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REVIEW

Cell-specific blood–brain barrier regulation in health and disease: a focus on hypoxia

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The blood–brain barrier (BBB) is a complex vascular structure consisting of microvascular endothelial cells that line the vessel wall, astrocyte end-feet, pericytes, as well as the basal lamina. BBB cells act in concert to maintain the characteristic impermeable and low paracellular flux of the brain vascular network, thus ensuring a homeostatic neuronal environment. Alterations in BBB stability that occur during injury have dire consequences on disease progression and it is clear that BBB cell-specific responses, positive or negative, must make a significant contribution to injury outcome. Reduced oxygenation, or hypoxia, is a characteristic of many brain diseases that significantly increases barrier permeability. Recent data suggest that hypoxia-inducible factor (HIF-1), the master regulator of the hypoxic response, probably mediates many hypoxic effects either directly or indirectly via its target genes. This review discusses current knowledge of physiological cell-specific regulation of barrier function, their responses to hypoxia as well as consequences of hypoxic- and HIF-1-mediated mechanisms on barrier integrity during select brain diseases. In the final sections, the potential of current advances in targeting HIF-1 as a therapeutic strategy will be overviewed.

Abbreviations

BBB, blood–brain barrier; HIF-1, hypoxia-inducible factor-1; TJ, tight junction; ECs, endothelial cells; TEER, transendothelial electrical resistance

The maintenance of CNS homeostasis is performed largely by the blood–brain barrier (BBB), which together with neurons and microglia form an organization referred to as the neurovascular unit (NVU). The BBB is dynamic performing both passive and active features of the brain endothelium essentially acting as a vascular gatekeeper that controls movement of substances from the circulating blood into the brain parenchyma – a role crucial for neuronal, and therefore CNS, homeostasis. Accumulating experimental evidence supports the hypothesis that opening of the BBB triggers a chain of events leading to neuronal dysfunction and damage resulting in neurological disease, and when coupled with previous insults BBB disruption could have serious detrimental consequences for patient outcome. Despite this knowledge, our understanding of physiological barrier function, as well as during disease, is very limited. In addition, the contribution of the perivascular cells that modulate barrier characteristics and their individual responses to injury is poorly characterized. This review will discuss the mechanisms through which hypoxia, a characteristic state of many brain diseases, disrupts barrier function and the importance of BBB cell-specific responses to barrier integrity. Additionally, consequences of hypoxia-mediated barrier modulation during brain disease and future therapeutic use of hypoxia-inducible factor-1 (HIF-1) modulators in the clinics will be reviewed.

Physiology

BBB organization and cell-specific function

The BBB is a complex structure consisting of microvascular endothelial cells (ECs) that line the vessel wall, astrocyte end-feet, pericytes, as well as the basal lamina (see Figure 1), which together with neurons and microglia form an organization referred to as the NVU. The basal lamina is an essential part of the BBB that surrounds the capillaries thereby anchoring the cells in place and providing a link with the resident

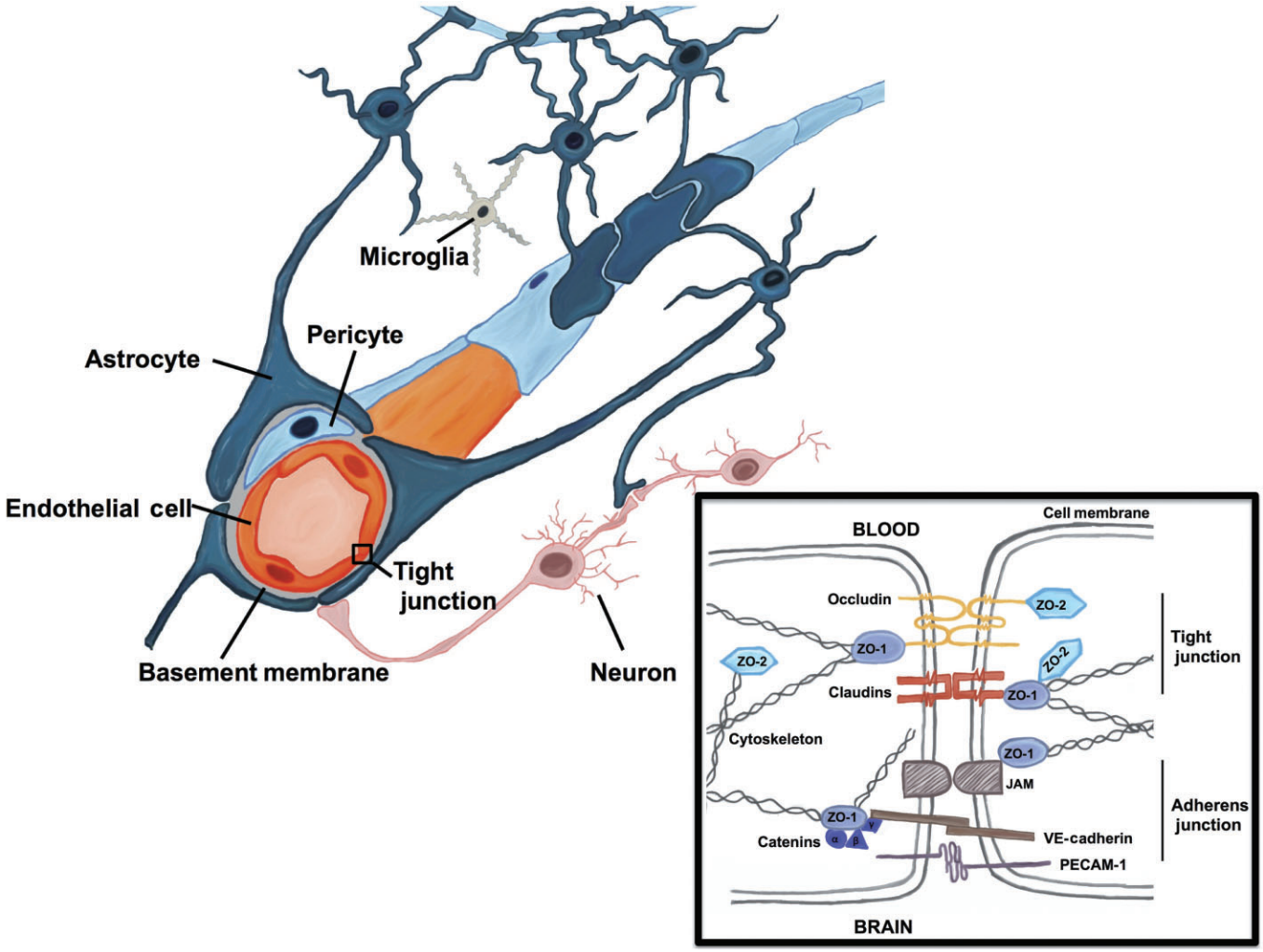

Figure 1

The BBB protects neurons and glial cells from systemically circulating agents as brain microvessels form a very tight barrier clearly distinct from vessels in other organs. The barrier is formed by ECs (red), which line the blood vessels, surrounded by pericytes (light blue), BM (grey) and astrocytic (dark blue) end-feet. Astrocytes provide the cellular link to the adjacent neurons (pink). The illustration also shows microglial cells (grey) in contact with astrocytes. Inset (black box) shows the cell–cell contact that takes place between two adjacent ECs at the BBB and demonstrates the basic organization of the BBB tight and adherens junctional proteins. Note the interactions of the junctional proteins with the cytoskeleton.

brain cells. The brain ECs are in direct contact with brain pericytes (Abbott *et al.*, 2010; Ogunshola and Al-Ahmad, 2012) and separated from the brain parenchyma by two layers of extracellular matrices (ECM). The inner layer is formed by the vascular basement membrane (BM) localized at the EC basolateral side and is shared with neighbouring pericytes (Hallmann *et al.*, 2005). The outer layer is formed by the glia limitans as a result of glial end-feet processes that surrounds or ensheaths the cerebral vascular tree (Paolinelli *et al.*, 2011). Perivascular glial end-feet form a direct interface between the vascular compartment and the brain parenchyma (see Figure 1) and are therefore thought to represent key checkpoints of brain metabolism and function (Wolburg *et al.*, 2009a).

BBB cells act in concert to maintain the characteristic impermeable and low paracellular flux of the vascular network that is chiefly mediated via high expression levels and localization of tight junction (TJ) proteins, namely, occludins, claudins, junctional adhesion molecules and their adaptor protein zonula occludens-1 (ZO-1; reviewed in Förster, 2008; Abbott *et al.*, 2010). TJs are elaborate structures that function as both a 'zipper' that separates the apical and basolateral cell membranes thus enabling asymmetric distribution of membrane constituents, and a 'fence' that limits paracellular permeability (see Figure 1 inset). Thus, stringent regulation of CNS homeostasis by severe restriction of the paracellular diffusional pathway between the ECs and substances and/or cells within the circulating blood is ensured. TJs are highly dynamic structures that rapidly undergo subcellular redistribution, expression level alterations as well as post-translational modifications, all of which affect protein–protein interactions (reviewed in Förster, 2008; Ogunshola, 2011). Cellular interactions and local release of factors (discussed in detail later), in addition to signals from circulating

substances, have marked effects on TJ expression and therefore barrier integrity. Brain ECs do not seem to spontaneously express high TJ levels but are induced by the surrounding perivascular cells, astrocytes and pericytes (Ballabh *et al.*, 2004; Abbott *et al.*, 2006; Ogunshola, 2011). Astrocytes are the most abundant cell in the brain and their long filamentous end-foot processes extend towards and envelope the vascular network. Astrocytes have long been known to play a central role in inducing expression of TJ proteins in microvascular ECs (Abbott, 2002; Ogunshola, 2011). Although pericytes have very close contact with ECs, lying juxtaposed to the capillary and sharing a BM, for a long time, the function of this cell in barrier maintenance was largely unknown. Scientific advances in tools and methodologies, however, have provided recent evidence from both *in vitro* and *in vivo* studies that pericytes also significantly contribute to barrier stability during development and adulthood.

