J Physiol 595.6 (2017) pp 1885–1902

JPn Neuroscience
The Journal of Physiology

TOPICAL REVIEW

Binaural blood flow control by astrocytes: listening to synapses and the vasculature

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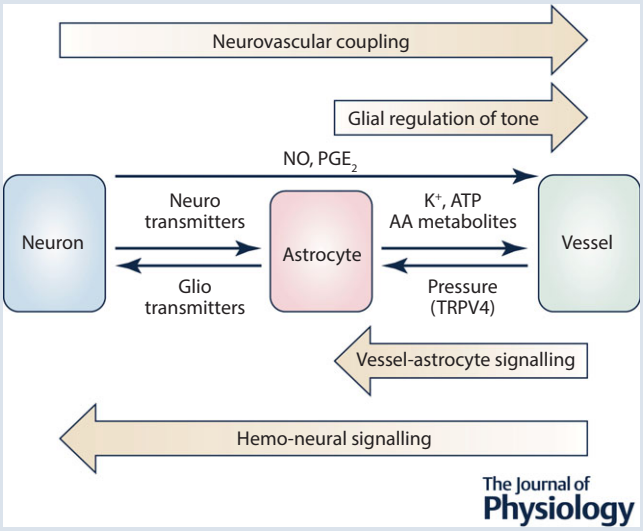

Abstract Astrocytes are the most common glial cells in the brain with fine processes and end-feet that intimately contact both neuronal synapses and the cerebral vasculature. They play an important role in mediating neurovascular coupling (NVC) via several astrocytic Ca^{2+}-dependent signalling pathways such as K^+ release through B_K channels, and the production and release of arachidonic acid metabolites. They are also involved in maintaining the resting tone of the cerebral vessels by releasing ATP and COX-1 derivatives. Evidence also supports a role for astrocytes in maintaining blood pressure-dependent change in cerebrovascular tone, and perhaps also in blood vessel-to-neuron signalling as posited by the 'hemo-neural hypothesis'. Thus, astrocytes are emerging as new stars in preserving the intricate balance between the high energy demand of active neurons and the supply of oxygen and nutrients from the blood by maintaining both resting blood flow and activity-evoked changes therein. Following neuropathology, astrocytes become reactive and many of their key signalling mechanisms are altered, including those involved in NVC. Furthermore, as they can respond to changes in vascular pressure, cardiovascular diseases might exert previously unknown effects on the central nervous system by altering astrocyte function.

Anusha Mishra started her research career in Kristen Harris's lab at the Medical College of Georgia, studying electron micrographs of the brain. She then did her PhD with Eric Newman at the University of Minnesota where she investigated changes in retinal neurovascular coupling in pathology and discovered a drug that reverses the loss of this response in diabetic animals. She is currently doing her postdoctoral training in David Attwell's lab at University College London, where she has been studying capillary level neurovascular coupling in health and disease and the role of astrocytes in mediating this response.

DOI: 10.1113/JP270979

This review discusses the role of astrocytes in neurovascular signalling in both physiology and pathology, and the impact of these findings on understanding BOLD-fMRI signals.

(Received 10 March 2016; accepted after revision 15 July 2016; first published online 13 September 2016)
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Abstract figure legend Putative signalling pathways between neurons, astrocytes and the vasculature.

Abbreviations AA, arachidonic acid; AD, Alzheimer's disease; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ATP, adenosine triphosphate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester); BOLD fMRI, blood oxygenation level-dependent functional magnetic resonance imaging; COX, cyclooxygenase; CNS, central nervous system; CYP, cytochrome P450; EETs, epoxyeicosatrienoic acids; fNIRS, functional near-infrared spectroscopy; GABA, γ-aminobutyric acid; GECI, genetically encoded calcium indicator; 20-HETE, hydroxyeicosatetraenoic acid; mGluR, metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; NVC, neurovascular coupling; PET, positron emission tomography; PGE_2, prostaglandin E2; RNA, ribonucleic acid; TRP, transient receptor potential; VSMC, vascular smooth muscle cell.

Introduction

Astrocytes are a major type of glial cell in the central nervous system (CNS). They uniformly tile the entire nervous system and occupy distinct minimally overlapping territories in the neuropil, referred to as astrocyte domains. These domains encompass thousands of synapses from many neurons (Bushong *et al.* 2002; Ogata & Kosaka, 2002; Bushong *et al.* 2004), suggesting that each astrocyte can sense the activity of a large number of neurons. Furthermore, these cells are coupled by gap junctions that allow direct cell-to-cell communication (Theis & Giaume, 2012), forming the astrocyte syncytium (Kirchhoff *et al.* 2001). This syncytium increases the spatial buffering capacity for K^+ to help regulate neuronal excitability (Kofuji & Newman, 2004). It may also allow astrocytes to convey glucose from blood vessels to neurons (Rouach *et al.* 2008), and to integrate information over large areas or act as a diffuse conduit to pass information to distant neurons (Araque *et al.* 2014) and, perhaps, to the vasculature to control cerebral blood flow.

Astrocytes have historically thought to be passive housekeeping cells, but research over the last few decades has revealed their multifaceted role in the development and maintenance of a healthy brain. They are necessary for synapse formation and maturation during development, a function likely to be carried over into adulthood, particularly in the context of learning, and of repair after injury (Pfrieger & Barres, 1997; Ullian *et al.* 2001). They respond to neuronal activity by releasing gliotransmitters such as glutamate, ATP and D-serine to modulate synaptic properties (Parpura *et al.* 1994; Henneberger *et al.* 2010; Panatier *et al.* 2011; Araque *et al.* 2014), although the mechanism underlying this release remains highly controversial (Hamilton & Attwell, 2010; Bazargani & Attwell, 2016). They maintain the temporal and spatial precision of synaptic transmission by taking up excess neurotransmitters (Barbour *et al.* 1988; Chatton *et al.* 2003) and buffering K^+ from the extracellular space (Newman *et al.* 1984). It is also becoming increasingly clear that astrocytes contribute significantly to the regulation of, and may even respond to changes in, cerebral blood flow (Moore & Cao, 2008; Attwell *et al.* 2010).

Cerebral blood flow

The brain has a very high energy demand compared to the rest of the body – although it comprises only 2% of the body weight, it commands 20% of the body's resting state energy usage (Kety, 1957; Sokoloff, 1960; Attwell & Laughlin, 2001; Harris *et al.* 2012). This high energy demand is supplied by an extensive cerebral vasculature. Large pial arteries, covered by multiple layers of vascular smooth muscle cells (VSMCs), lie on the surface of the cortex and branch into penetrating arterioles, ensheathed by a single layer of VSMCs, which enter the cortical parenchyma. These parenchymal arterioles further branch into the capillary network and are drained by ascending venules into the pial veins (Blinder *et al.* 2013).

