriers over a range of barrier sizes (3–8 μm), suggesting that once a cell

encounters a barrier it reduces its volume by the maximal extent possible. In glioma cells, this equates to about a 33% volume decrease and required the invading cells to shed essentially all 'free cytoplasmic water' that was not bound to proteins, nucleic acids and macromolecules⁴⁶. Given that at least 50% of a mammalian cell is occupied by organelles, this decrease in volume by 33% is close to the theoretical limit.

Cytoplasmic water fluxes across the cell membrane through aquaporin 1 (AQP1) or AQP4 (REFS 47–49) and follows the osmotic force provided by ions that flux through channels. The emerging hydrodynamic model then suggests that gliomas repurpose Cl⁻ and K⁺ channels (which are normally used to regulate the membrane potential) to regulate their shape and cell volume (FIG. 2). The underlying channels have been molecularly identified and belong to the CIC family of voltage-gated Cl⁻ channels and the KCa family of Ca²⁺-activated K⁺ channels⁵⁰.

The most interesting aspect of this model is that the energetic driving force for the movement of the cytosol comes from an unusual outwardly directed gradient for Cl⁻. In mature neurons, [Cl⁻]_i is around 10 mM and is at electrochemical equilibrium; hence, Cl⁻ will not move upon opening of ion channels. Glioma cells, by contrast, accumulate Cl⁻, which increases [Cl⁻]_i to ~100 mM, well above the equilibrium predicted by the resting potential⁵¹.

This is achieved by the sustained activity of the Na⁺-K⁺-Cl⁻ cotransporter 1 (NKCC1; also known as SLC12A2), which imports Cl⁻ against its concentration gradient by harnessing the transmembrane Na⁺ gradient. As a result of the high [Cl⁻]_i, glioma cells are 'primed' to shrink their volume upon the opening of any Cl⁻ channel in the membrane, and the insertion of a recombinant ligand-gated Cl⁻ channel proved that Cl⁻ efflux is indeed sufficient to achieve the volume decrease observed in *in vivo* experiments⁵¹. NKCC1 is constitutively expressed in gliomas, and this expression positively correlates with invasiveness⁵². Importantly, NKCC1 activity is regulated through phosphorylation by the protein kinase WNK3 (REF. 53), which is in turn regulated by the epidermal growth factor (EGF) through AKT (also known as protein kinase B)⁵². In addition to accumulating Cl⁻, NKCC1 can regulate migration speed through interaction with the actin cytoskeleton, thereby enhancing contractility as glioma cells migrate over a substratum⁵². These findings suggest that NKCC1 is a multimodal, anti-invasive target. Indeed, bumetanide (Bumex; Hoffmann-La Roche), an FDA-approved diuretic that inhibits NKCC1, significantly reduced glioma invasion in preclinical studies^{52,54}.

CIC3 is in turn responsible for the efflux of Cl⁻, which is followed by the efflux of water; this enables glioma cells to adjust their shape as they move. Although voltage-gated, increases in [Ca²⁺]_i are sufficient to activate CIC3 after Ca²⁺/calmodulin-dependent protein kinase II-dependent phosphorylation⁵⁵. If these Cl⁻ channels are inhibited by chlorotoxin (also known as TM-601), a 36-amino-acid peptide produced in the venom of the Israeli desert scorpion (*Leiurus quinquestriatus*), glioma cell migration is reduced^{56,57}. Importantly, not only does chlorotoxin block glioma cell migration through inhibition of CIC3 but it also specifically binds to human glioma cells and not to surrounding neural tissue^{56,58}. Evaluation of intracavitary administration of chlorotoxin in a Phase I/II clinical

trial (NCT00040573) in 18 adult patients demonstrated that the drug was well tolerated, with no significant toxicities, and bound specifically to the glioma for the entire 5-day monitoring period⁵⁹. Because of the specific binding, chlorotoxin is also being used in several studies to delineate tumour tissue from surrounding normal brain tissue, both in whole-brain imaging to measure tumour extent and intraoperatively to facilitate resection of malignant tissue^{60,61}. Development of similar inhibitors of other ion channels that are expressed by gliomas may be a worthy therapeutic strategy to restrict the spread of glioma cells.

To maintain electroneutrality, the CIC3-mediated Cl⁻ flux must be coupled with a cation conductance. Glioma cells express multiple members of the KCa channel family, which are also activated upon increases in [Ca²⁺]_i. In glioma cells, only KCa1.1 and KCa3.1 (also known as SK4 and the Gardos channel) form functional channels^{50,62–64}. Both KCa1.1 and KCa3.1 have an important role in maintaining a cation conductance and in balancing Cl⁻ efflux, and are crucially important for glioma cell migration, which can be induced by chemokines. Inhibition of either channel reduces glioma invasion *in vitro* and *in vivo*^{50,64–66}. For KCa3.1, the specific inhibitor senicapoc has been evaluated for treatment of sickle cell disease and could be explored in future glioma trials.

Ligands increase migration by increasing Ca²⁺-dependent activation of ion channels

Both Cl⁻ and K⁺ channels implicated in glioma invasion are sensitive to [Ca²⁺]_i. Therefore, any [Ca²⁺]_i increase results in a simultaneous increase in the conduction of Cl⁻ and K⁺, which in turn causes the hypothesized volume changes.