Barrier effectiveness is directly related to the ability of the perivascular cells to maintain their normal functional activities. As such, alterations or modifications of perivascular cell properties *per se*, as a result of disease progression or signalling pathway activation, will compromise barrier integrity and increase permeability. Despite this fact, the highly coordinated signalling mechanisms and dynamic interactions that exist between individual cells of the BBB remain largely unknown. Such knowledge is key to better understand barrier function under physiological as well as pathological conditions. This implies possible future development of cell-specific therapies and targeted treatments that could indeed represent an important way to stabilize barrier function during injury. The following sections deal with some of the cell-specific responses that occur particularly during oxygen deprivation and diseases characterized by hypoxia in more detail. Notably, because of the complexity related to studying BBB *in vivo*, much of the data comes from *in vitro* model systems, however, where possible reference to *in vivo* studies will also be given.

Astrocytes at the BBB

Similar to neurons and other glial cells astrocytes originate from the neuroectoderm (Allen and Barres, 2009). To date, 11 different types of astrocytes are known of which eight are specifically associated with blood vessels (Abbott *et al.*, 2006). Astrocytes are crucially involved in control of neuronal function through regulation of brain homeostasis, supply of neurons with energy and substrates for neurotransmission and recycling of neurotransmitters. Furthermore, they represent the connection between neurons and the brain vasculature, which they cover with their end-feet. Almost 50 years ago, the close proximity of the astrocytic end-feet to the brain endothelium raised the hypothesis that astrocytes contribute to BBB regulation (Davson and Oldendorf, 1967). Since then, a number of *in vivo* (Stewart and Wiley, 1981; Janzer and Raff, 1987; Willis *et al.*, 2004) and particularly *in vitro* studies have demonstrated the importance of astrocytes to BBB induction and regulation. Model systems using astrocyte-endothelial co-cultures (Dehouck *et al.*, 1990; Rist *et al.*, 1997; Fischer *et al.*, 2000; Al Ahmad *et al.*, 2009) as well as astrocyte-conditioned media (ACM) (Rubin *et al.*, 1991; Raub *et al.*, 1992) demonstrated the inductive potential of astrocytes on barrier tightness by increased transendothelial electrical resistance (TEER) and reduced permeability to low molecular weight tracers (for detailed review, refer to the excellent article by Deli *et al.*, 2005). There is now good evidence that barrier induction through astrocytes is mainly mediated via changes in the number, length and complexity of endothelial TJs (Tao-Cheng *et al.*, 1987), expression levels of TJ and associated-proteins like occludin and ZO-1, respectively (Siddharthan *et al.*, 2007; Colgan *et al.*, 2008), and the redistribution of junctional proteins such as platelet endothelial cell adhesion molecule-1, ZO-1 and claudin-5 at endothelial junctions (Colgan *et al.*, 2008; Al Ahmad *et al.*, 2011). In addition, astrocytes modulate expression and polarized localization of endothelial transporters, such as P-glycoprotein (P-gp) or multi-drug resistance protein (MRP) (Berezowski *et al.*, 2004; Al Ahmad *et al.*, 2011) as well as BBB-specific enzyme-systems such as γ-glutamyl transpeptidase (Meyer *et al.*, 1991; Hayashi *et al.*, 1997) and thus crucially regulate EC function.

Major efforts have been made to unravel how astrocytes modulate TJs, transporters and enzyme systems. Astrocytes are known to secrete a large number of substances including peptides, growth factors and chemokines (Nico and Ribatti, 2012) several of which modulate barrier function, such as basic fibroblast growth factor (bFGF), TGF-β1, glial cell-derived neurotrophic factor and src-suppressed C-kinase substrate (SSeCKS) (Igarashi *et al.*, 1999; Sobue *et al.*, 1999; Lee *et al.*, 2003; Reuss *et al.*, 2003; Dohgu *et al.*, 2004; Walshe *et al.*, 2009; Shimizu *et al.*, 2012). For instance, bFGF has demonstrated barrier-tightening effects *in vitro* by reducing barrier permeability (Sobue *et al.*, 1999) and increasing endothelial γ-glutamyl-transpeptidase and alkaline phosphatase activity (Hafny *et al.*, 1996). *In vivo* bFGF knockout increases BBB permeability to albumin and reduces the expression of ZO-1 and occludin, and coincides with reduced astrocyte differentiation (Reuss *et al.*, 2003). The effect of astrocyte-secreted TGF-β1 on barrier function is controversial. While some studies observed reduced BBB permeability and increased TJ expression in the presence of TGF-β1/TGF-β (Dohgu *et al.*, 2004; 2005; Walshe *et al.*, 2009), others reported adverse effects (Shen *et al.*, 2011). This discrepancy may be explained by the fact that TGF-β effects on the endothelium are highly dependent on the endothelial activation state and the tissue environment, especially the ECM as it mediates TGF-β activation (Cambier *et al.*, 2005).

Over the past 20 years, our understanding of the importance of astrocytes as regulators of BBB physiology and how they modulate barrier characteristics has dramatically increased but, realistically, unravelling of the complex pathways has just begun.

Pericytes at the BBB

The origin of CNS pericytes remains unclear with derivation from the mesoderm and neuroectoderm as well as from the monocyte lineage under general discussion (Sa-Pereira *et al.*, 2012). Diverse functions within the brain have been attributed to pericytes including regulation of brain homeostasis, angiogenesis, blood flow, immune and phagocytic activity, as well as being a source of pluripotent stem cells (Sa-Pereira *et al.*, 2012). The effect of pericytes on BBB induction and maintenance is less well characterized than astrocyte-induced responses. During development pericytes fulfil central roles in vessel stabilization through inhibition of EC proliferation

and migration as well as regulation of vessel maturation (Antonelli-Orlidge *et al.*, 1989; Hellström *et al.*, 2001; Al Ahmad *et al.*, 2011; Sa-Pereira *et al.*, 2012). Recent *in vivo* studies using platelet-derived growth factor (PDGF) receptor β (PDGFRβ) knockout mice have significantly improved our understanding. During embryonic angiogenesis, pericytes are recruited to the vessels via EC-derived PDGF-β. The impaired recruitment of pericytes to the brain microvasculature caused by inhibition of PDGF-β signalling, induced either by PDGF-β or PDGFRβ knockout, resulted in severe vascular consequences such as increased vessel diameter, formation of microaneurysms, endothelial hyperplasia and increased vessel permeability (Lindahl *et al.*, 1997; Hellström *et al.*, 2001; Daneman *et al.*, 2010). These alterations in vessel tightness were attributed to enhanced caveolae formation and transcytosis, abnormal TJ alignment and increased expression of permeability inducing factors like VEGF and angiopoietins (ANG) –2 (Hellström *et al.*, 2001; Daneman *et al.*, 2010). Corresponding studies in adult mice revealed that pericytes are also crucial for adult BBB maintenance, as reduced pericytic vessel coverage resulted in increased BBB permeability, altered endothelial gene expression and loss of astrocyte end-feet polarization (Armulik *et al.*, 2010). Age-dependent pericyte loss in PDGFRβ$^{+/-}$ mice elevated parenchymal accumulation of blood proteins and leakage of neurotoxic and vasculotoxic substances into the brain parenchyma and a reduction in the expression of ZO-1 and occludin protein (Bell *et al.*, 2010). *In vitro* studies using cells of human, murine, bovine and porcine origin further underlined the positive effect of pericytes on BBB tightness (Hayashi *et al.*, 2004; Dohgu *et al.*, 2005; Nakagawa *et al.*, 2007; Al Ahmad *et al.*, 2009; Daneman *et al.*, 2010). Similar to astrocytes, pericytes are capable of secreting factors that modulate BBB permeability under normal conditions, like ANG-1 (Hori *et al.*, 2004; Wang *et al.*, 2007), TGF-β1 (Dohgu *et al.*, 2005) and GDNF (Shimizu *et al.*, 2012) that increase ZO-1, claudin-5 or occludin *in vitro* (Hori *et al.*, 2004; Wang *et al.*, 2007; Shimizu *et al.*, 2011). Therefore, similar to astrocytes, pericytes seem capable of modulating TJs. In contrast, a few *in vitro* studies report that co-culture of ECs with pericytes reduces TEER via induction of matrix metalloproteinases (MMP)-2 and -9 activity and activation of VEGF-mediated signalling (Zozulya *et al.*, 2008; Thanabalasundaram *et al.*, 2010). Interestingly, this negative effect of pericytes seems to be dependent on their differentiation state (Thanabalasundaram *et al.*, 2011).

Tricellular interactions – ECs, pericytes, astrocytes

How ECs, astrocytes and pericytes influence each other and concertedly modulate barrier function is a highly interesting but challenging question. Due to the complexity of the BBB *in vivo* data on this topic is limited, but some *in vitro* studies have investigated the effect of simultaneous astrocyte and pericyte co-culture on ECs. The majority of these studies report increased TEER in triple cultures compared with co-culture or monoculture models (Nakagawa *et al.*, 2007; 2009; Al Ahmad *et al.*, 2009); however, decreased TEER in triple cultures due to the presence of pericytes was also reported (Hatherell *et al.*, 2011). Interestingly, only Hatherell *et al.* used human ECs. Importantly, the permeability to sodium-fluorescein, sucrose or 40 kDa FITC-dextran was not significantly altered in triple cultures compared with contact astrocyte co-cultures (Al Ahmad *et al.*, 2009; Nakagawa *et al.*, 2009). Nakagawa *et al.* indeed observed that claudin-5 and ZO-1 protein expression is increased and more restricted to cell–cell borders in triple cultures compared with endothelial monocultures (Nakagawa *et al.*, 2009). Using a novel three-dimensional culture model, our group showed that co-culture of ECs with astrocytes and pericytes also induces polarized, luminal localization of the ABC transporters P-gp and MRP-2 and that astrocytes and pericytes are indispensable for P-gp activity (Al Ahmad *et al.*, 2011). However, it is patently evident that many more studies and wider use of triple culture model systems is invaluable for understanding the multicellular crosstalk at the BBB.