To ensure sufficient supplies of oxygen and nutrients to the brain in the face of varying systemic blood pressure, cerebral blood flow is tightly controlled via a process termed autoregulation (Tzeng & Ainslie, 2014). Despite this regulatory mechanism, and due to a lack of major energy stores in the brain (besides some glycogen granules: Holmes & Holmes, 1926), increases in neuronal activity demand a further increase in energy supply, which is delivered by a corresponding increase in local blood flow. This coupling between neuronal activity and cerebral blood flow, termed functional hyperaemia or neurovascular coupling (NVC), was first described by Mosso in 1880, and further characterised by Roy and Sherrington a decade later (1890). NVC has now come to be accepted as a fundamental aspect of brain function and many non-invasive brain imaging techniques used in both clinical and research settings, such as blood oxygenation level dependent functional magnetic resonance imaging (BOLD fMRI), functional near-infrared spectroscopy

(fNIRS) and some forms of positron emission tomography (PET), exploit these changes in blood flow as a proxy measure of neuronal activity. Although the significance of neurovascular coupling for healthy brain function is widely accepted, the cellular mechanisms underlying this phenomenon have not yet been fully characterised.

Mechanisms of neurovascular signalling

Much of the initial efforts in NVC research focused on defining direct signalling from neurons to VSMCs. In many brain regions, glutamatergic activation of principal neurons results in the downstream production and release of nitric oxide (NO) and prostaglandins, vasoactive substances that can induce arteriole dilation (Meng *et al.* 1995*a*,*b*; Yang *et al.* 2000; Lacroix *et al.* 2015). The intracortical vasculature is also innervated by nerve terminals from subcortical regions that can release vasoactive neurotransmitters such as acetylcholine, serotonin, dopamine and noradrenaline (Krimer *et al.* 1998; Hamel, 2004, 2006). Adding another layer of complexity, pial arteries also receive nerve terminals from the peripheral nervous system which can release agents to dilate or constrict them (Hamel, 2006). However, this vascular innervation from subcortical and peripheral axons is distributed widely throughout the brain and pial vasculature, respectively, suggesting that this level of regulation will change vascular tone over large regions rather than mediate local NVC. A subset of subcortical neuronal inputs may also activate local interneurons as intermediaries in signalling to the vasculature (Cauli *et al.* 2004). Physiological activation of a cortical region would also activate the interneurons therein, and signalling through these interneurons may be one possible mechanism by which local NVC control can be gained. Indeed, interneurons are the primary neurons that express neuronal nitric oxide synthase (NOS) in the brain (Cauli *et al.* 2004; Jaglin *et al.* 2012). However, the contribution of interneurons to activity-evoked NVC is not completely understood and both dilatory and constrictory effects have been reported (Hamel, 2006). A detailed report of the neurogenic signals involved in regulating the cerebrovasculature can be found elsewhere (Hamel, 2006; Attwell *et al.* 2010; Cauli & Hamel, 2010). This review will focus instead on the regulation of cerebral blood flow by astrocyte-mediated signalling mechanisms.

The role of astrocytes in mediating NVC

Numerous studies in the last few decades have suggested a role for astrocytes in regulating cerebral blood flow. Astrocytes are characterised by a dense cloud of fine processes (Bushong *et al.* 2004) which contact hundreds of synapses (Ventura & Harris, 1999), while specialised astrocyte processes called endfeet enwrap the vascular network in the brain parenchyma (Simard *et al.* 2003; Mathiisen *et al.* 2010), making them ideally positioned to mediate NVC (Fig. 1). Based on observations of the astrocyte morphology, Ramón y Cajal speculated, as early as 1895, that they might regulate vascular diameter (Ramón y Cajal, 1895). More than a hundred years later, we now know that astrocytes can indeed respond to neuronal activity with rises in intracellular Ca^{2+} concentration (Cornell-Bell *et al.* 1990; Zonta *et al.* 2003; Hirase *et al.* 2004; Nimmerjahn *et al.* 2009) and, in turn, release various gliotransmitters (Parpura *et al.* 1994; Parri *et al.* 2001; Henneberger *et al.* 2010; Panatier *et al.* 2011; reviewed in Araque *et al.* 2014) and other factors into the microenvironment, some of which lead to the alteration of vascular tone.

One of the first hypotheses regarding astrocyte control of vascular diameter came from Paulson and Newman in 1987 (Paulson & Newman, 1987). Neuronal activity results in an increase in extracellular $[K^+]$ ($[K^+]_e$), which is taken up by astrocytes to buffer $[K^+]_e$ and thus regulate the excitability of nearby neurons. It was proposed that glial cells might release this K^+ in a directed manner via their endfeet processes, which are enriched in K^+ channels, thus raising the $[K^+]_e$ around the vessels and resulting in VSMC hyperpolarisation (via the unusual dependence of VSMC inward rectifier K^+ channels on $[K^+]_e$; Longden & Nelson, 2015) and thus dilation of blood vessels (Paulson & Newman, 1987). This K^+ siphoning hypothesis was later disproved by experiments showing that mice lacking in Kir 4.1 channels, the primary K^+ channels on glial endfeet around vessels, did not affect NVC (Metea *et al.* 2007). However, an alternative mechanism of

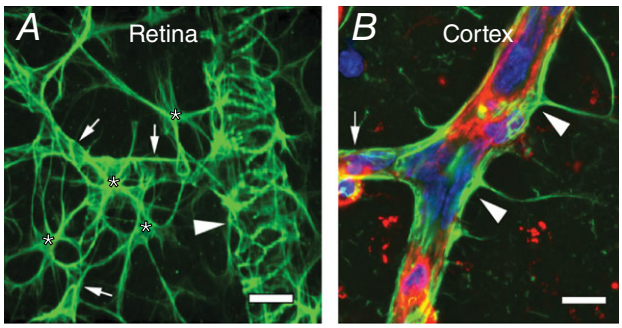

Figure 1. Astrocyte endfeet processes enwrap blood vessels in the central nervous system
Inner surface of a whole mount retina (*A*) and a cortical slice (*B*) immunolabelled for glial fibrillary acidic protein (in green) showing astrocytes with their endfeet around blood vessels. Stars indicate astrocyte somata, arrowheads point to endfeet surrounding large vessels, arrows point to endfeet surrounding capillaries. The cortical section is also stained with DAPI (blue) for nuclei and isolectin B_4 (red) for the vascular basement membrane. Scale bars = 10 μm. (Image by A. Mishra and Y. Chen.)

NVC dependent on K^+ release from astrocytes was brought forward by Nelson and colleagues (Filosa *et al.* 2006). Neuronal activity leads to a rise in astrocyte $[Ca^{2+}]_i$, which, in turn, can activate large conductance Ca^{2+}-activated K^+ (B_K) channels on astrocyte endfeet, leading to an efflux of K^+ onto the vasculature. This rise in $[K^+]_e$ results in smooth muscle hyperpolarisation and dilation of arterioles (Filosa *et al.* 2006).

A separate mechanism of astrocyte-mediated NVC was proposed in 1998, which depends on the synthesis and release of vasoactive agents from astrocytes (Harder *et al.* 1998). Astrocytes can metabolise membrane phospholipids to produce arachidonic acid (AA) (Stella *et al.* 1994), which can be used to synthesise vasodilatory substances such as prostaglandins and epoxyeicosatrienoic acids (EETs) within astrocytes (Amruthesh *et al.* 1993; Alkayed *et al.* 1996*b*), or it can be released onto VSMCs where it can be metabolised to the vasoconstrictor 20-hydroxyeicosatetraenoic acid (20-HETE) (Gebremedhin *et al.* 2000; Roman, 2002). Furthermore, altering the AA metabolism pathway was shown to reduce functional hyperaemia *in vivo* (Alkayed *et al.* 1996*a*). It was thus suggested that neuronal activation may lead to the release of astrocyte-derived AA metabolites to mediate NVC.