Interestingly, many ligands that increase glioma cell migration also increase [Ca²⁺]_i. For example, bradykinin, which is responsible for chemotactic attraction of gliomas to blood vessels (see above), raises [Ca²⁺]_i in glioma cells through the GPCR B2R^{41,42}. Cl⁻ and K⁺ channels expressed by glioma cells are activated by bradykinin, and inhibition of these channels is sufficient to block bradykinin-induced migration and tumour expansion^{50,66}. Specifically, gliomas express a splice isoform of the channel KCa1.1, which has increased sensitivity to Ca²⁺ (REFS 67,68). Given that KCa1.1 channels localize to the same lipid-raft microdomains as IP3R, the interaction between these proteins may play an important part in ligand-activated migration⁶². Aside from bradykinin, several other ligands, including lysophosphatidic acid (LPA), thrombin and sphingosine 1-phosphate, also bind to GPCRs, leading to IP3R-dependent [Ca²⁺]_i increases, thereby converging on a pathway that potentiates glioma cell migration through ion channel activation^{42,69}.

One of the most common groups of mutations in gliomas affects the gene encoding the EGF receptor (EGFR). In primary glioblastomas, ~40% have *EGFR* amplifications, ~60% have *EGFR* overexpression and ~30% have *EGFR* mutations that result in a gain of function⁷⁰. EGFR is a receptor tyrosine kinase that is activated by EGF and, in a similar manner to GPCRs, its activation leads to an IP3R-dependent increase in [Ca²⁺]_i in human glioma cells⁴². EGF also leads to downstream activation of transient receptor potential canonical channel 1 (TRPC1), a non-selective cation channel that is permeable to Ca²⁺ (REF. 71). Ca²⁺ influx through TRPC1 activates glioma Cl⁻ channels, which match the electrophysiological characteristics of CIC3, and inhibition of either significantly hampers migration^{71,72}. The ability of glioma cells to respond to EGF is similar to that of other

migratory cells in the adult brain^{73,74}. These studies demonstrate that an important mechanism by which chemotactic ligands increase migration is by promoting Ca²⁺-dependent activation of ion channels.

Thus, a coordinated cascade that begins with ligand-induced activation of ion channels, and which leads to the movement of Cl⁻ and K⁺ ions along with water, enables glioma cells to adjust their cell shape and volume to navigate the narrow extracellular spaces of the brain (FIG. 2).

Invading glioma cells disrupt brain function

In the healthy brain, astrocyte endfeet cover ~99% of the vascular surface^{75,76}, and the interaction between astrocytes and the vasculature is of functional importance for brain homeostasis, the coupling of blood flow and neuronal activity, and the BBB. The astrocyte–vascular interface is composed of endothelial cells forming the vascular walls, one or two basement membranes anchoring endothelial cells and astrocyte endfeet, which are also attached to the basement membrane. In addition, contractile cells are embedded between endothelial cells and astrocytes, either in direct contact with the vessel (pericytes at capillaries) or located between basement membranes (vascular smooth muscle cells at arterioles and arteries)⁷⁷.

Many astrocytic functions depend on the polarized membrane domain that is typical of astrocyte endfeet⁷⁸. This polarity is characterized by the enriched localization of several proteins in the endfoot. For example, the water channel AQP4 builds a complex with the inward rectifier K⁺ channel Kir4.1. Both are anchored into the membrane by the dystrophin-associated glycoprotein complex^{78,79} and are involved in K⁺ and water homeostasis⁸⁰. Glucose transporters localize to endfeet, where they take up glucose, which serves as an energy substrate for astrocytes and nearby neurons after conversion to lactate⁸¹.

Disruption of astroglial function by abluminal glioma cell migration

When human glioma cells are implanted into the rodent brain, a large majority of glioma cells associate with blood vessels and move along their surface to invade the unaffected brain^{45,82,83}. In this process, glioma cells surround blood vessels and seem to ‘lift up’ astrocytic processes as they degrade the perivascular basement membrane^{45,82,83}. In response to glioma-induced displacement, astrocytes shift into a reactive phenotype and withdraw processes from the vasculature^{82,84}. This perturbation of the physical interaction between vascular endothelial cells and astrocytes has two important consequences. First, it leads to a breakdown of the BBB and, second, it disrupts the neurovascular unit (FIGS 3,4). Although a leaky BBB is commonly observed in tumour masses in which newly generated vessels lack tight junction proteins, the focal breach of the BBB owing to local invasion is surprising and unexpected. Whether the displacement of astrocytic endfeet is responsible for a downregulation of tight junctional proteins such as claudin 5 and zonula occludens 1 (REF. 82) or whether it is the presence of the glioma cell per se remains to be shown. Also, whether this opening is transient or permanent is unknown. The consequence, however, is both troubling and potentially exciting. This focal breach enables motogens (thrombin, LPA and bradykinin, as discussed above), potentially toxic molecules and immune cells in the

bloodstream to enter the brain, but it also may permit the targeted delivery of anti-invasive chemotherapeutic drugs to invading cells, which hitherto had been thought to be protected from chemotherapeutics by the BBB.