Basement membrane

The BM represents an important but often neglected component of the BBB. Although the BM does not act as a diffusion barrier *per se*, it fulfils important functions for the BBB by providing structural support for the cells by anchoring them in place. Moreover, the BM provides an important platform for mediating signalling events that regulate cell differentiation, proliferation, migration and adhesion (Baeten and Akassoglou, 2011) and thus BBB function. The BM consists of structural matrix proteins that are secreted by the BBB cells with cell-specific differences (a detailed list is presented in Baeten and Akassoglou, 2011). BBB cells are anchored to the BM via ECM receptors, of which the best understood ones are the integrins and dystroglycan (Baeten and Akassoglou, 2011). More than just providing a physical link between the BM and the cells, the matrix receptors represent important modulators of signalling pathways that allow cellular adaptation to environmental changes. Perlecan, the dominating proteoglycan in the endothelial BM (Engelhardt and Sorokin, 2009), has been shown to interact with a number of different growth factors like VEGF, PDGF or TGF-β and retain them in the ECM thereby regulating cellular signal transduction to control cell responses and BBB maintenance (Roberts *et al.*, 2012).

Composition of the BM can also regulate BBB tightness (Tilling *et al.*, 1998). P-gp expression is increased when ECs are cultured on brain-derived ECM (Tatsuta *et al.*, 1994). ECMs derived from pericytes or astrocytes exhibit differential effects on EC impedance *in vitro* compared with controls grown on their endogenous ECMs (Hartmann *et al.*, 2007). Interestingly, ECs cultured on astrocyte-derived ECM displayed a higher resistance than those cultured on pericyte-derived ECM. Astrocyte-derived ECM has been shown to up-regulate the expression of endothelial-specific γ-glutamyl-transpeptidase and activity (Mizuguchi *et al.*, 1994; Hayashi *et al.*, 1997). Several BM-associated proteins have been shown to be important for BBB maintenance *in vivo*. Depletion of perlecan *in vivo* is lethal due to deterioration of brain vesicles and myocardial BM (Baeten and Akassoglou, 2011), whereas specific depletion of perlecan in the endothelial BM results in microvessel bleeding and endothelial dilations (Hallmann *et al.*, 2005). Agrin, another proteoglycan, accumulates at brain microvessels at the time of BBB tightening (Wolburg *et al.*, 2009b) and its depletion results in TJ disruption (Rascher *et al.*, 2002) and depolarization of astrocytic end-feet mainly through redistribution of aquaporin 4 (AQP4;

Wolburg *et al.*, 2009b). Osada *et al.* showed that blocking of β1-integrin using a neutralizing antibody results in altered claudin-5 localization and increased endothelial permeability *in vitro* and *in vivo* (Osada *et al.*, 2011).

Taken together, these data indicate that the BM composition participates in BBB regulation; however, the mechanisms are only poorly understood and need to be better addressed.

Hypoxia and the BBB

Mechanisms of hypoxic BBB disruption

Hypoxia, when the oxygen demand of tissues is not met, acts as an initial trigger for pathophysiological changes at the BBB such as altered distribution of water and ions, inflammatory events and oxidative stress, oedema formation, infiltration of peripheral immune cells and leakage of blood proteins into the brain. In addition, hypoxia induces major alterations in vessel structure as it stimulates proliferation of ECs leading to formation of new blood vessels and furthermore promotes activation and proliferation of astrocytes (reviewed by Ogunshola and Al-Ahmad, 2012; Stanimirovic and Friedman, 2012). A large number of *in vivo* and *in vitro* studies have demonstrated that hypoxia is a major stress factor inducing BBB disruption (Schoch *et al.*, 2002; Kaur *et al.*, 2006; Al Ahmad *et al.*, 2009; Lochhead *et al.*, 2010). Regarding the temporal course of hypoxic barrier opening, detailed *in vivo* studies are rare. Increased BBB permeability to Evans blue was observed in mice 6 h after onset of hypoxia (7% O_2; Li *et al.*, 2011). After 24 and 48 h of hypoxic exposure to 8% O_2, increased BBB leakage to sodium fluorescein was demonstrated in mice (Schoch *et al.*, 2002; Bauer *et al.*, 2010). Another study by Witt and colleagues demonstrated that exposure to 6% O_2 for 1 h with subsequent re-oxygenation in a rat model resulted in a biphasic opening of the BBB within the first hour and again after 6–24 h of re-oxygenation (Witt *et al.*, 2008). For *in vitro* models, a generalized statement about the course of barrier opening is almost impossible due to different culture systems, cell sources, oxygen concentrations and read-outs. However, decreased endothelial tightness has been observed from the first 30 min of hypoxic exposure for up to 48 h (Abbruscato and Davis, 1999a; Fischer *et al.*, 1999; Yamagata *et al.*, 2004; Fleegal *et al.*, 2005; Kuhlmann *et al.*, 2007; Al Ahmad *et al.*, 2009).

The mechanisms of hypoxic barrier disruption have been studied intensively and it is evident that disruption of the BBB occurs on many different molecular levels. TJ complexes are major targets of hypoxic BBB disruption. At the molecular level, hypoxia modulates protein expression levels and subcellular redistribution of occludin, ZO-1 and claudin-5 (Fischer *et al.*, 2002; Mark and Davis, 2002; Koto *et al.*, 2007; Bauer *et al.*, 2010; Willis *et al.*, 2010). The redistribution critically regulates TJ integrity and is probably mediated via phosphorylation changes at serine, threonine and tyrosine residues and by caveolae-mediated endocytosis that determines their localization at the plasma membrane and interaction with other proteins at the TJ (Luissint *et al.*, 2012). PKC enzymes, myosin light chain kinase, and RhoA regulate TJ protein phosphorylation and contribute to hypoxic or inflammatory barrier disruption (for review, refer to Luissint *et al.*, 2012). Increased transcellular and pinocytic activity of brain ECs represent additional events contributing to augmented barrier permeability during hypoxia (Plateel *et al.*, 1997; Cipolla *et al.*, 2004). Hypoxia-mediated alterations and breakdown of the BM also aggravate BBB opening and are particularly important during ischaemic events (Candelario-Jalil *et al.*, 2009; Stanimirovic and Friedman, 2012). Direct and indirect contribution of pericytes and astrocytes to hypoxic-mediated BBB permeability is discussed below.

HIFs as mediators of the hypoxic response

Hypoxia requires an immediate response of the affected tissues/cells to sustain their function and prevent cell death. The most important tasks of the adaptation process are to decrease energy consumption and increase oxygenation. This is mainly achieved through metabolic adaptation and temporal cell cycle arrest as well as induction of angiogenic and erythropoietic genes to increase oxygen delivery to the hypoxic tissues (Wenger *et al.*, 2005). Different signalling pathways are involved in these events including the unfolded protein response, mammalian target of rapamycin signalling, and HIF-mediated gene regulation (Majmundar *et al.*, 2010). HIFs are considered master regulators of the hypoxic response and are heterodimeric transcription factors composed of an oxygen-sensitive α-subunit and a constitutively expressed β-subunit. Under normoxia, the HIFα subunits are constitutively transcribed but constantly targeted for proteasomal degradation through a cascade of hydroxylation of conserved proline residues via prolyl hydroxylases (PHDs), subsequent recognition by the Von Hippel–Lindau (VHL) protein, ubiquitination and degradation by the proteasome. As oxygen tension drops, the PHD enzymes are inhibited and the lack of hydroxylation results in cytoplasmic stabilization of the α-subunits. After phosphorylation, HIFαs translocate to the nucleus and dimerize with ARNT (also known as HIF-β) and co-activators forming a functional HIF transcription factor (Wenger *et al.*, 2005; Fandrey and Gassmann, 2009). By binding to hypoxia-responsive elements in promoter regions, HIFs induce the expression of target genes involved in cellular adaptation to hypoxic stress-regulating erythropoiesis, angiogenesis, proliferation and cellular metabolism (Ogunshola and Al-Ahmad, 2012; see Figure 2).

Three different HIFs have been identified, namely, HIF-1, -2 and -3 of which HIF-1 is the most widely studied. More than 100 HIF-1 target genes are known to date (Semenza, 2003). HIF-1 signalling is considered essential for cellular adaptation and survival during hypoxia. Several HIF-1 target genes are neuroprotective and act as pro-survival factors such as erythropoietin and VEGF. Moreover, HIF-1 signalling regulates the expression of several proteins implicated in glycolysis such as phosphofructokinase or enolase-1 and glucose transporter 1 (GLUT1), thus regulating metabolic adaptation to low oxygenation. Besides its positive effects, HIF-1 also accounts for detrimental effects observed during hypoxia/ischaemia by activation of prodeath genes, such as BNIP3, COX2 or p53 stabilization (reviewed in Singh *et al.*, 2012). At present, it is not clear what determines whether the initiated HIF-1-mediated response is protective or detrimental. However, it is likely that the severity of the insult and its duration, as well as cell type-specific differences and paracrine