Evidence soon emerged from *in vitro* slice experiments that neuronal stimulation-evoked vasodilation of cortical arterioles was dependent on prostaglandin E_2 production and associated with rises in astrocyte $[Ca^{2+}]_i$ (Zonta *et al.* 2003). In hippocampal slices, direct activation of astrocytes, by Ca^{2+} uncaging or activation of metabotropic glutamate receptors (mGluRs), was shown to evoke 20-HETE-dependent arteriole vasoconstriction (Mulligan & Macvicar, 2004). Interestingly, in the retina, light-evoked neuronal activation led to both dilation (by EETs) and constriction (by 20-HETE) of retinal arterioles in a manner dependent on glial activation (Metea & Newman, 2006). Around the same time, an *in vivo* study reported that cortical arterioles dilate in response to both neuronal and glial activation in a prostaglandin-dependent manner (Takano *et al.* 2006). Furthermore, spontaneous glial Ca^{2+} waves in the retina were also observed to evoke vasoconstriction when they propagated across an arteriole (Kurth-Nelson *et al.* 2009), although the molecular mechanism for this is as yet unknown.

The fact that glial activation evoked dilations in some studies but constrictions in others opened up the possibility of bidirectional control of the vasculature by astrocytes and raised new questions regarding the conditions that define the polarity of the neurovascular response. These studies also suggested possible regional differences in the molecular signals mediating NVC, for example EETs might mediate dilation in the retina but prostaglandin E_2 might be the predominant dilating agent in the brain (Attwell *et al.* 2010; Macvicar & Newman, 2015). More recent studies, however, have also found a role for glial-derived PGE_2 in mediating retinal arteriole dilation (Mishra & Newman, 2010) and EETs in mediating cortical arteriole dilation (Peng *et al.* 2002, 2004; Lecrux *et al.* 2011; Liu *et al.* 2011).

To further establish the role of astrocytes in NVC, some researchers have also used models of glial ablation. In one such study, increases in cerebral blood flow produced by basal forebrain stimulation were reduced by ~50% when the gliotoxins fluorocitrate and fluoroacetate (Paulsen *et al.* 1987) were used to damage astrocytes in the cortex (Lecrux *et al.* 2011). Based on pharmacology, the authors concluded that basal forebrain stimulation evokes the release of EETs onto blood vessels by activating astrocytes (Lecrux *et al.* 2011). Another study found that ablation of glial cells with L-2-aminoadipic acid, another potent gliotoxin (Huck *et al.* 1984), significantly reduced light-evoked retinal blood flow without altering electroretinograms in the cat retina *in vivo* (Song *et al.* 2015). Although these studies support the idea of astrocyte-mediated NVC, they should be interpreted with caution as ablation of glial cells will also undoubtedly have some indirect effects on neurons due to changes in homeostatic mechanisms such as glutamate–glutamine cycling (Hassel *et al.* 1992), K^+ buffering (Lian & Stringer, 2004*a,b*) and extracellular pH regulation (Stringer & Aribi, 2003). These neuronal effects, however, are likely to be minimal or only present at high doses (Paulsen *et al.* 1987; Virgili *et al.* 1991; Hassel *et al.* 1992), which is also demonstrated by the maintenance of the electroretinogram in the study by Song *et al.* (2015).

Taken together, these findings have established astrocytes as being important players in regulating cerebrovascular blood flow (Fig. 2) and lent credence to the concept of neuro-glio-vascular coupling, as originally posited by Cohen *et al.* (1997). However, they also raise a number of new questions. (i) How is the polarity of the astrocyte-mediated NVC determined? (ii) How is the balance between dilation *vs.* constriction regulated to achieve the desired response? (iii) What is the relative contribution of B_K channels *vs.* AA metabolites to astrocyte-mediated NVC? (iv) What is the precise role of astrocyte $[Ca^{2+}]_i$? (v) What is the relative contribution of neurons vs astrocytes in the haemodynamic response?

Additionally, it is worth noting that although functional hyperaemia has been traditionally thought to be mediated exclusively at the arteriole level, evidence now suggests that pericyte-mediated dilation of capillaries also contributes to this response (Peppiatt *et al.* 2006; Hall *et al.* 2014; Kornfield & Newman, 2014). Given the large relative resistance of capillaries in the vascular network *in vivo* (Blinder *et al.* 2013), they may contribute significantly to neurovascular coupling at the local level compared to arterioles alone (Hall *et al.* 2014). The contribution of

astrocytes to capillary blood flow regulation has yet to be explored in more detail.

Bidirectional control of blood vessel diameter by astrocytes

Why brain activity should sometimes result in vasoconstriction as observed by several groups (Zonta *et al.* 2003; Mulligan & Macvicar, 2004; Metea & Newman, 2006), conceivably producing a reduction in energy supply, became a point of contention in the field. Some of these findings might be due to the variables of slice work. In the retina, light-evoked dilation of retinal arterioles was enhanced and constriction was suppressed when the NO concentration in the tissue was lowered (Metea & Newman, 2006). This seems counterintuitive at first because NO is an established vasodilating agent, having been initially identified as the endothelium-derived relaxing factor (Palmer *et al.* 1987). However, it can be explained when the effect of NO on AA metabolism is considered. The enzymes that metabolise AA (epoxygenases which produce EETs, cyclooxygenases which produce prostaglandins and ω-hydroxylases which produce 20-HETE) are susceptible to modulation by NO (Roman, 2002; Attwell *et al.* 2010). It is possible that damage caused during dissection induces an increase in