In addition, the displacement of astrocytic endfeet from the vessel wall abolished the functional regulation of vessel diameter by astrocytes. Astrocytes are responsible for a local activity-dependent adjustment of blood flow, whereby the astrocytes translate neuronal activity into vasodilation or vasoconstriction⁸⁵. However, vessels encased by glioma cells were rendered unresponsive to astrocyte-derived vasoactive substances that regulate blood flow⁸². Interestingly, no harm seems to be done to the underlying smooth muscle, as vessels remain fully responsive to direct smooth muscle stimulation. Nonetheless, the absence of astrocyte-derived signals to the smooth muscle on tumour-ensheathed vessels may compromise adequate delivery of oxygen and glucose to neurons.

The biological advantage gained by glioma cells migrating along vessels and displacing astrocytic endfeet is probably multifactorial. We propose that glioma cells at the tumour border seek this interaction to facilitate proliferation and invasion while simultaneously disrupting astrocyte physiological functions that are crucial for brain homeostasis. Glioma cells probably seek the perivascular space to better position themselves to extract important nutrients from the bloodstream. While inhabiting this space, glioma cell-driven displacement of astrocytic endfeet can interrupt several astrocyte–vasculature processes. These include the astrocyte–neuron lactate shuttle, arteriolar cerebral blood flow regulation and BBB maintenance.

Glutamate in glioma biology

Glutamate has been shown to have many important roles in glioma biology: for example, acting as a multifaceted growth factor⁸⁶, a space-vacating excitotoxin⁸⁷ and a mitogenic stimulant^{88,89}. In the vicinity of a tumour, extracellular glutamate can reach neurotoxic concentrations⁹⁰. Glutamate is produced by the tumour from glutamine and released through the system x_c^- cysteine–glutamate antiporter, which is highly expressed by human gliomas^{90–92}. The sustained toxic increases in extracellular glutamate concentration lead to neuronal hyperexcitability and eventual death, and recent studies suggest that increased extracellular glutamate concentration is responsible for the tumour-associated seizures^{93,94} that occur in up to 80% of patients with primary brain tumours. With regards to glioma invasion, the extracellular release of glutamate through system x_c^- also has an autocrine effect, which promotes invasiveness. Specifically, glioma cells lack the GluR2 subunit of the AMPA receptor, which renders the channels permeable to Ca^{2+} (REFS 88,89). Thus, glutamate released from glioma cells activates these Ca^{2+} -permeable AMPA channels, leading to oscillations in $[Ca^{2+}]_i$ (REF. 88), which drive cell motility. When either system x_c^- -mediated glutamate release or Ca^{2+} -permeable AMPA channels were inhibited, both Ca^{2+} oscillations and migration through spatial barriers were inhibited⁸⁸. In addition, when AMPA channels were made impermeable to Ca^{2+} , glioma cells invaded the brain less robustly, leading to smaller tumour volume^{89,95}. Although it is clear that glutamate-dependent Ca^{2+} signalling is critical for the preservation of a pro-migratory phenotype, it is still unknown whether this Ca^{2+} signalling is necessary for system x_c^- -mediated glutamate

release. Taken together, these data suggest that glutamate released by glioma cells not only has extrinsic effects on neuronal excitability but also intrinsically promotes glioma invasiveness in a Ca^{2+} -dependent manner.

Glutamate release can be inhibited using sulfasalazine, an FDA-approved drug for the treatment of inflammatory bowel disease, through inhibition of system x_c^- (REFS 88,96). In rodent models of gliomas, sulfasalazine reduced the frequency of electrographic epileptic activity through inhibition of glutamate extrusion^{93,94}. Sulfasalazine also decreased the autocrine pro-migratory effects of glutamate on glioma cells by interfering with Ca^{2+} -permeable AMPA receptor-dependent cell invasion and restrained *in vivo* tumour spreading by 50% (REF. 88). Thus, sulfasalazine should be considered as a therapeutic agent for gliomas, both to reduce seizure activity and to control invasive growth in patients. However, a preliminary study of ten patients with recurrent gliomas suffering from advanced disease found that sulfasalazine administration lacked clinical efficacy⁹⁷. Regrettably, this study was under-powered and performed in patients who were already in very poor health. Future studies should evaluate sulfasalazine as an adjuvant therapy to prevent tumour-associated seizures in patients who have not already progressed to end-stage disease, and one such study is underway (NCT01577966).

Conclusions and future challenges

Recent genomic analysis from The Cancer Genome Atlas Research Network and others illustrates a much broader molecular heterogeneity of malignant gliomas than previously appreciated⁴. This should have probably been expected in light of the multiple lineages from which gliomas can arise in the adult brain. However, the propensity of glioma cells to move and invade the brain via the same extracellular routes used by neural cells along existing structures such as blood vessels seems to be shared among all of the malignant gliomas. Despite being cancerous, numerous biological traits inherited from their neural ancestors remain active and important in the disease process. These biological traits and the interactions with neurons, other glia and endothelial cells have thus far not been exploited therapeutically, but these uniquely neural biological traits of gliomas are ripe for further exploration, and several of the aforementioned targets along with available drugs are summarized in TABLE 1. Gliomas notoriously develop drug resistance and/or upregulate compensatory pathways in response to monotherapies; hence, use of these agents as adjuvants or as a part of a drug cocktail may meet greater clinical success. Targeting ion channels and transporters that mediate hydrodynamic shape and volume changes, which enable cells to navigate the extracellular space of the brain efficiently, is proof of concept that anti-invasive strategies are possible. The finding that invading glioma cells focally breach the BBB suggests a role for early administration of specific anti-invasive drugs. Similarly, chemotaxis towards bradykinin could be exploited by blocking B2Rs using specific inhibitors, such as icatibant.