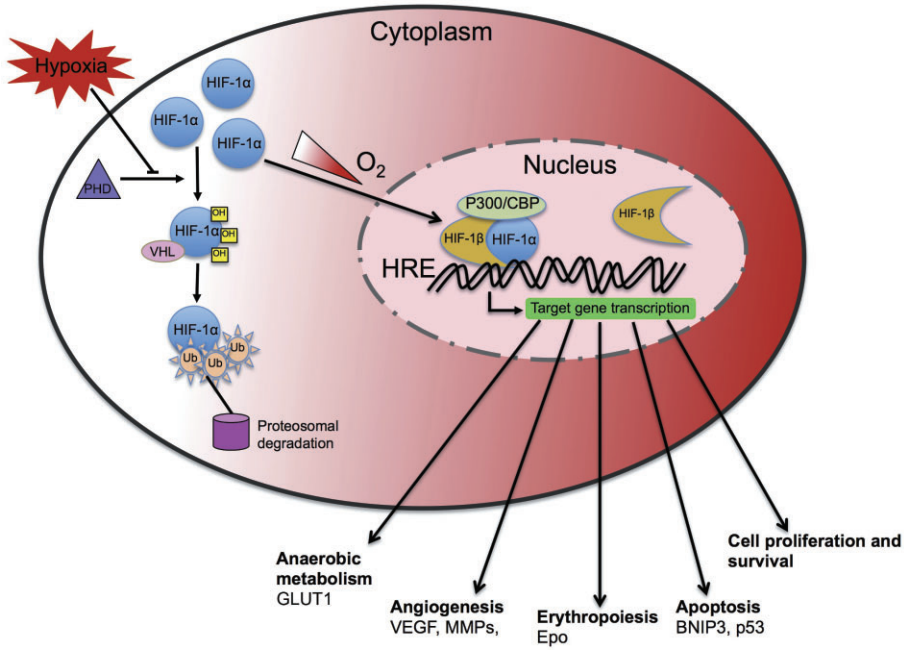

Figure 2

Schematic diagram illustrating the mechanism of HIF-1 regulation under normoxic and hypoxic conditions. HIFs are heterodimeric transcription factors composed of an oxygen-sensitive α-subunit and a constitutively expressed β-subunit. Under normoxia (white gradient), the HIF-1α subunit is constitutively transcribed but constantly targeted degradation through hydroxylation of conserved proline residues by PHDs leading to recognition by VHL protein, ubiquitination and subsequent degradation by proteasome. As oxygen tension drops (red gradient), the PHD enzymes are inhibited and the lack of hydroxylation results in cytoplasmic stabilization of the α-subunits. After phosphorylation, HIF-1α translocates to the nucleus and dimerizes with HIF-1β (also known as ARNT) and co-activators such as p300/CBP forming a functional HIF-1 transcription factor. HIF-1 then binds to hypoxia-responsive elements (HREs) in the promoter regions of its many targets inducing expression of genes involved in cellular adaptation to hypoxic stress by regulating erythropoiesis, angiogenesis, proliferation and cellular metabolism in order to reduce O_2 consumption and increase O_2 delivery to tissues.

signalling play a critical role. Thus, HIF-1 signalling is a key determinant of functional outcome.

HIF-1 regulates BBB permeability

Growing evidence suggests that HIF-1 could play a pivotal role in the changes that occur at the BBB during hypoxia. Good insights have been obtained from cerebral ischaemia and reperfusion studies as well as *in vitro* work (reviewed by Ogunshola and Al-Ahmad, 2012). The HIF-1 inhibitors 2-methoxyestradiol and YC-1 reduce oedema formation (directly correlating to BBB permeability) and infarct volumes after ischaemia or ischaemia reperfusion (Yeh *et al.*, 2007; Chen *et al.*, 2008a), whereas rapid DMOG (dimethyloxalylglycine)-induced HIF-1α stabilization increased oedema formation. In both studies, these effects were attributed to modulation of VEGF production (Chen *et al.*, 2008a). Our own *in vitro* work using brain ECs also suggest that HIF-1 stabilization is directly linked to barrier disruption and that inhibition of HIF-1 can significantly improve barrier stability (Engelhardt *et al.* unpubl. data). Likewise, HIF-1 inhibition using siRNA in a rat focal ischaemia model showed less BBB disruption and a better outcome for the animals and is correlated with decreased VEGF levels, as well as reduction of caspase-3 and p53 expression (Chen *et al.*, 2009). These studies suggest that HIF-1 stabilization is likely to trigger increased BBB permeability through activation of its multiple target genes and signalling cascade.

Contribution of astrocytes and pericytes to hypoxic BBB regulation

The importance of non-neuronal cells in brain diseases associated with hypoxia has long been neglected. The next section discusses the response of BBB cells to hypoxia/ischaemia and their subsequent effect on BBB function.

Astrocytes

Information on the basal responses of astrocytes to reduced oxygenation is limited and far from complete. We know that astrocytes are more resistant to hypoxia and ischaemia than neurons, which is probably due to their higher capacity to metabolically adapt to hypoxia/ischaemia by using alternative energy sources and switching to anaerobic glycolysis thereby ensuring maintenance of the ATP levels (Schmid-Brunclik *et al.*, 2008; Turner and Adamson, 2011). Astrocytes are activated *in vivo* (Kaur *et al.*, 2006) and *in vitro* (Schmid-Brunclik *et al.*, 2008) following hypoxic/ischaemic injury and secrete elevated levels of a large number of factors that can be neurotoxic as well as neuroprotective (reviewed in Trendelenburg and Dirnagl, 2005; Vangeison and Rempe, 2009). Modulation of a variety of proteins suggests a highly

complex tempering of barrier status. Indeed, a microarray study recorded more than 1100 hypoxia-responsive genes in human astrocytes with five times more genes up-regulated than suppressed. Notably, many of the up-regulated genes were glycolytic enzymes and angiogenic molecules (Mense *et al.*, 2006). Astrocytes co-cultured with ECs results in mutual up-regulation and preservation of antioxidant enzymatic activity and reduced radical-induced EC injury (lipid peroxidation) compared with endothelial monocultures (Schroeter *et al.*, 1999; Ogunshola and Al-Ahmad, 2012).

Different *in vitro* studies have shown that astrocyte co-culture or treatment of ECs with ACM improves EC performance and maintenance of barrier function during hypoxic insults (Fischer *et al.*, 2000; Brown *et al.*, 2003; Al Ahmad *et al.*, 2009). Consistent with these observations astrocytes/ACM preserve the junctional localization of TJ proteins, like ZO-1 or claudin-5 (Fischer *et al.*, 2000; Al Ahmad *et al.*, 2011) and hypoxia/glycaemia-dependent down-regulation of the adherens junction protein E-cadherin is partially reversed in the presence of astrocytes (Abbruscato and Davis, 1999b). Media from hypoxic and re-oxygenated astrocytes contain ANG-1 that increases occludin expression and reduces endothelial proliferation thereby stabilizing the vasculature (Song *et al.*, 2002). In addition, co-culture with astrocytes attenuates endothelial caspase-3 activation during hypoxia (Al Ahmad *et al.*, 2009) thereby reducing cell death and caspase-3-mediated TJ disruption (Zehendner *et al.*, 2011).

Astrocytes are the major source of VEGF in the brain and respond to hypoxic stimuli with increased induction and secretion of VEGF that acts as an endothelial survival factor (Chow *et al.*, 2001; Kaur *et al.*, 2006; Mense *et al.*, 2006; Schmid-Brunclik *et al.*, 2008). However, VEGF is also a prominent angiogenic molecule and thus a strong inducer of vascular permeability *in vitro* and *in vivo* (Fischer *et al.*, 1999; Schoch *et al.*, 2002). Our group has shown that despite a beneficial effect of astrocyte co-cultures on hypoxic barrier maintenance, inhibition of VEGF signalling (with the VEGF receptor inhibitor SU1498) further improved maintenance of barrier characteristics (Al Ahmad *et al.*, 2011). In agreement with this result, astrocyte-derived VEGF was also shown to drive BBB disruption after hypoxic exposure (Kaur *et al.*, 2006) and in CNS inflammatory disease (Argaw *et al.*, 2012). Thus, the effect of VEGF secretion during insult is multifaceted. VEGF binds and activates two TK receptors, namely, VEGFR1 (also known as FLT1) and VEGFR2 (also known as KDR or FLK1). Hypoxia-induced endothelial hyperpermeability seems to be mediated predominantly by activation of VEGFR1 (Vogel *et al.*, 2007), in agreement with the finding that hypoxia up-regulates both the expression of VEGFR1 and the binding of VEGF to VEGFR1 (Fischer *et al.*, 1999). Excellent reviews on hypoxia- and HIF-1-mediated VEGF permeability have been published (Fan *et al.*, 2009; Nakayama and Berger, 2013). However, it has also been shown that VEGF splicing in the terminal exon results in variants, termed $VEGF_{xxx}b$ ($VEGF_{165}b$), that act as anti-angiogenic dominant negative splice isoforms (Ladomery *et al.*, 2007; Nowak *et al.*, 2008). Notably, high homology means these anti-angiogenic isoforms may have been mistakenly identified as the more canonical species in many studies. Although the ratio of the b-isoforms to the canonical species could be highly relevant, investigations on the effects of stimuli such as hypoxia on these splice variants are yet to be performed. Astrocytic secretion of MMPs during hypoxia also causes BM reorganization and weakens barrier function. Increased activity of MMP-2, MMP-9 and MMP-13 was detected in hypoxic astrocyte supernatants and treatment of ECs with those supernatants led to MMP-13-dependent delocalization and proteolysis of ZO-1 and disruption of VE-cadherin (Lu *et al.*, 2009).

Astrocytes additionally express various cytokines in response to hypoxic stimulation. For example, IL-1β is a HIF-1 target gene (Zhang *et al.*, 2006) that can activate HIF-1α and VEGF expression in a NFκB-dependent manner in astrocytes and cause down-regulation of the vessel-stabilizing factor SSeCKs (Argaw *et al.*, 2006). Also, large amounts of the monocyte chemoattractant proteins (MCP) MCP-1 and MCP-5 are produced by hypoxic astrocytes in a HIF-1-dependent manner (Mojsilovic-Petrovic *et al.*, 2007). Apart from its main function of recruiting leukocytes at sites of inflammation, MCP-1 was shown to increase the paracellular permeability of endothelial monolayers via TJ redistribution mediated by Rho signalling (Stamatovic *et al.*, 2003) and is involved in formation of vasogenic oedema *in vivo* (Stamatovic *et al.*, 2005). Clearly, hypoxic responses of astrocytes are critical during injury and disease and particularly affect the stability of the BBB.

