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Review

Role of Astrocytes in Neurovascular Coupling

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Neural activity is intimately tied to blood flow in the brain. This coupling is specific enough in space and time that modern imaging methods use local hemodynamics as a measure of brain activity. In this review, we discuss recent evidence indicating that neuronal activity is coupled to local blood flow changes through an intermediary, the astrocyte. We highlight unresolved issues regarding the role of astrocytes and propose ways to address them using novel techniques. Our focus is on cellular level analysis in vivo, but we also relate mechanistic insights gained from ex vivo experiments to native tissue. We also review some strategies to harness advances in optical and genetic methods to study neurovascular coupling in the intact brain.

Introduction

Normal brain activity depends on a continuous supply of oxygen and glucose through cerebral blood flow (CBF). Although cerebral energetic demands are very high, the brain has very little means of energy storage (Attwell and Laughlin, 2001). Therefore, local brain activity has to be matched by a concomitant increase in local CBF—a phenomenon referred to as functional hyperemia or neurovascular coupling.

Understanding the mechanisms underlying functional hyperemia is important for several reasons. First, noninvasive functional brain imaging methods, which have provided insight into the human brain at work at unprecedented detail (Raichle, 1998), rely on this coupling—in particular, the greater rise in CBF compared to oxygen consumption—to map brain activity (Lauritzen and Gold, 2003; Logothetis, 2003; Raichle and Mintun, 2006; Villringer and Dirnagl, 1995). Therefore, the more we know about the signaling pathways, the better we will understand what kind of underlying brain activity these techniques reflect. Second, perturbed functional hyperemia is involved in the pathophysiology of several neurological diseases (discussed below) (Attwell et al., 2010; Girouard and Iadecola, 2006; Iadecola, 2004), and identifying key steps in functional hyperemia may facilitate alleviation or treatment of these disorders.

Recently, astrocytes have been proposed as important conduits between neuronal and vascular activity. In this review, we will discuss the role of astrocytes in functional hyperemia, highlight the unresolved issues regarding astrocytes, and propose how they can be addressed by novel techniques. Our focus is on analysis of cells in their native environment in vivo, but we also discuss the role of molecular pathways gleaned from ex vivo studies. For aspects of functional hyperemia not related to astrocytes, and for astrocytic functions other than functional hyperemia, we refer the reader to a number of excellent and recent reviews (Barres, 2008; Halassa and Haydon, 2010; Iadecola, 2004; Lauritzen, 2005; Sofroniew and Vinters, 2010; Volterra and Meldolesi, 2005). We have taken the liberty of combining information from different species and brain regions, hoping to identify common principles.

A Macroscopic View of Cerebrovascular Regulation

Most of cerebrovascular regulation takes place on the arterial side of the cerebral vasculature, which can be divided into large pial arteries, derived from arteries branching off the circle of Willis, penetrating arterioles delving into the tissue, and capillaries, where most of the oxygen diffusion into the parenchyma occurs (Figures 1A–1D). Local CBF changes are induced by constriction or relaxation of smooth muscle cells in arteries and arterioles. As penetrating arterioles are located within regions of synaptic activity (Figure 1D) and, together with surface arteries, account for a large part of cerebrovascular resistance (Faraci and Heistad, 1990), they are probably the main targets of local neuronal and glial pathways regulating functional hyperemia. This functional network of neurons, glia, and vascular cells has been termed the neurovascular unit (Figure 1D). In addition, upstream dilation of surface arteries and larger penetrating arterioles is also necessary for adequate and sufficient downstream CBF increase (Erinjeri and Woolsey, 2002; Iadecola et al., 1997; Tian et al., 2010). Since these larger upstream vessels are separated from neurons and astrocytes by the Virchow-Robin space, it has been postulated that intramural (Dietrich et al., 1996) or flow-mediated signals (Fujii et al., 1991) convey intraparenchymal changes to these surface vessels, although the glia limitans, a network of astrocytic processes covering the brain's surface, may also contribute to neurovascular signaling (Xu et al., 2008). Finally, although capillaries lack smooth muscle cells, they are surrounded by pericytes (Figure 1D), which contribute to microvascular CBF (Bell et al., 2010), and which may have the ability, at least in vitro, to actively regulate capillary diameter (Kawamura et al., 2003; Peppiatt et al., 2006), although their contribution to functional hyperemia in vivo remains uncertain (Fernández-Klett et al., 2010).

In summary, signaling from neurons in activated brain regions to local penetrating arterioles (and possibly also capillaries) and a coordinated response of surface vessels, are necessary for local CBF to increase during neuronal activation.