The vast majority of current clinical trials approach gliomas from a traditional oncological perspective, focusing on the tumour per se. Future therapies should be more neurocentric and consider the brain-specific interaction of gliomas with their host organ. Maintenance of the BBB could reduce leakage of serum components into parenchyma, thereby controlling

oedema. Also, prevention of glutamate release through system x_c^- may decrease excitotoxic neuronal death, leading to less space for tumour expansion. For a sea-change in our treatment of gliomas to occur, novel aspects of glioma biology must be targeted.

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Box 1**Shared migratory traits between neural progenitor and glioma cells****Migration along blood vessels and white matter tracts**

Glioma cells actively seek out blood vessels⁴¹ and migrate along them⁸² (panel a of the figure). Human glioma cells (HuN) implanted intracranially into mice are shown invading along blood vessels (panel b of the figure). Indeed, the perivascular space is an important invasion pathway and is recognized as one of Scherer's secondary structures⁹⁸ (BOX 2). Like glioma cells, neural progenitor cells or neuroblasts generated in the subependymal zone, one of the neurogenic zones still active in the adult brain of rodents, migrate over long distances. In rodents, progenitor cells migrate in tangentially orientated chains along the rostral migratory stream (RMS) into the olfactory bulb. The RMS contains a high density of parallel blood vessels that serve as a scaffold for neuroblast migration^{99,100} (panel c of the figure). After injury, the brain seems to regain more plasticity, therefore subependymal and subgranular zone neurogenesis is increased and several cell types putatively migrate towards a lesion site in an attempt to restrict or repair tissue damage. Whereas astrocytes are usually stationary even after injury¹⁰¹, neuron-glia antigen 2 (NG2) cells — glial progenitor cells destined to form oligodendrocytes — readily migrate into lesion sites^{102,103}. In addition, neuroblasts are recruited from the subependymal zone, and in contrast to NG2 cells, migrate over long distances to injury sites in the cortical grey or white matter using blood vessels or white matter tracts (panel d of the figure) as a substrate¹⁰⁴.

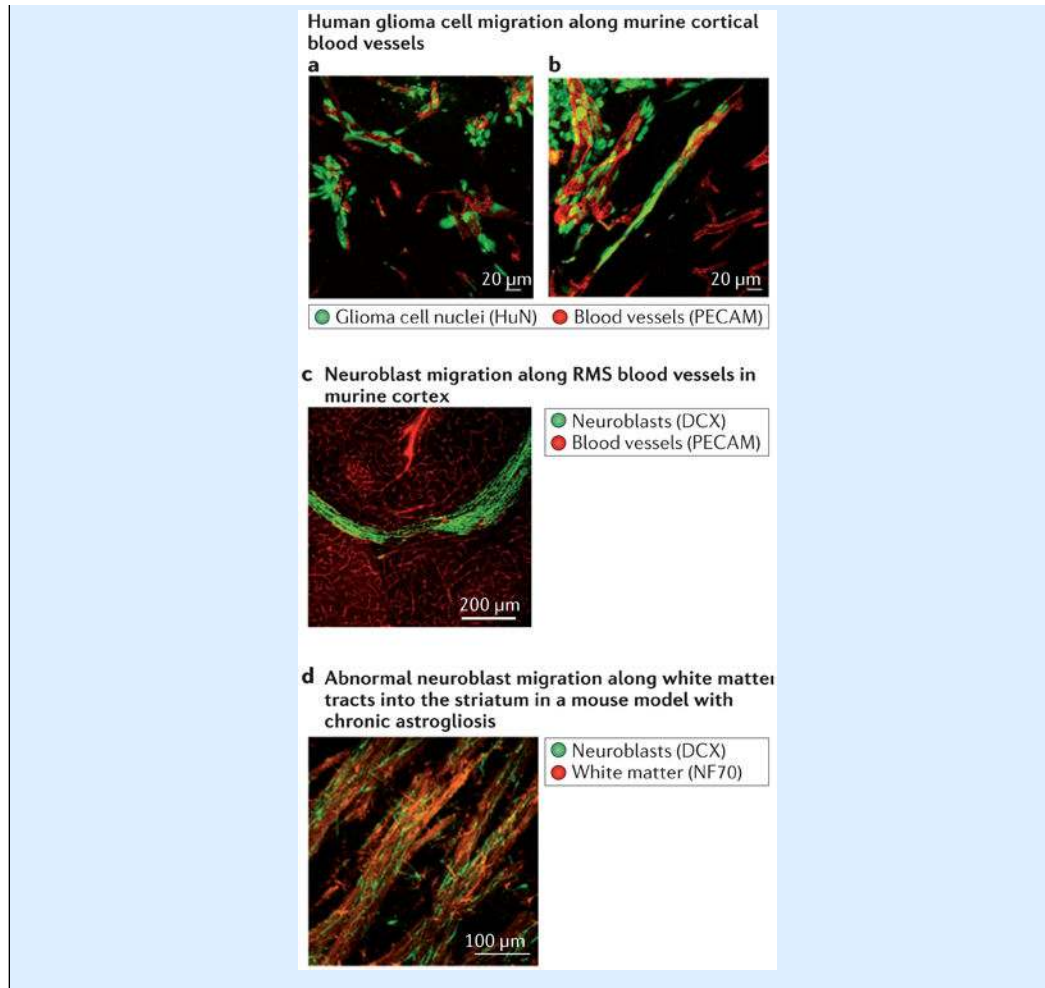
Glutamate-mediated Ca²⁺ changes promotes motility