Pericytes in hypoxic BBB regulation

Only a few studies have investigated the survival of pericytes after hypoxic and ischaemic insults. Our *in vitro* data suggest that pericytes have comparable sensitivity to astrocytes. We did not observe any impairment of mitochondrial activity in pericytes exposed for up to 48 h in 0.2% oxygen reflecting no loss of viability, whereas ischaemic conditions reduced mitochondrial function only after 24 h of exposure (Engelhardt *et al.*, unpubl. obs.). Likewise, it was shown that ECs are much more susceptible to ischaemia than astrocytes and pericytes *in vitro* (Ceruti *et al.*, 2011). *In vivo* pericytes were observed to migrate away from microvessels in response to traumatic brain injury (TBI; Dore-Duffy *et al.*, 2000) and hypoxic stimuli already 2 h after onset of the insult and preceding any changes in vessel structure (Gonul *et al.*, 2002). Furthermore, a reduction of pericyte-to-EC ratio was reported after 1 week of hypobaric hypoxia (Dore-Duffy *et al.*, 2007). Although the cell status during these events remains unclear, the changes occurring during pathologies associated with increased BBB permeability suggest that pericyte loss *per se* could contribute to augmented barrier leakage. Indeed, *in vitro* models have shown that the presence of pericytes protects endothelial monolayers from hypoxic barrier disruption (Hayashi *et al.*, 2004; Al Ahmad *et al.*, 2009), particularly during prolonged and severe oxygen deprivation, by maintaining TJ protein localization and reducing endothelial caspase-3 activation (Al Ahmad *et al.*, 2009; 2011). Interestingly, under severe conditions, co-culture of pericytes with ECs maintained TEER and reduced paracellular flux of labelled substances better than astrocyte co-cultures (Al Ahmad *et al.*, 2009).

Like astrocytes, pericytes respond to hypoxia by up-regulating various growth factors. Park *et al.* demonstrated that in retinal pericytes ANG-1, but not ANG-2, mRNA is significantly elevated (Park *et al.*, 2003) again suggesting a

vessel-stabilizing effect. Interestingly, the induction of ANG-1 could be mimicked through treatment of the pericytes with recombinant VEGF (Park *et al.*, 2003). An *in vivo* study suggested that hypoxic pericytes rapidly increase VEGF levels within 24 h, whereas astrocytic VEGF production was observed after 4 days (Dore-Duffy *et al.*, 2007). In our studies, we observed different outcomes when inhibiting VEGF signalling using SU1498; inhibition was only beneficial in EC pericyte co-cultures during 1% O_2 but not during more severe oxygen deprivation (0.1% O_2; Al Ahmad *et al.*, 2009). Although temporal-spatial VEGF-mediated induction of ANG-1 may be a plausible explanation for some of the barrier-stabilizing effects of pericytes during hypoxia, it is unlikely that elevated VEGF expression does not largely contribute to hypoxic barrier disruption, indeed VEGF signalling by other brain cells may also have an effect. Like astrocytes, pericytes also secrete MMPs and their expression is augmented through pro-inflammatory cytokines like TNF-α (Takata *et al.*, 2011). Additionally, pericytes can induce MMP expression in ECs, although the link between pericyte MMP expression and hypoxic barrier disruption is still to be properly demonstrated. Taken together, however, the data indicate that timing, severity and cellular crosstalk is likely to be very complex.

Overall, despite limited information, similar to astrocytes, pericytes appear to fulfil important barrier-stabilizing functions but in response to stress also secrete molecules that can cause BBB remodelling and increased permeability. Thus, more studies are required to understand the multiple roles of this elusive perivascular cell.

Pathophysiology

Hypoxia-mediated BBB dysfunction is associated with many neurological diseases such as stroke, TBI and Alzheimer's disease (AD) (Ballabh *et al.*, 2004). As discussed earlier, paracrine interactions between brain ECs, astrocytes and pericytes play a crucial role in maintaining the BBB. However, their contribution to various brain diseases by secreting factors that modulate the barrier during injury is frequently overlooked. The next section of this review will focus on direct or indirect influences of hypoxic, and particularly HIF-1-mediated BBB cell-specific responses to progression of select diseases namely stroke, TBI and AD (see Figure 3).

Stroke/cerebral ischaemia

Stroke is a major cause of morbidity and mortality across all industrialized countries. The most commonly occurring type of stroke is ischaemic (Bamford *et al.*, 1990), which accounts for approximately 80% of the total incidences. Ischaemic stroke results from occlusion of a major cerebral artery either by thrombosis or an embolus. In either case, this restricts the delivery of substrates to the tissue, particularly oxygen and glucose, which leads to a cascade of ischaemic events (Dirnagl *et al.*, 1999). Within the core of the ischaemic region, deficits in blood flow, decreased energy stores (ATP levels), metabolic failure and ionic imbalance are severe and cell death progresses within minutes. The region surrounding the compromised area, called the ischaemic penumbra, suffers milder insults and is salvageable if blood flow is restored quickly. However, the reperfusion of ischaemic tissue can also lead to

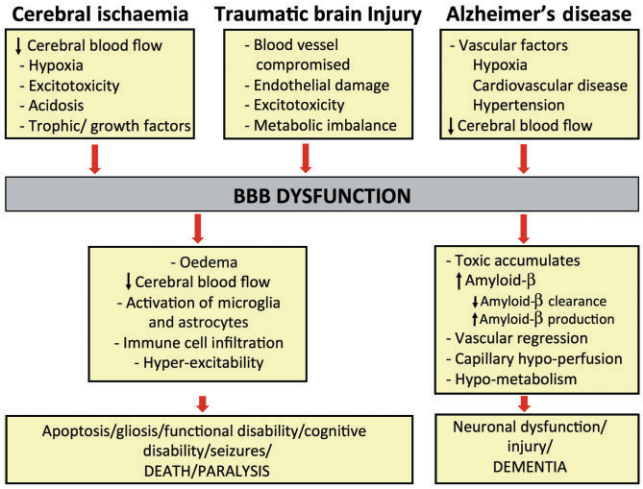

Figure 3

Flow chart of the events leading to BBB disruption and the following events occurring later in stroke, TBI and AD.

secondary damage caused by the rapid re-exposure to oxygen and inflammatory responses in the damaged tissue. Restoration of blood flow also increases the pressure on the damaged ECs and their TJs (Sandoval and Witt, 2008). Additionally, reperfusion results in a fresh supply of leukocytes that translocate into the parenchyma triggering a cascade of cytokine release (Dirnagl *et al.*, 1999). Activated astrocytes and pericytes modulate blood flow, maintain ion homeostasis and contribute to water homeostasis, as well as participate in the inflammatory cascade and release a number of substances that may be either neuroprotective or harmful during ischaemia. This wide variety of responses results in a number of effects including loss of TJ expression and localization, increase in permeability, degradation of BM, loss of integrins, oedema and further inflammation (Sandoval and Witt, 2008; Kwon *et al.*, 2009), all of which contribute to BBB disruption (see Figure 3). Although the trigger for the pathological changes is hard to establish they are probably interdependent. Cerebral ischaemia induced by transient occlusion of middle cerebral artery evokes a marked biphasic opening of the BBB. Early opening of the BBB takes place within the first half hour of reperfusion and is followed by partial closing. This initial acute opening is described as a 'haemodynamic' BBB opening resulting in cytotoxic oedema (reviewed by Nagy *et al.*, 1979; Kuroiwa *et al.*, 1985). Subsequently, a second delayed, but progressive, opening occurs between 24 and 48 h post-reperfusion and results in vasogenic oedema (Huang *et al.*, 1999). Notably, taking advantage of this later progressive BBB opening may aid the delivery of low permeability neuroprotective therapeutic agents.

Brain ECs maintain impermeability of the BBB largely through their TJs. Notably, ZO-1 and occludin expression were reduced in a microsphere-induced cerebral embolism model of cerebral ischaemia (Kago *et al.*, 2003). Although not many studies have evaluated TJ changes post-ischaemia, some reports have demonstrated changes in BBB cell contacts partly due to BM degradation. Astrocyte end-feet are well known to swell post-ischaemia (Kimelberg, 2005) and degra-

dation of BM also causes their detachment from the vessel wall leading to increased permeability (Kwon *et al.*, 2009). In addition to swelling, surviving astrocytes in the vicinity of the ischaemic damage also undergo a process of hypertrophy referred to as reactive astrogliosis. Similarly, pericytes have also been shown to migrate away from vessels in animal models of ischaemia by breaking through the BM that ensheaths them (Dore-Duffy *et al.*, 2000; Melgar *et al.*, 2006). This indicates that in addition to changes in BBB permeability induced by TJ alterations, contact with BBB-supportive cells is also modified and probably enhances BBB disruption. It is reported that ischaemia followed by reperfusion in rats results in BBB disruption and breakdown of BM collagen IV (Hamann *et al.*, 2002; Scholler *et al.*, 2007). Likewise, ischaemic/reperfusion injury also results in a decrease in laminin and fibronectin (Hamann *et al.*, 1995). Other BM proteins, such as agrin and perlecan are also degraded after ischaemia (Fukuda *et al.*, 2004; Baumann *et al.*, 2009). Fukuda and group were the first to provide direct evidence that active proteases, which are known to degrade the BM are generated in ischaemic cerebral tissue (Fukuda *et al.*, 2004). The most widely studied proteases, MMPs, are released by astrocytes (Zhang *et al.*, 2004) and microglia (Del Zoppo *et al.*, 2007) post-ischaemic injury. MMP-9 and -2, in particular, are major contributors of ischaemic pathology. Many groups have demonstrated the detrimental role of MMP-9 in ischaemic models by using inhibitors of MMP-9 or MMP-9 knockout animals and reported reduction in BBB damage, brain water content, infarct size and neurological deficit (Romanic *et al.*, 1998; Asahi *et al.*, 2001; Jiang *et al.*, 2001; Park *et al.*, 2009; Cui *et al.*, 2012). Therefore, inhibiting MMPs may be highly beneficial for treating cerebral ischaemia, as reviewed elsewhere (Cunningham *et al.*, 2005; Morancho *et al.*, 2010).