Neuronal Regulation of Local Blood Flow

Because brain research has traditionally been centered on neurons, and neuronal activity can easily be measured by

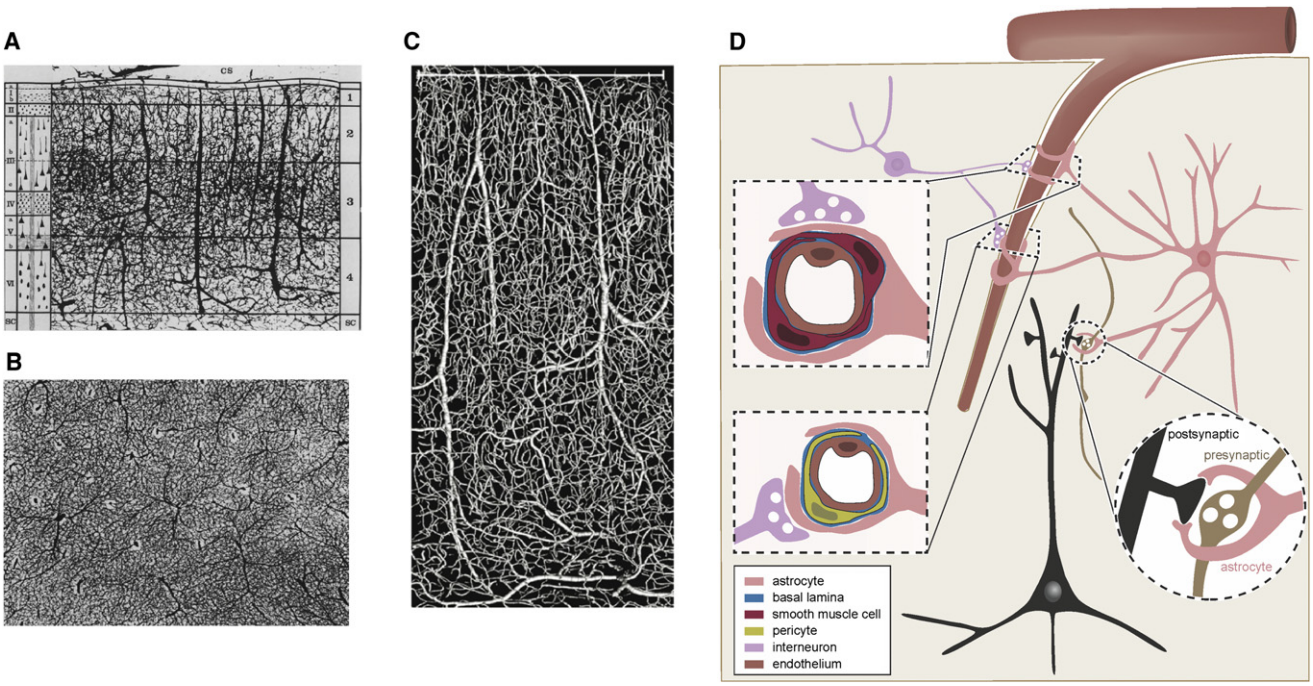

Figure 1. Organization of the Cerebral Vasculature

(A) The architecture of cerebral vasculature exemplified by casts obtained from human brains. Note the penetrating vessels, from which capillaries emerge to perfuse brain tissue. Cortical layers are indicated at the left along with cartoons of neuronal cell bodies.

(B) Tangential section through layer 4 from human brain showing the relatively homogeneous coverage of capillaries, except the regions immediately surrounding arteries.

(C) Three-dimensional volume rendering of human vascular network reconstructed from India ink injected into blood vessels of human brain. Pial surface is on top and the scale bar is 1 mm.

(D) Schematic of the various elements of the neurovascular unit. Penetrating arterioles and capillaries are completely covered by astrocytes. Interneurons targeted at the vasculature synapse onto astrocytes. Astrocytes also detect synaptic activity at neuronal synapses.

Panels (A) and (B) are reproduced with permission from Duvernoy et al. (1981) and (C) from Lauwers et al. (2008).

electrophysiological techniques, there has been the long-held view that neuronal activity directly triggers functional hyperemia. Neuronal processes are indeed closely associated with all parts of the vasculature. Pial arteries and large surface arterioles are innervated by nerve fibers that originate in autonomic and trigeminal sensory ganglia (Hamel, 2006). In the brain parenchyma, penetrating arterioles and capillaries are contacted by local interneurons (Figure 1D) as well as by processes of intrinsic neurons originating from subcortical centers (Golanov et al., 2001; Hamel, 2006; Rancillac et al., 2006; Yang et al., 2000). In addition, centrifugal brainstem fibers may also indirectly affect functional hyperemia by modulating glutamate release from excitatory synapses (Petzold et al., 2009).