Gliomas assiduously release glutamate, which acts as a multifunctional growth factor that supports growth and invasion⁸⁶. Glutamate acts as an autocrine and paracrine ligand to promote invasion by inducing oscillatory intracellular Ca²⁺ changes⁸⁸ through activation of Ca²⁺-permeable AMPA receptors⁸⁹. This mechanism is remarkably similar to that used by migratory neurons during the development of the cerebellum¹⁰⁵, during which Ca²⁺ oscillations mediated by NMDA receptors guide granule cell migration.

Ca²⁺-activated K⁺ channels are essential for migration

One of the targets of these Ca²⁺ oscillations in migrating cells is the Ca²⁺-activated K⁺ channel KCa3.1. This channel is required for the migration of neuroblasts along the RMS¹⁰⁶ but is equally utilized by invading gliomas⁵⁰.

The similar biological traits of gliomas and neural stem cells or neural progenitor cells may provide further support for these being a likely cell-of-origin for gliomas. DCX, doublecortin; NF70, neuronal filament 70; PECAM, platelet endothelial cell adhesion molecule.

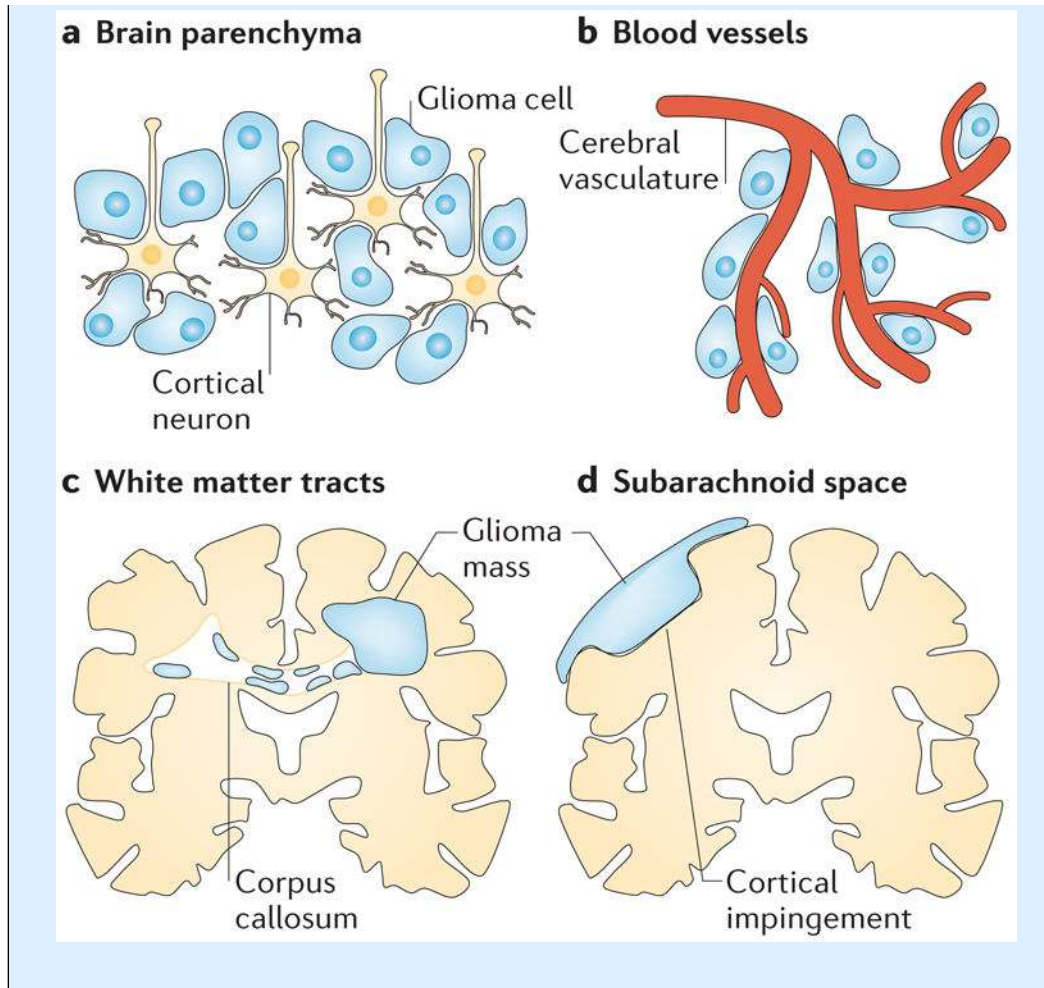


Box 2**A historical perspective: Scherer's structures**

The extensive invasion of gliomas within the brain has fascinated pathologists and neurosurgeons for the past century. In 1938, Hans Joachim Scherer, a German neuropathologist, published a widely referenced manuscript in which he serially sectioned the brains of 100 patients with glioma and carefully examined the tumour and the surrounding brain⁹⁸. He concluded that gliomas migrate along existing brain structures that he called 'secondary structures', which are formed by the interaction of glioma cells with the neural microenvironment. In appreciation of his pioneering work, these are now frequently called 'Scherer's structures' and include the following pathways of glioma cell invasion: the brain parenchyma (part **a** of the figure), pre-existing blood vessels (part **b** of the figure), white matter tracts (either perifascicularly intrafascicularly or interfibrillary) (part **c** of the figure) and the subarachnoid space below the meningeal covering of the brain (part **d** of the figure).