BBB disruption after ischaemia increases permeability to macromolecules allowing fluid movement from intravascular to extravascular spaces and leading to vasogenic oedema (Sandoval and Witt, 2008). Aquaporins (AQPs) are highly permeable water channels that play a crucial role in fluid balance. AQP4 in particular participates in the maintenance of brain water homeostasis and is highly concentrated on astrocyte end-feet (Nielsen *et al.*, 1997). The role of AQP4 has been explored in various models of ischaemic stroke where it contributes to the formation of cerebral oedema. In disease models, such as acute cerebral ischaemia and water intoxication (Manley *et al.*, 2000), water moves into the cell resulting in cytotoxic brain oedema. Deletion of AQP4 was shown to prevent cellular water uptake in these models, as demonstrated by reduced brain water content, lower intracranial pressure values, infarct size and lesion volume (Manley *et al.*, 2000; Zeng *et al.*, 2012). In chronic ischaemia or TBI models that result in vasogenic oedema due to BBB disruption, deletion of AQP4 exacerbated cerebral oedema formation assessed by water content measurements and intracranial pressure (Papadopoulos *et al.*, 2004). Based on these findings it can be concluded that AQP4 plays a differential role in modulating brain water homeostasis and is dependent on the duration and type of insult.

VEGF, one of the most important factors induced by hypoxia, performs a major role in driving BBB disruption leading to oedema formation in the acute phase following ischaemia (Zhang *et al.*, 2000; Chi *et al.*, 2007; 2008). Elevated VEGF expression appears as early as 1 h after the onset of cerebral ischaemia (Abumiya *et al.*, 1999; Plate *et al.*, 1999b). Indeed, early administration of VEGF increases BBB permeability, increases infarct size and worsens neurological outcome following an ischaemic insult (Zhang *et al.*, 2000). Similarly, inhibition of endogenous VEGF in the acute phase reduces BBB permeability (Mayhan, 1999; Valable *et al.*, 2005; Yeh *et al.*, 2007). This is in line with findings that suggest VEGF is not only an angiogenic factor but also a permeability factor (Plate, 1999a). Apart from its role at the vasculature, there are several studies that have also examined the effect of VEGF on cerebral infarct size. Van Bruggen *et al*. reported that antagonism of VEGF reduces ischaemia reperfusion-related brain oedema and injury in mice (van Bruggen *et al.*, 1999). Similarly, VEGF administered i.v. increased infarct size when given early after ischaemia in rats but, in contrast, improved neurological function when given at later stages 2 days post-ischaemia (Zhang *et al.*, 2000). These studies suggest that despite enhancing angiogenesis and reducing neurological deficits during late-stage stroke recovery, inhibition of VEGF at the acute stage of injury may reduce BBB compromise. The negative side of VEGF could perhaps be achieved partly by counteracting its permeabilizing effect in the acute phase of ischaemia. Indeed, systemic adenoviral gene delivery of ANG-1, a known anti-permeability factor that maintains and stabilizes mature vessels by promoting interaction between ECs and surrounding support cells (Hori *et al.*, 2004), causes resistance to vascular leakage induced by VEGF in the brain after cerebral ischaemia (Zhang *et al.*, 2002). Conversely, ANG-2 that leads to de-stabilization of vessels and dissociation of pericytes, is up-regulated by hypoxia and cytokines including VEGF (Mandriota and Pepper, 1998; Valable *et al.*, 2005). Thus, the co-ordination of VEGF and the ANG is crucial for maintaining the integrity of existing vessels and modulating growing vessels for better outcome following ischaemia.

In summary, the exact mechanisms causing brain vascular disruption are hard to establish but factors like VEGF and MMPs released from BBB-supportive cells are likely to play an instrumental role. Further confirmation of these conclusions using cell-specific conditional knockout animal experiments is necessary for better understanding of the role of these cells at the BBB post-ischaemia.

Traumatic brain injury

TBI results from an outside force causing instant mechanical disruption of brain tissue and delayed pathogenic events that collectively mediate widespread neurodegeneration (reviewed by Gaetz, 2004). Subsequently, the traumatized brain is highly susceptible to secondary brain injuries, which can be caused by hypoxia (Hellewell *et al.*, 2010) and seizures (Bao *et al.*, 2011). Reports from animal model studies and significant clinical data both suggest a central role for vascular integrity in TBI outcome, especially BBB breakdown which can last from minutes to several days to weeks, or even years after the acute event (Unterberg *et al.*, 2004; Hawkins and Davis, 2005; Abbott *et al.*, 2006; Tomkins *et al.*, 2008). This kind of disruption might also influence the progression of long-term TBI complications, such as AD, which include delayed neuronal dysfunction and cell death (see Figure 3).

Studies of animal models of TBI have also demonstrated a biphasic increase in BBB permeability to albumin and other high molecular weight proteins peaking at 4 to 6 h as well as 2–3 days after injury (Başkaya *et al.*, 1997; Hicks *et al.*, 1997). The first peak in BBB permeability generally overlaps with increased production of a number of secretory factors that include chemokines, cytokines, MMPs and VEGF, which contribute to BBB dysfunction and also invasion of neutrophils. A thorough review on molecular pathogenesis of BBB breakdown in TBI has been recently published (Nag *et al.*, 2011).

Chemokines not only play a key role in post-traumatic recruitment of leukocytes, but may also modify the permeability of the BBB. After injury recruitment of leukocytes into the brain parenchyma releases a number of inflammatory cytokines that worsen the outcome of the disease state. For example, as stated earlier MCP-1 is up-regulated post-hypoxia and very recently, Semple *et al*. reported its important role in reducing lesion size and secondary damage and improving functional outcome in mice following TBI (Semple *et al.*, 2010). This improvement may be attributed to decreased leukocyte accumulation resulting in an environment more advantageous for neuronal survival. Increased levels of cytokines are correlated to poorer clinical outcomes. TNF-α mRNA and protein is elevated acutely after experimental TBI (Bermpohl *et al.*, 2007) as well as in clinical settings (Csuka *et al.*, 1999) and is known to exacerbate BBB disruption post-TBI (Bermpohl *et al.*, 2007). Similarly, a rapid increase of IL-1β is observed early enough after experimental TBI (Wang and Shuaib, 2002). Patel *et al.*, using intracerebral or intraventricular administration of exogenous IL-1β, demonstrated significant exacerbation of injury (Patel *et al.*, 2003). In addition, transgenic mice, in which the IL-1β is overexpressed in astrocytes alone, were observed to have a leaky BBB (Shaftel *et al.*, 2007). Thus, BBB dysfunction mediated via cytokines released from BBB cells can worsen injury outcome in the acute stages and may be hypoxia-dependent. In contrast, the mechanisms involved in the delayed (second phase) opening of the BBB are presently unclear and require further investigation.

Although the role of VEGF and its receptors has been studied extensively in ischaemia over the last years, only a few groups have performed studies elucidating the role of this molecule post-TBI. Using different models of TBI, it was reported that astrocytes as well as inflammatory cells react to injury by increasing VEGF expression (Papavassiliou *et al.*, 1997; Sköld *et al.*, 2002). In contrast, pericytes were shown to undergo cell death post-TBI using TUNEL assay (Dore-Duffy *et al.*, 2007). Morgan *et al*. demonstrated a substantial increase in angiogenesis, based on BrdU-positive nuclei within the endothelium post-TBI via increased expression of VEGF and its receptor (Morgan *et al.*, 2007). This induction of angiogenesis could be mediated by the up-regulation of astrocytic VEGF, which in turn increases both astrocyte proliferation and facilitates the expression of other growth factors, which support late-stage recovery (Krum *et al.*, 2008). Corticosteroids that are usually used for treating early vasogenic oedema after acute BBB compromise have been demonstrated to stabilize the BBB by decreasing the expression of VEGF (Kim *et al.*, 2008). Again, the data suggest that during disease early VEGF release from glial cells can be harmful to BBB function and further worsen the outcome post-TBI. Similarly, MMPs, in particular MMP-2, MMP-3 and MMP-9, are up-regulated following TBI (Shigemori *et al.*, 2006; Vilalta *et al.*, 2008; Hayashi *et al.*, 2010). As found in cerebral ischaemia, genetic deletion of MMP-9 or pharmacological inhibition of the activity of MMPs using TIMPs significantly reduced the extent of tissue damage and improved functional outcome in murine models of TBI (Wang *et al.*, 2000). In addition, mice overexpressing the human TIMP1 gene subjected to TBI showed reduced levels of MMP-9 synthesis and a less leaky vasculature accompanied by decreased brain tissue damage compared with control animals (Tejima *et al.*, 2009). All these observations indicate that secretion of VEGF and MMPs by BBB cells could indeed represent a potential target for pharmacological intervention in TBI and ischaemia. Nonetheless, it should be emphasized that as these factors also play an important role in the repair process at later stages, delayed inhibition of their activity may actually have adverse therapeutic effects (Nag *et al.*, 1997; Zhao *et al.*, 2006).