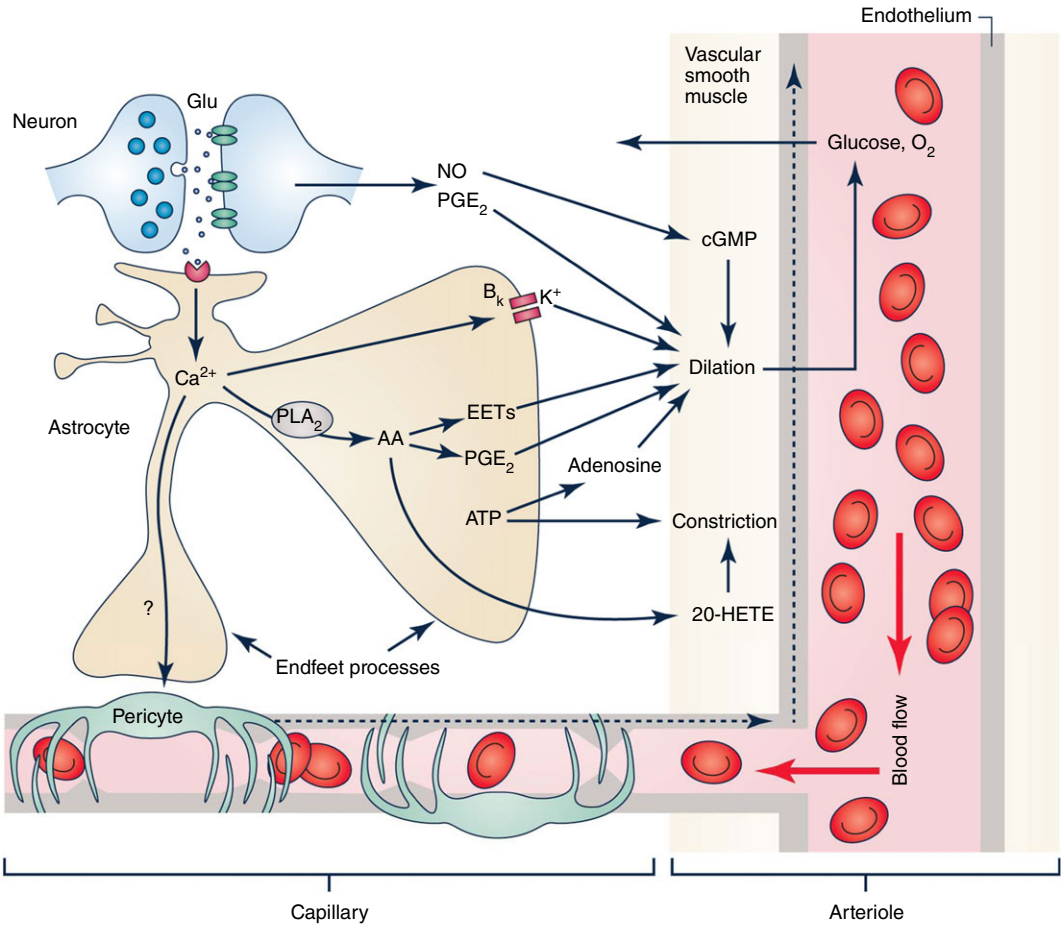

Figure 2. Pathways mediating NVC
Neuronal activity results in synaptic release of glutamate, activating both postsynaptic neurons and astrocytes. Neuronal messengers such as NO and PGE_2 may directly dilate cerebral arterioles. Activation of astrocytes results in a cascade of several signalling pathways. Ca^{2+}-dependent activation of B_K channels can release K^+ to dilate vessels. Ca^{2+} can also activate phospholipase A_2, leading to the synthesis and release of AA metabolites such as EETs and PGE_2 to dilate vessels (both arterioles via VSMCs, and capillaries via pericytes). AA can also be released from astrocytes onto vascular smooth muscle cells, where it can be metabolised to the vasoconstrictor 20-HETE. Release of ATP from astrocytes can directly cause constriction of the vessels, or it can be metabolised by ectonucleotidases to raise the level of adenosine, a vasodilator. A dilation evoked at the capillary level may propagate to upstream arterioles via gap junctions between endothelial cells (dashed arrows). Some of the astrocyte-generated vasoactive factors (e.g. ATP and PGE_2) may contribute to the maintenance of basal tone, whereas others (EETs, PGE_2, 20-HETE, K^+ efflux from B_K channels) are involved in activity-evoked changes in vascular diameter, which lead to increases in blood flow to bring O_2 and glucose to supply the energy demand of active neurons. (Image by A. Mishra.)

the activity of nitric oxide synthases (NOS) in the retina, resulting in abnormal NO levels and altering the relative balance of AA metabolites produced.

Furthermore, these aforementioned studies were conducted in 95% O_2-bubbled perfusate, a widely accepted practice in neuroscience, which results in the abnormally high tissue $[O_2]$ of > 500 mmHg (Mishra *et al.* 2011) compared to physiological measurements ranging between ∼15–20 mmHg in the cortex (Metzger *et al.* 1971), 12.7–64.4 mmHg in the cerebellum (Offenhauser *et al.* 2005) and ∼5–40 mmHg in the retina (Cringle & Yu, 2010). When slices are exposed to 20% O_2, tissue $[O_2]$ ranges between 10–20 mmHg at depths of 50–90 μm in the hippocampus (Gordon *et al.* 2008) and 30–60 mmHg in the retina (Mishra *et al.* 2011), approximating physiological concentrations. When neurovascular coupling experiments were repeated under these conditions mimicking physiological $[O_2]$, astrocyte stimulation led to dilation of hippocampal arterioles (Gordon *et al.* 2008) while also inducing an increase in glycolytic metabolism and raising the concentration of lactate in the tissue. Lactate inhibits the PGE_2 transporter (Chan *et al.* 2002) and thus raises the extracellular PGE_2 concentration, leading to dilations (Gordon *et al.* 2008). On the other hand, O_2 is also a strong modulator of haem enzymes (see Attwell *et al.* 2010 for review) and when experiments were conducted in 20% O_2, 20-HETE-mediated constrictions were suppressed and a prostaglandin-mediated dilation component was revealed in the retina (Mishra *et al.* 2011). This is probably because of the different O_2 affinities of the enzymes synthesising 20-HETE and prostaglandins (Attwell *et al.* 2010). Thus, it appears that O_2 can modulate NVC by changing the metabolic state of the tissue and altering the AA metabolites synthesised. However, high levels of inspired O_2 *in vivo* did not alter the neurovascular response in either the retina (Mishra *et al.* 2011) or the cortex (Lindauer *et al.* 2010), perhaps because autoregulatory mechanisms constrict the vasculature and reduce cerebral blood flow upon exposure to high $[O_2]$ in order to maintain tissue P_{O_2} within the physiological range despite high arterial P_{O_2} (Torbati *et al.* 1978; Omae *et al.* 1998; Floyd *et al.* 2003; Lu *et al.* 2009; Mishra *et al.* 2011).

Given these effects of NO and O_2 on NVC, it is noteworthy that the NOS enzymes themselves are also sensitive to $[O_2]$ (Stuehr *et al.* 2004) and that NO can act as either a mediator (in the cerebellum: Akgören *et al.* 1996; Yang *et al.* 2000) or a modulator of NVC (in the cortex: Lindauer *et al.* 1999), depending on the brain region being studied. We must also consider the possibility that other factors that are altered in slice preparations bathed with artificial cerebrospinal fluid, such as glucose and other metabolic products (Yamanishi *et al.* 2006; Puro, 2007), may also alter NVC. Even more importantly, vessels in brain slices completely lack perfusion pressure and physiological tone. A few studies have overcome this limitation by artificially perfusing the vasculature in slices via cannulation (Lovick *et al.* 2005; Kim & Filosa, 2012); however, due to the difficult nature of this technique, it is not widely practiced. Thus, although slice experiments are a necessity for the ease of carrying out pharmacological experiments and observing active dilation of the vasculature (as opposed to passive dilation resulting from an upstream increase in blood flow), the pitfalls and confounds of *in vitro* experiments should be taken into account in data interpretation.