Importantly, Scherer suggested that invading glioma cells assume the physical shape of the structure they occupy rather than each cell having a characteristic form¹⁰⁷. Hence, cells from the same tumour do not form a single type of Scherer's structure; instead, cells can appear very different in shape, depending on whether they migrate along blood vessels, nerve tracts or the subarachnoid space. This is an important observation that is of relevance to the recently discovered hydrodynamic mechanism of cell movement discussed in this Review.

These early observations were derived from static images, which do not permit examination of the dynamic changes that occur during the natural history of disease. As such, they leave several important questions regarding mechanisms of glioma cell migration unanswered. For example, is this migration a stochastic process, with cells randomly reaching these 'secondary structures', or do these structures actively attract gliomas and provide a distinct advantage for their invasion? Is it possible that these structures simply provide the path of least resistance? As discussed in this Review, glioma cells respond to a range of chemical cues, including growth factors, peptides, chemokines and extracellular matrix components, making it likely that they actively seek out a preferential microenvironment for invasion.



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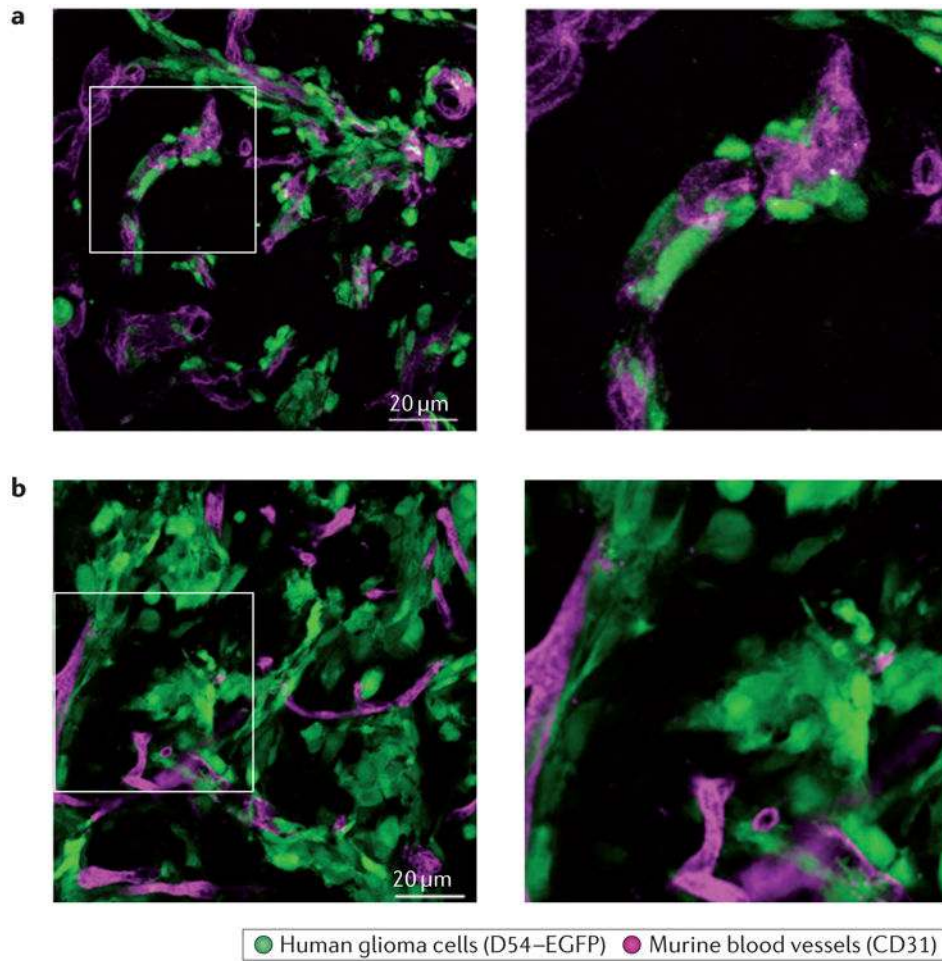


Figure 1. Glioma cells associate with blood vessels in the brain

Most glioma cells that have migrated away from the main tumour mass into the brain parenchyma can be found on blood vessels. Examples of the patient-derived glioma line GBM22 (part **a**) and of the human glioma cell line D54 (part **b**) are shown, with high-magnification zooms of the boxed areas on the right. In both examples, glioma cells closely associate with blood vessels in the brain. Human glioma cells are labelled with enhanced green fluorescent protein (EGFP), and murine vessels are labelled with a CD31-specific antibody.