AQP4 expression is markedly modified in both experimental and clinical TBI (Manley *et al.*, 2000; Papadopoulos and Verkman, 2007; Tang *et al.*, 2010; Fukuda and Badaut, 2012). Initial studies implied that induction of AQPs in a model of TBI promoted oedema formation (Manley *et al.*, 2000) and that therapeutic inhibition of AQP4 could be beneficial in controlling oedema (Taya *et al.*, 2008). Indeed, Fukuda *et al*. used siRNA against AQP4 to demonstrate improvements that were associated with decreased oedema formation, increased microglial activation and decreased BBB disruption post-TBI (Fukuda *et al.*, 2013). However, it subsequently became apparent that the modifications in AQP4 expression are dependent on the type of oedema and its regional distinction (Sun *et al.*, 2003; Papadopoulos *et al.*, 2004). For instance, in an ischaemic model that results in cytotoxic oedema, inhibition of AQP4 expression was associated with decreased oedema, reduced infarct area and an improvement in functional outcome (Fazzina *et al.*, 2010). In contrast, in a cold lesion injury model of vasogenic brain oedema, AQP4-deficient mice had a markedly worse clinical outcome (Papadopoulos *et al.*, 2004). These findings suggest that AQP4 is mainly essential for the clearance of vasogenic oedema but its other effects depend on the type of injury and the time point being measured.

Finally, BBB disruption also triggers a chain of events that not only affects vascular cells but also neuronal cells by causing epilepsy (Tomkins *et al.*, 2007; 2008). This theory seems plausible since the studies cited suggest that damage to the microvasculature might result in the extravasation of serum proteins such as albumin, which leads to the activation of astrocytes as the first step in the epileptogenic process. This interaction suggests a key functional role of activated astrocytes in neuronal excitability. Hardly any work has been performed on the specific role of pericytes in TBI, although endothelin-1-induced pericyte-mediated vasoconstriction was recently reported (Dore-Duffy *et al.*, 2011). Many more studies on cell-specific responses to TBI are surely warranted.

Alzheimer's disease

AD is a progressive and irreversible neurodegenerative disorder characterized by the accumulation of amyloid β-peptide (Aβ) in the CNS, the presence of hyper-phosphorylated tau

filaments and cerebrovascular changes that lead to cerebral amyloid angiopathy (Greenberg *et al.*, 2004). AD is another neurodegenerative disease that is characterized by hypoxia, a state that is believed to only aggravate disease progression (Ogunshola and Antoniou, 2009; Peers *et al.*, 2009; Figure 3).

At present, the cellular and molecular basis by which systemic hypoxia influences AD are not completely understood. However, the emerging evidence indicates that prolonged hypoxia induces formation of Aβ that accumulates over years and probably contributes to vascular alterations (Selkoe, 2001). Hypoxia induces Aβ processing through mechanisms that increase the activity of two enzymes crucial for Aβ formation, β-secretase and γ-secretase (Sun *et al.*, 2006; Zhang *et al.*, 2007; Li *et al.*, 2009). HIF-1α mediates a transcriptional increase in β-secretase expression (Zhang *et al.*, 2007) and presenilin-1 levels – presenilin being the major protein of the γ-secretase complex (Pluta, 2007). In addition, hypoxia also promotes down-regulation of neprilysin, an Aβ-degrading enzyme (Nalivaevaa *et al.*, 2004), which can lead to alterations in the expression of vascular-specific genes in brain ECs (Wu *et al.*, 2005). All of these effects can be considered pro-amyloidogenic, that is, would predispose to increased Aβ levels either through increased production or reduced degradation. Recently, Wilcock and colleagues used a transgenic mouse model to demonstrate that during progression of an AD-related pathology the numbers of astrocytic processes in apparent contact with cerebral vasculature were reduced. Furthermore, amyloid accumulation caused significant reductions in AQP4 and potassium channels associated with cerebral vessels in both the mouse model and individuals with AD (Wilcock *et al.*, 2009). This observation seems to agree with studies performed in cerebral ischaemia or TBI models during the vasogenic phase suggesting that the presence of AQP4 is beneficial for functional outcome. Clearly, AQ4 plays a significant role in controlling brain water homeostasis; however, many more studies are required to define how its modulation will affect functional outcome of disease progression in different pathologies. An excellent review on aquaporins and AD has been published (Foglio and Rodella, 2010). Emerging evidence suggests that the Aβ accumulation that causes vascular alterations ultimately leads to hypo-perfusion (Sagare *et al.*, 2012). Pericytes could probably influence this hypo-perfusion in conditions of severe ischaemia and oxidative stress, as they contract not only during the hypoxic insult but remain contracted even after reperfusion (Yemisci *et al.*, 2009). Hypoxia also promotes phosphorylation of tau through the MAPK pathway (Fang *et al.*, 2010). Tau is a microtubule-associated protein that plays a major role in stabilizing microtubules, found mainly in neurons but has also been shown in low levels in astrocytes (Lee *et al.*, 2001). Aβ and/or hypo-perfusion can induce hyperphosphorylation of tau, leading to the protein structural changes found in AD patients, which affects its binding with tubulin and causes destabilization of the cytoskeleton (Michaelis *et al.*, 2002). This leads to formation of neurofibrillary tangles and suggests that tau pathology develops secondary to Aβ injury and can be modulated via hypoxia-mediated signalling.

Alterations in vascular permeability and BBB disruption are detected in the brains of AD patients (Claudio, 1996). However, deposition of Aβ aggregates in cerebrovasculature and the brain is less understood and the mechanisms that cause changes in permeability are not clear. The BBB regulates the entry of plasma-derived Aβ into the brain and clears brain-derived Aβ through the receptor for advanced glycation end-products (RAGE) and low-density lipoprotein receptor-related protein respectively (Shibata *et al.*, 2000; Deane *et al.*, 2003). Previous reports showed increased levels of free Aβ in plasma of AD patients or mouse models (Kawarabayashi *et al.*, 2001; Zhou *et al.*, 2012). Through these studies, one can speculate that Aβ may disrupt the TJs of BBB via interaction with RAGE as a specific mediator. In agreement with this hypothesis, a recent study performed by Kook *et al.* using cultured ECs showed Aβ-induced structural alterations and reduction in protein levels of ZO-1 as well as increased permeability. Furthermore, a neutralizing antibody against the extracellular domain of RAGE effectively blocked Aβ-induced alterations in ZO-1 distribution suggesting that Aβ–RAGE interactions are critical for TJ integrity (Kook *et al.*, 2012). It is well known that calcium influx is induced by Aβ in the cells (Kawahara *et al.*, 2000) and increased intracellular calcium also leads to TJ alterations as well as inducing MMP expression (Stuart *et al.*, 1996; Rosenberg, 2009; Kook *et al.*, 2012). Thus, MMP activation could further accelerate degradation of the BM as observed post-ischaemia and worsen the outcome of AD.

Thus, although the amyloid hypothesis for the pathogenesis of AD suggests this peptide initiates a cascade of events leading to neuronal injury and loss and eventually dementia, from the studies mentioned above, vascular alteration may significantly contribute to the disease as well. Recently, Zlokovic presented an alternative, two-step vascular hypothesis as discussed in the review (Zlokovic, 2011). The hypothesis suggests that in the first step, the primary damage to brain vasculature initiates a non-amyloidogenic pathway of neuronal dysfunction and injury, which is mediated by BBB dysfunction. This is correlated with leakage and secretion of multiple toxic molecules and/or reduced blood flow, which causes multiple focal ischaemic or hypoxic injuries. In the second step, BBB dysfunction also leads to increased Aβ generation and impairment of Aβ clearance. Both these processes contribute to accumulation of Aβ species in the brain and toxicity. It is clear from this hypothesis that phenomena like vascular regression and hypo-metabolism occur secondary to vascular and/or Aβ injury but also that BBB alterations probably make a sizeable contribution to disease progression. Temporal-spatial BBB disruption in senescence-accelerated mouse prone 8 mice, considered by some researchers as a model of AD, was recently reported (Del Valle *et al.*, 2009). However, the role of the perivascular cells, astrocytes and pericytes, and the mechanisms that caused the changes in permeability are not clear and require further investigation.

Vascular regression is a phenomenon observed in AD patients (Zlokovic, 2005; Grammas, 2011) as well as in APP transgenic mice – a model for late-stage AD (Paris *et al.*, 2004). For example, reduction in brain capillary length in the hippocampus correlated well with increased clinical dementia rating scores in AD patients (Bouras *et al.*, 2006). The reason for this phenomenon is unclear but could be explained partly by the anti-angiogenic activity of Aβ (Thomas *et al.*, 1996). Aβ counteracts the pro-angiogenic effects of VEGF and bFGF in ECs by sequestering VEGF in the plaques (Yang *et al.*, 2004).

Reduced angiogenic effects in AD are also caused by TGF-β1 (Tesseur and Wyss-Coray, 2006). Transgenic overexpression of TGF-β1 in astrocytes stimulates Aβ deposition in brain vessels, along with alterations in regional cerebral blood flow and AD-like vascular degeneration and brain metabolic activity. Furthermore, elevated levels of TGF-β1 in human AD correlate with increased Aβ deposition in brain vessels (Wyss-Coray *et al.*, 2001). However, it is noteworthy that TGF-β1 can also be neuroprotective and promote Aβ clearance as shown in microglia (Wyss-Coray *et al.*, 2001). These findings raise an interesting question of whether vascular regression and/or degeneration in AD results from unsuccessful vascular repair and/or remodelling.

Another phenomenon that occurs during AD progression that may be modulated by BBB cells is hypo-metabolism. Neurons are incapable of synthesizing or storing glucose and are dependent on glucose transport across the BBB, a process that is mediated by glucose transporters (GLUTs). GLUT1 is also located on cerebrovascular ECs and astrocytes (Vannucci *et al.*, 1997). In AD, dysfunctional cerebral ECs express less GLUT1 as well as HIF-1, a major regulator of GLUT1, thereby reducing glucose uptake in the brain (Liu *et al.*, 2008). Furthermore, studies using 18F-fluorodeoxyglucose PET have identified reductions in glucose uptake in individuals with a high risk of dementia (Herholz, 2010). Decreased glucose uptake across the BBB, as seen by PET, may also precede brain atrophy (Herholz, 2010). This suggests that hypo-metabolism due to BBB dysfunction is a contributing factor for disease progression and occurs at a later stage after vascular and/or Aβ injury. Whether swelling and retraction of astrocyte endfeet inhibits glucose transport to neurons and thereby facilitates neuronal hypo-metabolism needs further study.