If neurons and blood vessels are closely associated anatomically, what signals are then responsible for the functional transfer of information between the two? Early hypotheses focused on the relation between neuronal metabolism and local circulation and proposed that increased energy use and/or oxygen consumption of neurons directly trigger vasodilation (Siesjo, 1978). However, changes in hemodynamics can appear within 1–3 s of increased neural activity, while metabolic changes occur more slowly than this (Lou et al., 1987), indicating that the nature of neuron-to-vessel signaling is more complex. In addition, neurovascular coupling remains unchanged in the face of experimental variations of oxygen and glucose supply (Mintun et al., 2001; Powers et al., 1996), and oxygen consumption occurs in a much smaller area than the subsequent CBF increase (Attwell and Iadecola, 2002; Malonek and Grinvald, 1996). These studies indicated that blood flow changes occur through several intermediate steps, rather than by direct activation through products of cerebral energy metabolism. Indeed, later studies demonstrated that a large fraction of functional hyperemia can be attributed to actions of the excitatory neurotransmitter glutamate (Lauritzen, 2005). For example, ionotropic glutamate receptor activation may mediate functional hyperemia by calcium-activated synthesis of nitric oxide (NO), prostaglandins, and epoxyeicosatrienoic acids in neurons (Akgören et al., 1994; Faraci and Breese, 1993; Lindauer et al., 1999; Niwa et al., 2000a; Peng et al., 2002). In addition, the vasculature-targeted information is conveyed by anatomically discrete local interneurons (Figure 1D), which either can be activated locally by presynaptically released glutamate, as in stellate neurons of the cerebellum (Rancillac et al., 2006; Yang et al., 2000), or can act as relays for remote brainstem nuclei, such as the cholinergic basal forebrain nucleus or the serotonergic raphe nuclei (Cauli et al., 2004).

In sum, although there is a close relationship between oxygen consumption and functional hyperemia (Hoge et al., 1999; Lin

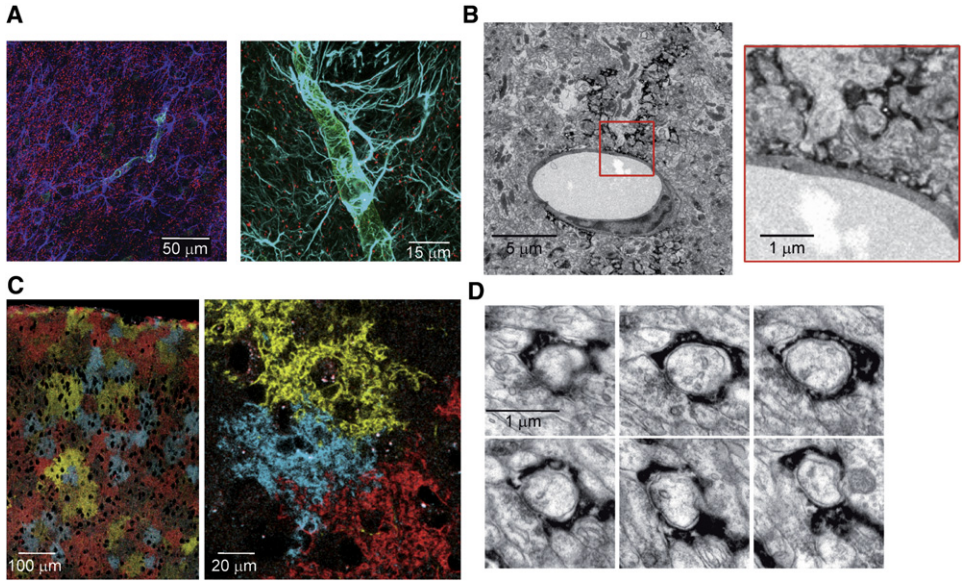

Figure 2. Astrocytes Are Key Elements of Neurovascular Coupling

(A) GFAP stain (blue or cyan) highlights the larger processes of astrocytes, some of which envelop a small arteriole labeled with laminin (green). Also shown are glutamatergic synapses, labeled with antibodies against vesicular glutamate transporter 1 (VGluT1; red).

(B) Electron micrograph from cerebellum showing Bergmann glia contacting blood vessel. In this image, the glial process is highlighted by photoconversion of GFP, which is expressed only in a subset of Bergmann glia in this line of mice. Magnification of the boxed region clearly illustrates the close contact between astrocytic processes and blood vessel.

(C) Astrocytes from the cortex of a "brainbow" mouse showing their tiling, and the private territory each occupies. Dark ovoid regions are mainly neuronal cell bodies. In the right image, a small region is shown at higher magnification, revealing the boundaries between neighboring astrocytes.