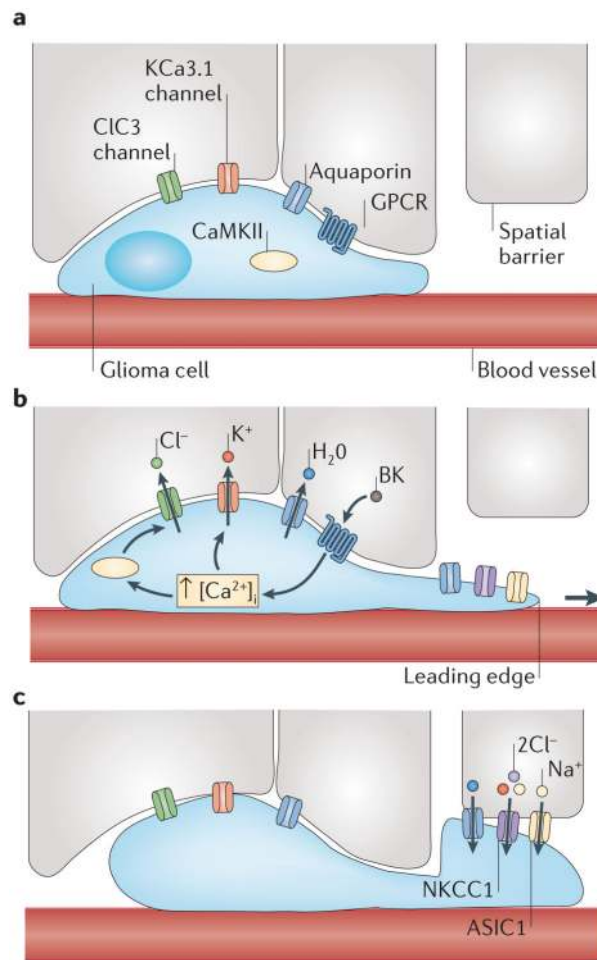


Figure 2. A hydrodynamic model of glioma cell migration

a | Glioma cells encounter spatial barriers (for example, other cells) as they migrate along blood vessels through the brain. Gliomas express various channels, including Cl^- channel protein 3 (CIC3; a voltage-gated Cl^- channel), Ca^{2+} -activated K^+ channel KCa3.1 and aquaporins. **b** | Upon stimulation of G protein-coupled receptors (GPCRs), there is a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in glioma cells, leading to Ca^{2+} -dependent activation of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII)-dependent CIC3 and KCa3.1 channel opening. This leads to the efflux of Cl^- and K^+ , obligating water to flow down its osmotic gradient and leave the cell. This volume decrease enables glioma cells to decrease cytoplasmic volume and squeeze into small spaces. **c** | After passing a spatial barrier, glioma cells can regain volume through ion influx through the Na^+ - K^+ - Cl^- cotransporter 1 (NKCC1) and acid-sensing ion channel 1 (ASIC1). BK, bradykinin.

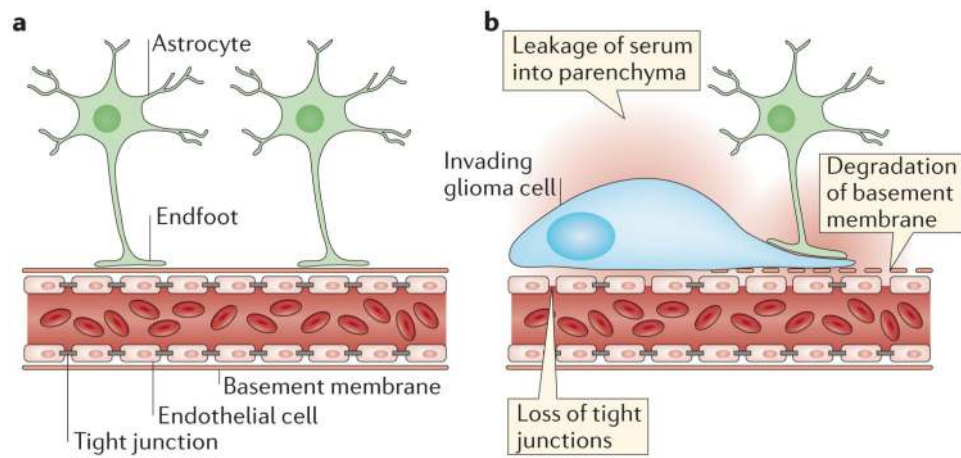


Figure 3. Glioma cells break down the blood–brain barrier

a | In the healthy brain, astrocytic endfeet surround blood vessels. Vascular endothelial cells form the blood–brain barrier (BBB) through tight junctions, trapping cells and serum components in the vascular lumen. **b** | Glioma cells migrate along blood vessels, leading to displacement of astrocytic endfeet, degradation of tight junctions on endothelial cells and breakdown of the basement membrane surrounding blood vessels. In turn, serum components leak into the neural parenchyma.