It is clear that many more studies are needed to fully understand the effect of hypoxia-mediated changes on neurodegenerative pathologies with respect to BBB function and contribution of astrocytes and pericytes. And the question remains, are we missing important mechanistic roles of the BBB under pathophysiological conditions by studying the nervous system in isolation from the influence of the vascular system? The likely answer is yes. Thus, more research based on the BBB and the NVU as a whole is warranted to provide important insights in the future.

Pharmacology

HIF modulators as therapeutic targets

The important role for HIF-1 in the mediation of the adaptive processes to hypoxia means that it could potentially represent an important target to prevent cellular damage during disease progression. Targeting HIF-1 now represents a potential therapeutic strategy in numerous physiological including myocardial ischaemia and cerebrovascular diseases. In this regard, a wide variety of PHD inhibitors that cause stabilization of HIF-1 have been developed as erythropoiesis stimulating agents and neuroprotective drugs. Additionally, activity relating to development of HIF-1 inhibitors to prevent solid tumour angiogenesis has also exponentially increased in the last years but may also be useful to prevent BBB alterations during injury conditions. Some promising advances in these areas are discussed below.

HIF stabilizers

The therapeutic potential of development and use of small molecule HIF stabilizers to improve cell survival after injury is gaining popularity in many different fields. In particular PHDs, the enzymes that regulate HIF-1 stabilization, are now being recognized as important targets for future medical intervention. PHD enzymes require iron and 2-oxyglutarate (2-OG) in order to catalyse HIF prolyl hydroxylation (Jaakkola *et al.*, 2001). hus iron chelators and competitive inhibitors impair the activity of PHD enzymes and other iron-dependent enzymes. deferoxamine, an iron chelator, and cobalt chloride ($CoCl_2$), a competitive inhibitor of iron, are routinely used both *in vitro* and *in vivo* to inhibit PHD enzyme activity and thus stabilize HIF. Both seem to be protective in preconditioning preclinical models of cerebral ischaemia (Prass *et al.*, 2002; Siddiq *et al.*, 2005; Aminova *et al.*, 2008; Jones *et al.*, 2008). Indeed, *in vivo* high concentrations of iron, stored in the cytoplasmic protein ferritin, are released during ischaemia (Harten *et al.*, 2010) and could lead to radical-mediated damage of cellular components (Sorond and Ratan, 2000). Thus, sequestration of iron in general may be beneficial in certain injuries as well as making a major contribution to the neuroprotective action of iron chelators such as $CoCl_2$. The 2-OG analogues L-mimosine, DMOG and 3,4-dihydroxybenzoate can also be used to inhibit PHD enzymes (reviewed by Harten *et al.*, 2010). Some small-molecule activators have also been identified. A potent and effective activator of the HIF pathway is tilorone. Tilorone appears to be able to cross the BBB, stabilize HIF-1α protein and confer significant resistance to stroke and spinal cord injury by an as yet unknown mechanism (Ratan *et al.*, 2008). However, as its use is associated with the accumulation glycosaminoglycans, its future use in humans is debatable (Harten *et al.*, 2010). Finally, the race is on for development of novel PHD inhibitors, particularly for the treatment of ischaemic diseases such as stroke, and a number of compounds has been developed by Fibrogen (reviewed by Harten *et al.*, 2010). A selection of the most promising PHD inhibitors currently being considered for therapeutic strategies has recently been reviewed (Nagel *et al.*, 2010; Karuppagounder and Ratan, 2012). However, it must be emphasized that, although HIF stabilizers mimic the neuroprotective effects of preconditioning in both *in vitro* and *in vivo* ischaemic models, the use of PHD inhibition as a post-injury treatment remains somewhat controversial. In mice, post-ischaemic PHD inhibition offered less protection than pre-ischaemic treatments (Baranova *et al.*, 2007) and early activation of HIF-1α using DMOG accentuated brain oedema and BBB disruption compared with ischaemia alone (Chen *et al.*, 2008b). Importantly, in both cases, these effects were attributed to increased VEGF levels with astrocytes being the most likely culprits (Vangeison *et al.*, 2008).

HIF inhibitors

Although identification of agents that specifically increase hydroxylation activity of PHDs – and thus HIF-1 degradation – is relatively unexplored, the fact that HIF-1 induction is closely linked to BBB permeability means putative inhibitors may have a relevant application preventing barrier changes associated with hypoxic and/or ischaemic-induced injury progression. Indeed, in the cancer field, targeting of HIF-1 in

hypoxic cells is an attractive therapeutic strategy that is already being actively pursued, since HIF-1 is tightly linked to the metastatic potential of many tumours, treatment resistance and poor prognosis (Hu *et al.*, 2012; Meijer *et al.*, 2012; Xia *et al.*, 2012). To date, a number of molecules that inhibit the HIF-1 pathway have been identified predominantly using cell-based screening systems and research of HIF-1 activity in cancer cell lines. Supraphysiological supplementation of iron and ascorbate enhances PHD activity and results in HIF-1 degradation (Harten *et al.*, 2010) and has already been used in the clinics. In addition, both are considered relatively safe, inexpensive and readily available. 2-OG and its derivatives, the rate-limiting co-substrate for PHDs, stimulate PHD activity and reduce basal HIF-1α protein levels suggesting they may also have a therapeutic role (Ban *et al.*, 2011). Moreover, targeted screening recently identified a compound, KRH102053, as a PHD2 activator and showed it reduced levels of HIF-1 and its downstream target genes, leading to inhibition of hypoxia-induced responses including metastasis and glucose metabolism *in vitro* (Choi *et al.*, 2008). A number of other inhibitors have also been developed – for excellent overviews of putative HIF-1 inhibitors in development as well as clinical and preclinical trials see Ban *et al.* (2011); Xia *et al.* (2012). Although various small molecules have been developed as HIF-1 inhibitors, the mechanisms by which they work are still unclear in many cases. The fact that none of them seem to exclusively target HIF-1α signalling suggests they can also have multiple off-target effects. Perhaps an exception is EZN-2968, an RNA antagonist that targets HIF-1α mRNA and thereby directly inhibits HIF-1α expression (Greenberger *et al.*, 2008). Tumour reduction was found in nude mice implanted with DU145 human prostate cancer cells treated with EZN-2968 and a phase I clinical study in patients with advanced malignancies revealed that EZN-2968 was well tolerated (Ban *et al.*, 2011). EZN-2968 is currently discussed as the best potential for specific HIF-1α inhibition and promising results of the clinical studies are expected (Ban *et al.*, 2011).

In conclusion, the future looks bright for the use of HIF inhibitors as therapeutics, but overall, it is apparent that future design of more specific agents is required for better drugs to be developed. Perhaps such drugs will also be useful to prevent BBB disruption particularly during the acute phase after injury in the future.

Targeted BBB cell-specific treatment

The major focus of therapeutic targets has always been neuronal mechanisms of injury. Notably, attention to dysfunction or loss of non-neuronal cell types, that is, the cells that maintain the neuronal homeostatic environment, are avenues not well explored even though they may significantly increase the chances of success. As discussed above, HIF stabilization, and/or PHD inhibition, compromises barrier stability and as such targeting HIF-1 may be a way to maintain barrier stability during injury. Using therapeutic targets that activate a broad programme of genes in different cell types, as discussed above, may stimulate biological effects better than the separate application of growth factors or other repair proteins (reviewed by (Ratan *et al.*, 2007), especially in this case as an endogenous programme of adaptation is augmented. However, the wide range of HIF targets could have the caveat that modulation of multiple processes could potentially have a negative outcome on whole body physiology. For example, clinical syndromes associated with excessive activation of the HIF pathway exist (Gassmann *et al.*, 2003) and whereas some studies indicate that HIF-1 is neuroprotective, others demonstrate negative outcomes. Clearly, the effects on the NVU in particular must be better understood to ascertain the general widespread application of such therapeutics. Since different cell types of the BBB and CNS show diverse and sometimes opposing responses to HIF-1 modulation, targeting the pathway in a cell-specific manner will circumvent adverse side effects that can be expected of general HIF-1 modulators. Cell-specific therapeutics (e.g. endothelial-, astrocyte- or pericyte-specific drugs) may also result in more efficacious treatment and better tailoring to the injury in question. Targeting and augmenting vascular EC function directly will provide the significant advantage that such drugs would not be required to cross the BBB, that is, be easier targets than current neuron-directed research. Thus, detailed knowledge of cell-specific responses during injury is required to support development of more specific therapeutic agents. Additionally, in-depth knowledge of the side effects of long-term or high-dose treatments must be well defined prior to their clinical implementation. It must also be emphasized that the consequence of increased levels of HIF-1 in different cell types is highly divergent and dependent on the type of injury. Indeed, it is now becoming clear that the PHDs may also have specific endogenous substrates as well as divergent cell-specific roles (Smirnova *et al.*, 2012). Thus, an additional challenge will be whether development of isoform-specific inhibitors will be more efficacious and safe than a more global approach. Clearly, BBB cell-specific responses and their contribution to injury progression are instrumental factors that need to be carefully considered, and the balance of HIF-1 neuroprotective effects with the putative negative outcome on BBB function must be weighed before implementing any treatment strategy.

Conclusion

Overall, hypoxia and HIF-1 significantly contribute to BBB dysfunction in various neurological diseases. The influence of BBB cell specificity to outcome remains very unclear but deserves significantly more investigation since this knowledge will enable development of more effective therapeutic strategies to combat disease progression. Clearly, balancing HIF-1 beneficial and deleterious effects with cell-specific responses, disease profiles, and windows of opportunity will be very difficult.

Conflict of interest

No conflict of interest.

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