Bidirectional modulation of NVC may also be explained, in part, by the extracellular $[K^+]$ that the vascular smooth muscle cells are exposed to. Although moderate increases in $[K^+]_e$ induces VSMC hyperpolarisation and thus dilation (Filosa *et al.* 2006), concentrations above 20 mM can result in VSMC depolarisation and constriction of cerebral arterioles (Knot *et al.* 1996; Horiuchi *et al.* 2002), which is expected from the $[K^+]_e$ dependence of the current that flows through VSMC inwardly rectifying K^+ channels (Farr & David, 2011; Longden & Nelson, 2015). Following neuronal stimulation or astrocyte Ca^{2+} uncaging, moderate increases in astrocyte $[Ca^{2+}]_i$ correlate with dilation but large increases correlate with vasoconstriction (Girouard *et al.* 2010), suggesting that the magnitude of the astrocyte $[Ca^{2+}]_i$ signal may determine the polarity of vasomotor response, possibly by altering $[K^+]$ in the extracellular space surrounding the vessels. It has also been suggested that vasoconstriction might be physiologically relevant in certain instances *in vivo*, whereby increases in blood flow to active brain regions may be assisted by the active constriction of blood vessels in the surround or the contralateral hemisphere, a hypothesis further supported by the observation of negative BOLD signals in the periphery of activated regions (Devor *et al.* 2007).

The controversies surrounding the role of astrocytes in NVC

Astrocyte-mediated mechanisms of NVC are believed to be dependent on astrocyte Ca^{2+} signalling. Activation of neurons results in the synaptic release of neurotransmitters such as glutamate which, in addition to acting on postsynaptic receptors, can also activate astrocytic group I metabotropic glutamate receptors ($mGluR_1$ and $mGluR_5$), leading to inositol trisphosphate (IP_3)-dependent Ca^{2+} release from internal stores (Panatier & Robitaille, 2015). Until recently, this was thought to be the main neuron-to-astrocyte signalling mechanism responsible for the downstream release of vasoactive agents. However, recent studies have questioned this conclusion. Whisker

stimulation-evoked haemodynamic responses were found to be independent of $mGluR_5$ activation (Calcinaghi *et al.* 2011), a finding supported by the lack of group I mGluR expression in adult astrocytes (Sun *et al.* 2013). Furthermore, mice lacking IP_3R_2, the predominant IP_3 receptor in astrocytes, were shown to have normal NVC (Takata *et al.* 2013; Bonder & McCarthy, 2014) and fMRI responses (Jego *et al.* 2014), challenging the idea that astrocytes are required for NVC. There is also controversy regarding whether astrocyte Ca^{2+} signals, when they do occur, are fast enough (Nizar *et al.* 2013) or reliable enough (Winship *et al.* 2007) to generate functional hyperaemia.

However, it is important to consider the possibility that astrocyte Ca^{2+} signals important for neurovascular signalling might be mediated by pathways other than mGluR-mediated IP_3 release, or may not have been detected using traditional methods of imaging using bulk loading of Ca^{2+} indicator dyes. In addition, most previous reports investigated $[Ca^{2+}]_i$ changes within astrocyte cell bodies alone, but the signals that are important for neuro-glial communication and NVC probably occur in their fine processes near synapses and/or endfeet. Indeed, astrocyte fine processes display Ca^{2+} transients and localised process-wide Ca^{2+} waves that are not always reflected by soma measurements (Di Castro *et al.* 2011; Panatier *et al.* 2011; Shigetomi *et al.* 2013*a*) and are poorly detected by bulk loaded indicators (Reeves *et al.* 2011). Observations made *in vivo* have achieved some degree of success in demonstrating stimulation-evoked rapid $[Ca^{2+}]_i$ rises in astrocyte cell bodies and endfeet in the somatosensory cortex (Lind *et al.* 2013). The development of genetically encoded calcium indicators (GECIs) such as GCaMP has further aided the detection of these astrocyte Ca^{2+} signals. Using astrocyte specific expression of GCaMP6f, it was recently shown that although large Ca^{2+} fluctuations in astrocyte soma are absent in the IP_3R_2 knockout mouse, Ca^{2+} transients in astrocyte processes are still present (Srinivasan *et al.* 2015), indicating that alternative mechanisms that raise astrocyte $[Ca^{2+}]_i$ must exist. Similarly, using GCaMP3 expression, Otsu *et al.* (2015) also detected the presence of mGluR-independent $[Ca^{2+}]$ signals in astrocyte processes in the olfactory bulb of adult mice and found them to precede neuronally evoked vessel dilation (Otsu *et al.* 2015). In contrast, another study using GCaMP6f found that neuronal stimulation-evoked arteriole dilations were preceded by a $[Ca^{2+}]_i$ rise in astrocyte processes only when the stimulation was above a certain threshold (Institoris *et al.* 2015), implying that NVC may be mediated by astrocytes in conditions of high activity but is generated perhaps by neurons directly following mild activation. It has also been proposed that the initiation and maintenance of the NVC response might be mediated differentially, whereby neuronal or Ca^{2+}-independent astrocyte signalling pathways may initiate vascular dilation and Ca^{2+}-dependent astrocyte signalling component may be important only in the maintenance phase of the response (Martindale *et al.* 2005; Calcinaghi *et al.* 2011; Rosenegger & Gordon, 2015). A computational model of neuro-glio-vascular coupling also suggested an astrocytic contribution only during high levels of neuronal activity (Blanchard *et al.* 2016), supporting this hypothesis. However, this study only included astrocytic neurotransmitter uptake in their model. Incorporating astrocytic mechanisms involved in NVC such as K^+ buffering and AA production/release into such models may offer more significant insights into this process.

In addition, astrocyte $[Ca^{2+}]_i$ signalling can be driven by other mechanisms that do not require a dependence on IP_3, including AMPA receptors (Seifert & Steinhauser, 1995), NMDA receptors (Lalo *et al.* 2006), purinergic receptors (Lalo *et al.* 2008), TRPA1 channels (Shigetomi *et al.* 2013*b*), Na^+–Ca^{2+} exchangers and ryanodine receptors (Kirischuk *et al.* 1997). These mechanisms should be considered as possible alternatives when investigating neuroglial communication. In particular, ATP has long been known to raise astrocyte $[Ca^{2+}]_i$ (Fellin *et al.* 2006; Newman, 2006) and is often used as a positive control in experiments studying astrocyte $[Ca^{2+}]_i$ (Sun *et al.* 2013; Otsu *et al.* 2015). Glial Ca^{2+} waves are primarily propagated by ATP release from glial cells acting on purinoceptors on neighbouring cells (Newman, 2001; Bowser & Khakh, 2007), and ATP also plays a role in direct neuroglial communication, as demonstrated in the retina (Newman, 2006), cerebellum (Piet & Jahr, 2007) and the cortex (Ase *et al.* 2010; Lalo *et al.* 2011). Recently, overexpression of an ectonucleotidase to break down extracellular ATP was shown to significantly decrease the BOLD signal evoked by electrical forepaw stimulation (Wells *et al.* 2015), suggesting that purinergic signalling may be involved in NVC. However, the source(s) of endogenously released ATP, and the specific receptor(s) involved in mediating its possible neurovascular effects, have yet to be defined.

It is also important to keep in mind that NVC may involve Ca^{2+}-independent astrocyte signalling. Due to their electrically passive nature, the biology of astrocytes was largely unexplored until the middle of the twentieth century when researchers started characterising them electrophysiologically (Hild *et al.* 1958; Tasaki & Chang, 1958; Kuffler & Potter, 1964), but the field really bloomed only in the 90s when Ca^{2+} indicators were used to show astrocyte Ca^{2+} waves in response to glutamate and neuronal stimulation (Cornell-Bell *et al.* 1990; Dani *et al.* 1992). Measuring Ca^{2+} is, to date, the best method we have to study astrocyte activity, but this should not lead us to believe that this is the only way that signalling within or via astrocytes can be achieved. The development of new methods to study astrocytes is therefore required

to improve our understanding of their function, both Ca^{2+}-dependent and -independent.