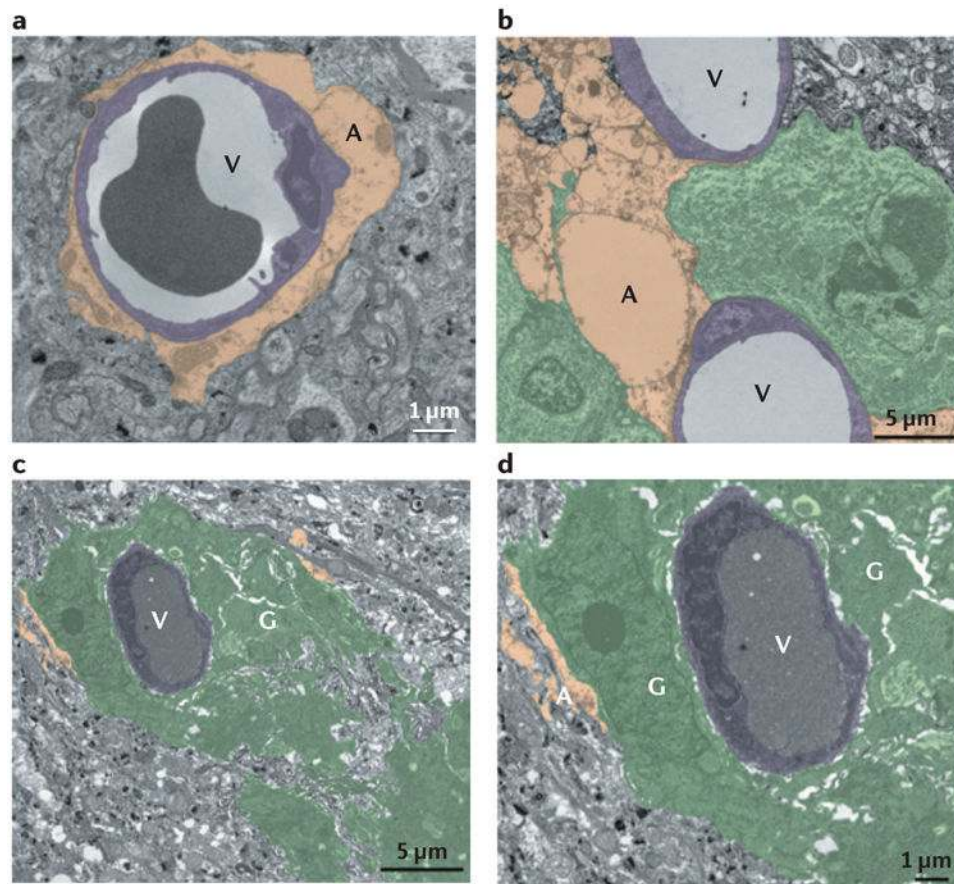


Figure 4. Glioma cells displace astrocytic endfeet from blood vessels

Electron micrographs of blood vessels (V) that are normally covered by astrocyte endfeet (A) (part **a**) can be partially (part **b**) or completely (parts **c** and **d**) ensheathed by patient-derived glioma cell lines GBM14 (part **b**) and GBM22 (parts **c** and **d**) glioma cells (G).

Table 1
Possible neural targets for clinical management of malignant gliomas

Protein	Normal function in nervous system	Presumed mechanism in glioma biology	Drug	Trial
MMP2, MMP3, MMP9	Synaptic plasticity ¹⁰⁸ ; neuroblast migration along RMS ¹⁰⁹	Digestion of extracellular matrix	• Prinomastat • Chlorotoxin	• Phase II • Phase I/II
Integrin	Neuroblast, oligodendrocyte, microglial and astrocytic migration (for a review, see REF. 110); cell signalling	Cell-cell and cell-matrix contact	Cilengitide	Phase III
AQP1–AQP4	Water and ion homeostasis ⁸⁰ ; production of CSF (for a review, see REF. 111)	Cell volume regulation		
CIC3	Regulation of LTP ¹¹² and GABAergic quantal size ¹¹³	Cell volume regulation	Chlorotoxin	Phase I/II
KCa1.1	Repolarization after action potential ^{114,115}	Volume change, pro-invasive		
KCa3.1	Neuroblast migration along RMS ¹⁰⁶	Volume change, pro-invasive	Senicapoc	Phase II (sickle cell)
NKCC1	Neuronal $[Cl^-]_i$ regulation ¹¹⁶ ; glial volume regulation ¹¹⁷	Cl^- accumulation serves as energy for volume changes for invasion and proliferation	Bumetanide	FDA-approved diuretic
Bradykinin receptor 2	Vasodilation	Blood vessel association, chemotaxis	Icatibant	Orphan drug status, Phase I (angioedema)
System x_c^-	Glial oxidative homeostasis (for a review, see REF. 118)	Autocrine glutamate signalling	Sulfasalazine	Phase 0

AQP, aquaporin; $[Cl^-]_i$, intracellular Cl^- concentration; CIC3, Cl^- channel protein 3; CSF, cerebrospinal fluid; FDA, US Food and Drug Administration; KCa, Ca^{2+} -activated K^+ channel; LTP, long-term potentiation; MMP, matrix metalloproteinase; NKCC1, Na^+ - K^+ - Cl^- cotransporter 1; RMS, rostral migratory stream.