Astrocyte control of pial arterioles

Although pial vascular tone is largely mediated by endothelial and myogenic factors, astrocytes have also been implicated in neurovascular signalling to pial arteries. Neuronally evoked dilation of pial arterioles is partly dependent on purinergic signalling from the glia limitans, a layer of specialised astrocyte processes that form a barrier around the brain and abut pial vessels (Xu *et al.* 2008). In this study, interrupting the signalling along the astrocytic syncytium, by damaging the glia limitans with the gliotoxin L-2-aminoadipic acid, reduced neuronally evoked pial arteriole dilation, but endothelial damage induced by photoactivation of an intravascular dye had little effect (Xu *et al.* 2008). In contrast, a more recent study reported that damaging endothelial cells locally using a similar photoactivated dye method stopped the propagation of dilation along pial arterioles beyond the region of damage and, when the damage is induced over a broader region, caused a reduction in the haemodynamic response (Chen *et al.* 2014). Perhaps the different observations reported by these two studies can be explained by differences in stimulation protocol or the anaesthesia regime, but, at this point in time, the relative contribution of astrocytes and the endothelium in propagating dilations to upstream arterioles is still unclear and a role for astrocytes cannot be ruled out. In addition, factors other than ATP have also been suggested in astrocyte signalling to pial arterioles, for example AA metabolites (Ellis *et al.* 1990), and have yet to be investigated in more detail.

Astrocytes and resting vessel tone

Cerebrovascular tone is generated largely by autoregulatory means, including pressure-induced (Faraci *et al.* 1989) and flow-induced (Pohl *et al.* 1991) myogenic mechanisms. Regulation of cerebral vessels by perivascular nerve terminals (both peripheral, in the case of extracerebral pial vessels, and subcortical, in the case of parenchymal vessels) also contributes to neurogenic vascular tone (Hamel, 2006). Recently, however, a role for astrocytes in maintaining the resting tone of arterioles has also been unveiled. In the *in vivo* retina, increasing extracellular ATP constricted arterioles while breakdown of extracellular ATP, inhibition of purinergic receptors and application of the gliotoxin fluorocitrate all dilated arterioles (Kur & Newman, 2014). A purinergic signalling mechanism also contributed to the development of pressure-evoked vasomotor tone in the cortex (Kim *et al.* 2015). The development and maintenance of this vascular tone was found to be dependent on TRPV4-mediated astrocytic $[Ca^{2+}]_i$ rises both *in vivo* and *in vitro*, presumably leading to ATP release from astrocytes to constrict vessels (Kim *et al.* 2015).

Rosenegger *et al.* (2015) reported that Ca^{2+}-dependent mechanisms in astrocytes contribute to COX1-mediated tonic dilation of cortical arterioles. When they used the Ca^{2+} chelator BAPTA to arrest Ca^{2+} fluctuations within the astrocyte syncytium surrounding arterioles, the resting diameter of the arterioles decreased significantly (Rosenegger *et al.* 2015). However, another group found that similarly arresting astrocyte Ca^{2+} with BAPTA-filling from a patch pipette led to a decrease in pressure-induced vascular tone, i.e. dilation (Kim *et al.* 2015). It is highly probable that these opposing mechanisms (COX1-mediated dilation and purinergic constriction) may operate together to maintain the right balance of constrictory and dilatory factors to stabilise vascular tone in the face of changing microenvironment (Fig. 2), both within the brain and the blood, an effect that is well known elsewhere in the vascular system (Meininger & Davis, 1992) and in extracerebral pial arteries (Faraci *et al.* 1989).

Vascular signalling to astrocytes

Cortical astrocytes have been reported to respond with a $[Ca^{2+}]_i$ rise to pressure changes in arterioles both *in vitro* and *in vivo* (Kim *et al.* 2015). This suggests two interesting possibilities: (i) the nervous system can sense vascular changes and may be able to adjust them in turn; and (ii) the cardiovascular system may be able to alter neuronal function. In the Kim *et al.* study, changes in vascular pressure raised $[Ca^{2+}]_i$ in astrocytes, which contributed to the maintenance of pressure-evoked cerebrovascular tone, thus providing some support to the first conjecture (Kim *et al.* 2015). There is also evidence that astrocytes in the brain stem sense blood pH to activate chemoreceptor neurons and alter breathing rate (Gourine *et al.* 2010), and those in the subfornical organ sense blood Na^+ levels to control salt intake behaviour (Shimizu *et al.* 2007). Similarly, it is possible that astrocyte sensing of vascular pressure may, directly or via astrocyte-mediated neuronal activation, lead to the regulation of cardiovascular function. On the other hand, signals from blood vessels might also be altering neuronal function. An example of direct signalling from blood vessels to neurons via NO production and release has been demonstrated in the optic nerve (Garthwaite *et al.* 2006). As Ca^{2+}-dependent signalling within astrocytes can regulate neuronal activity by releasing gliotransmitters (Parpura *et al.* 1994; Henneberger *et al.* 2010; Panatier *et al.* 2011; Araque *et al.* 2014), it is plausible that vascular-evoked changes in astrocyte Ca^{2+} may modulate neuronal function, as proposed by the 'hemo-neural hypothesis' (Moore & Cao, 2008; Kim *et al.* 2015). All these possibilities ought to be further explored, especially

in the context of altered vascular tone in cardiovascular diseases such as hypertension that accompany, or may lead to, neurodegenerative disorders (Bloch *et al.* 2015).

Astrocytes and cerebrovascular dysfunction in CNS pathology

Astrocytes respond to almost all forms of CNS dysfunction with a process termed reactive astrogliosis (Barres, 2008; Anderson *et al.* 2014), during which their morphology and protein expression patterns change drastically (Hamby *et al.* 2012; Zamanian *et al.* 2012). This suggests that the function of astrocytes may evolve during disease, although it is unclear whether these changes contribute to the progression of pathology or exist as protective mechanisms. Challenging cultured astrocytes with inflammatory mediators alters the expression of many proteins involved in neuron-to-astrocyte signalling and, accordingly, astrocyte Ca^{2+} signalling is markedly altered (Hamby *et al.* 2012). Reactive astrocytes change their expression of neurotransmitter receptors and transporters such as mGluRs (Aronica *et al.* 2000) and GLT-1 (Rothstein *et al.* 1995; Bramlett & Dietrich, 2004). They also alter the expression of the inwardly rectifying K^+ channels, altering their ability to buffer extracellular K^+ (Bordey *et al.* 2000, 2001; Tong *et al.* 2014). Such changes in astrocyte physiology, particularly in their Ca^{2+} dynamics and K^+ buffering ability, are expected to alter their role in the regulation of cerebral blood flow with possible negative effects on neuronal function. The need to address the neurovascular unit as a therapeutic target in the context of diseases with a vascular component, including Alzheimer's disease (AD), cortical spreading depression, traumatic brain injury and hypertension, has been recently highlighted (Calcinaghi *et al.* 2013; Iadecola, 2013; Bloch *et al.* 2015; Lok *et al.* 2015; Ostergaard *et al.* 2015). Astrogliosis-induced changes in NVC deserve further attention in this regard. Indeed, a recent report showed that an angiotensin II type 1 receptor blocker can improve the cognitive and cerebrovascular deficits in AD by reducing astrogliosis and inflammation, while leaving Aβ pathology intact (Ongali *et al.* 2014). Another study reported that glioma cells invade the space between astrocyte endfeet and the vasculature, disrupting NVC (Watkins *et al.* 2014). Such structural changes are likely to alter the generation of vasoactive signals as well as the response of the neurovascular unit to these signals and perhaps also lead to blood–brain barrier dysfunction, although these ideas have not yet been investigated. Together, these findings imply that CNS pathology may partly comprise a deficit in gliovascular coupling and commands more attention.

In diabetic patients, a reduction in functional hyperaemia precedes clinical retinopathy (Garhofer *et al.* 2004; Mandecka *et al.* 2007). In a rodent model of diabetes, a similar deficit in NVC was reported. This seemed to be mediated by altered glial AA metabolism due to high NO levels produced by increased inducible NOS (iNOS) expression (Mishra & Newman, 2010). This deficit could be pharmacologically reversed in diabetic animals by acute I.V. or chronic oral administration of an iNOS blocker (Mishra & Newman, 2011). As iNOS upregulation is associated with inflammatory responses that occur after injury throughout the body, including in the brain following stroke (Iadecola *et al.* 1995; Garry *et al.* 2015), and can alter glial derived vascular messengers (Metea & Newman, 2007; Mishra & Newman, 2010), its role in glial-mediated NVC in neuropathology needs to be further explored.

Another factor to consider in disease is the tissue O_2 level. Although high levels of O_2 exposure have no effect on NVC responses in the healthy brain *in vivo* (Lindauer *et al.* 2010; Mishra *et al.* 2011) because of autoregulatory mechanisms that maintain tissue P_{O_2} within an acceptable range (Torbati *et al.* 1978; Omae *et al.* 1998; Floyd *et al.* 2003; Lu *et al.* 2009; Mishra *et al.* 2011), this might be altered in disease states. In injuries such as ischaemic stroke, subarachnoid haemorrhage and traumatic brain injury, a breakdown of the blood–brain barrier often ensues (Doczi, 1985; Germano *et al.* 2000; Price *et al.* 2016; Ueno *et al.* 2016), and indeed is being recognised as another common hallmark of CNS pathology (Zhao *et al.* 2015). It is plausible that this breakdown, combined with a lack of autoregulatory compensation, exposes the brain to pathological levels of O_2 and contributes to the observed reduction (Fordsmann *et al.* 2013) or inversion (Koide *et al.* 2012; Pappas *et al.* 2015) of the neurovascular response. In light of the astrogliosis following brain injury and the ensuing changes in their $[Ca^{2+}]_i$ dynamics (Hamby *et al.* 2012) and K^+ uptake (Bordey *et al.* 2000), astrocyte-mediated neurovascular pathways such as those dependent on B_K channels (Pappas *et al.* 2015) or 20-HETE synthesis (Fordsmann *et al.* 2013) might be altered in disease. This is a particularly attractive hypothesis in the context of reperfusion injury, where reinstatement of blood flow after a stroke beyond a limited therapeutic time window results in further damage to the nervous tissue (Bai & Lyden, 2015; Marshall, 2015). The sudden rise in tissue oxygenation produced by the return of blood, especially in the absence of a healthy blood–brain barrier, is known to induce oxidative stress (Bai & Lyden, 2015) but it might also result in the production of glial-derived vasoconstricting factors (Fordsmann *et al.* 2013), limiting the supply of oxygen and glucose to neurons and resulting in further injury.

Furthermore, autoregulation and NVC are both dependent on the resting tone of cerebral vessels (Aaslid *et al.* 1989; Blanco *et al.* 2008). Given that astrocytes play a role in both these processes and that the resting tone of vessels is altered or disrupted in CNS pathologies such as

subarachnoid haemorrhage (Terpolilli *et al.* 2015), glioma (Watkins *et al.* 2014) and cortical spreading depression (Ayata & Lauritzen, 2015), understanding the role of astrocytes in cerebrovascular function in both healthy and pathological conditions is essential for unravelling disease mechanisms and developing novel therapeutic targets.

Development of the neuro-glio-vascular unit

Although neurogenesis is largely complete before birth in rodents, their connections take longer to mature. Astrocytes and the vasculature, integral members of the neurovascular unit, begin development late in the embryo and continue for the first few weeks of life (Caley & Maxwell, 1970; Bayraktar *et al.* 2015). Astrocyte density and morphology in the rat matures to adult levels differentially in different brain regions: at about postnatal day 8 in cortical layer I (Stichel *et al.* 1991), 2–3 weeks in the hippocampus (Nixdorf-Bergweiler *et al.* 1994) and cortical layer VI (Stichel *et al.* 1991), and approximately 6 weeks in cortical layers II–V (Stichel *et al.* 1991). This is reflected in the K^+ buffering capacity of the brain, which does not reach mature levels until 4 weeks after birth (Nixdorf-Bergweiler *et al.* 1994). The diffuse cloud of fine astrocyte processes mature and form astrocyte territories during the third week after birth (Bushong *et al.* 2004) and synthesis of vasoactive mediators such as AA metabolites in the brain also reaches adult levels at the same time, probably as a result of the establishment of adult levels of enzyme expression in the astrocytes (Seregi *et al.* 1987). Existence of the morphologically complete neurovascular unit, comprising the vasculature, neurons and the fine processes and endfeet of astrocytes, is only apparent 3 weeks after birth in the cortex (Caley & Maxwell, 1970). Accordingly, hindpaw stimulation in rodents younger than 2 weeks (mimicking neonatal age in humans) evokes a decrease in blood flow and pial arterial constrictions *in vivo* (Zehendner *et al.* 2013; Kozberg & Hillman, 2016). This response evolves to display stimulation-evoked localised increases in blood flow and pial dilations in a time course parallel to the structural development of the neurovascular unit, starting at around 3 weeks of age (Zehendner *et al.* 2013; Kozberg & Hillman, 2016). This early inverted vascular response is thought to be an important factor in the development of a healthy, mature cerebrovascular architecture and perhaps also the blood–brain barrier (reviewed in Lacoste & Gu, 2015).

Besides the inversion of the neurovascular response, other age-related factors may also contribute to the discrepancies found in NVC research. Studies that use *in vitro* experiments to investigate NVC are largely conducted on tissue from young animals, while *in vivo* studies primarily use adult animals, presenting a co-confound of age and experimental preparation. For example, mGluR-dependent astrocyte-mediated NVC was demonstrated in slices from young animals (P9–21; Zonta *et al.* 2003; Mulligan & Macvicar, 2004; Gordon *et al.* 2008) but, perhaps owing to the developmental downregulation of mGluR I expression in astrocytes (Sun *et al.* 2013), this mechanism did not appear to play a role in adults animals *in vivo* (Calcinaghi *et al.* 2011). Therefore, the age of animals should be carefully considered in the design and interpretation of NVC studies.

Implications of astrocyte control of NVC for BOLD signals

The BOLD signal, used in most functional imaging studies as a proxy measure of brain activity, measures the relative concentration of deoxyhaemoglobin, which rises during activity due to oxygen consumption by active neurons but falls as the increase in blood flow brings in more oxyhaemoglobin. Because the blood flow response is much greater than the fall in deoxyhaemoglobin, BOLD signals primarily reflect the increase in blood flow evoked by neuronal activation (Attwell & Iadecola, 2002). As outlined in this review, astrocytes play a complex role in generating the neurovascular response as well as maintaining the baseline tone of the cerebral vasculature, which influences the magnitude of the BOLD response. This has important implications for the interpretation of functional imaging studies, particularly with reference to different arousal states, disease and development stages. Subcortical neuronal mechanisms that are responsible for modulating brain activity during sleep or arousal states also exert control of the cerebral parenchymal arterioles over large regions of the cerebrum (Hamel, 2004, 2006) and appear to be at least partly mediated by glial cells (Cauli & Hamel, 2010; Lecrux *et al.* 2011). Depending on the arousal state of the subject, the relationship between neuronal activity, astrocyte activity and vascular responses might be differentially modulated by these subcortical nerve terminals, and therefore impact the BOLD signal.

In most CNS diseases, neuronal activity is noticeably altered, but less perceptible are the changes that occur in astrocytes and vascular reactivity. This is partly due to our lack of complete understanding of the disease processes and partly because these components of the nervous system have not been studied in as much detail as neurons. It is very likely that neurovascular signalling pathways might be altered in disease due to altered signalling from reactive astrocytes (as discussed above) or a change in vascular rigidity or reactivity (Hamel, 2015; Tong & Hamel, 2015), leading to a reduction in the coupling or even a complete uncoupling of neuronal activity from vascular responses. Furthermore, conditions like tissue damage and oedema might change the water component of the tissue, which can also alter the BOLD signal (Krings

et al. 2002; Kim & Ogawa, 2012). BOLD may still be a powerful tool in identifying a disease or measuring signals that correlate with symptoms, but it would be impractical to use BOLD signals as a measure of neuronal activity per se under these conditions.

The use of BOLD signals to study developmental processes also comes with severe limitations (Harris *et al.* 2011; Lacoste & Gu, 2015). As discussed above, the development of the neuro-glio-vascular unit, which is essential for the proper execution of neurovascular signals that give rise to activity-evoked blood flow changes, continues for weeks after birth in rodents. In humans, gliogenesis is essentially complete within the first few months after birth (Roessmann & Gambetti, 1986), but synaptogenesis and synaptic pruning continue into the teenage years while myelination can last into the twenties in humans (reviewed in Semple *et al.* 2013). Anatomical studies suggest that although the human brain is 95% of its adult size by age 6, it is still developing into the teenage years (Lenroot & Giedd, 2006). The 'mature' neuro-glio-vascular unit in the rat is only apparent after 3 weeks of age and continues to develop until at least 6 weeks of age (Caley & Maxwell, 1970). Using a simplistic model to compare the developmental stages of rodent and human brains, this suggests a minimum age of 1.5 years before the neurovascular response can be expected to mature in humans (http://www.translatingtime.net). Indeed, activity-evoked blood flow and BOLD responses are inverted in young rodents and neonatal humans (Anderson *et al.* 2001; Born *et al.* 2002; Zehendner *et al.* 2013; Kozberg & Hillman, 2016), probably reflecting the large oxygen consumption of the developing brain combined with a decrease in blood flow (Lacoste & Gu, 2015). This developmental negative relationship between neuronal activity and blood flow is thought to be important in patterning the cerebrovasculature and, ultimately, brain maturation (Lacoste & Gu, 2015). Given these developmental changes, our ability to effectively decipher BOLD signals during childhood and adolescence is severely hampered by a lack of systematic studies investigating the neurovascular relationship during the course of early life in humans (further discussed in Harris *et al.* 2011) and should be carefully considered in interpreting data from these populations.

The transcriptome as a resource for future directions

Many aspects of the astrocytic regulation of cerebral blood flow are as yet uncharted territory. One possible strategy to develop new hypotheses and guide future experiments would be to use published transcriptome analyses (Cahoy *et al.* 2008; Hamby *et al.* 2012; Zamanian *et al.* 2012; Zhang *et al.* 2014). A quick study of the genes expressed at a $>$ 15-fold higher level in astrocytes than in all other cell types in the transcriptome published from the Barres lab (Zhang *et al.* 2014) identified the adenosine receptor A_{2b}, $mGluR_3$, small and intermediate conductance K^+ channels, many enzymes (of particular interest were phospholipases, epoxide hydrolases and CYP enzymes) and a large number of transporters including those for glutamate, GABA, glucose, zinc and cationic amino acids. Many of these proteins, such as mGluRs, adenosine receptors, K^+ channels and enzymes involved in AA metabolism, are already known to be expressed in astrocytes and play important roles in maintaining homeostasis of the extracellular environment and NVC, as discussed in this review. Many others probably play an important role in astrocyte-specific functions and would be of interest to future neuroglial and neurovascular studies. Furthermore, astrocyte-specific genes that help them associate with the vasculature, such as vascular cell adhesion molecule 1, or play a role in their response to disease, such as pentraxin 3 and fibroblast growth factors (particularly FGF8 and FGF3 and their respective receptors) (Rosenman *et al.* 1995; Ravizza *et al.* 2001; Rubio *et al.* 2010; Kang *et al.* 2014), might be of particular interest with respect to alterations in cerebrovascular tone and NVC following disease. Although the transcriptome provides a measure of the level of RNA transcribed and not protein expression, it may prove to be an important starting point in understanding the functions of astrocytes and other glial cells.

Conclusion

The evidence undeniably favours a multifaceted role for astrocytes in cerebrovascular regulation. NVC occurs to supply the energy demand of active neurons and, therefore, it is indisputable that neurons initiate the response, but increasing evidence supports a role for astrocytes as mediators of neurovascular signals. However, controversies still exist regarding the relative contribution of vasoactive signals arising directly from neurons (such as NO and prostaglandins) *versus* those released by astrocytes (AA metabolites, K^+ release), as well as the location and timing of astrocytic Ca^{2+} signalling. The pattern of astrocyte Ca^{2+} signalling (for example, localised transients in processes compared to global intracellular waves) may also be important in defining the vascular response generated. We must endeavour to develop better experimental designs and $[Ca^{2+}]$ detection techniques to resolve these outstanding questions. Astrocytes also play a role in regulating the resting tone of arterioles by releasing both dilating and constricting agents. They can respond to alterations in cardiovascular factors such as pressure and flow which, given their role in neuronal homeostasis, may alter neuronal activity. This suggests a novel binaural role for astrocytes whereby they listen to signals from both neurons and vessels and, accordingly, orchestrate signalling to the cerebral vasculature to maintain a healthy

blood supply to the brain. Their role in maintaining this intricate balance in healthy brains, and how it is altered in pathology, must be explored further with a view to developing new therapeutic targets for diseases of the CNS where the cerebrovasculature is affected.

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Additional information

Competing interests

The author declares no competing interests.

Acknowledgements

I thank David Attwell, Grant R. Gordon, Renaud B. Jolivet, Steven J. Sullivan and Yang Chen for their help in the preparation of this manuscript.