(D) Serial sections show glial processes (dark regions with photoconverted GFP) in close proximity to a synapse.

Panels (A), (B), and (D) are courtesy of Dr. Akari Hagiwara (unpublished data). Panel (C) shows images courtesy of Dr. Jean Livet and based on published data (Livet et al., 2007).

et al., 2010; Offenhauser et al., 2005), metabolic byproducts do not directly trigger blood flow changes. Instead, the same neurotransmitters that mediate neuron-to-neuron information exchange also initiate polysynaptic signaling pathways that ultimately trigger functional hyperemia.

Astrocytes Are Anatomical Intermediaries between Neurons and Blood Vessels

The intraparenchymal vasculature is extensively covered by astrocytic endfeet (Mathiisen et al., 2010; McCaslin et al., 2011) (Figure 2A and 2B), which may serve as functional intermediaries between neurons and blood vessels. This intimate anatomical relationship between astrocytes and blood vessels was already noted in some of the earliest descriptions of astrocytic morphology by Cajal and Golgi (Golgi, 1886; Ramon y Cajal, 1895). Recent analyses of astrocytic morphology have revealed that the vascular external surface is almost completely covered by astrocytic endfeet (Mathiisen et al., 2010; Nielsen et al., 1997; Petzold et al., 2008; Simard et al., 2003). Moreover, perivascular astrocytic endfeet (Figure 2B) are important and highly specialized cellular compartments that are enriched in astrocyte-specific proteins such as aquaporin-4, connexin 43, purinergic receptors, and potassium channels (Price et al., 2002; Simard et al., 2003). Finally, at the ultrastructural level, the processes of many vasoactive interneurons, in particular those expressing noradrenaline, synapse onto astrocytes rather than directly onto blood vessels (Hamel, 2006).

These morphological and functional data indicate that, with the possible exception of gaseous transmitters, all signaling molecules targeted to the vasculature must first act on or pass through astrocytes in order to reach the smooth muscle cells in the vessel wall (Figure 1D). The organization of astrocytes into separate domains (Halassa et al., 2007) (Figure 2C) and the very close anatomical and functional relationship between astrocytes and neuronal synapses (Barres, 2008; Haydon, 2001) (Figure 2D) make these cells ideal candidates to convey changes in neuronal activity levels to the vasculature and to be common executors of neurovascular pathways.

A Historical Perspective of Astrocytic Regulation of Vascular Tone

The first recorded person to implicate astrocytes in functional hyperemia was Ramón y Cajal, who hypothesized in 1895 that constriction of astrocytic endfeet would trigger vasoconstriction and endfeet relaxation would induce vasodilation (García-Marín et al., 2007). About a century later, Paulson and Newman proposed astrocytic potassium "siphoning"—i.e., influx of potassium ions into astrocytes near active synapses, and efflux of potassium from astrocytic endfeet into the perivascular space and subsequent potassium-induced vasodilation—as a mechanism of functional hyperemia (Paulson and Newman, 1987). Moreover, Harder and colleagues noted that astrocytes express all proteins necessary to detect neuronal activity and, facilitated by astrocytic calcium elevations,

potentially convert these signals into vasodilation (Harder et al., 1998).

Since astrocytes, unlike neurons, are electrically inexcitable, they are relatively inert to traditional electrophysiological methods. Therefore, studies of astrocytic activity were only possible after the introduction of calcium dyes (Tsien, 1988) and their delivery into identified astrocytes (Kang et al., 2005; Nimmerjahn et al., 2004). Most data on astrocytic influences on CBF so far have been obtained in acute brain slices, because they offer excellent experimental control, are technically practical, and allow relatively easy merging of imaging and electrophysiological techniques (Figure 3A).

Cellular imaging of neurons and astrocytes together with CBF recordings in single vessels in vivo in living animals was achieved only relatively recently, using multiphoton microscopy of fluorescently labeled blood vessels and multicell bolus loading of calcium indicators (Helmchen and Kleinfeld, 2008; Kleinfeld et al., 1998; Stosiek et al., 2003) (Figures 3B–3D). A particularly valuable development has been the ability to monitor blood flow in individual capillaries by following the movement of erythrocytes (Chaigneau et al., 2003; Dirnagl et al., 1992; Kleinfeld et al., 1998) (Figures 3B and 3D), enabling simultaneous recording of CBF and cellular activity with high spatial and temporal resolution.

Astrocytic Activation Changes Vascular Tone in Acute Brain Slices

The different pathways involved in the vascular changes following astrocytic activation in brain slices, which are, together with findings obtained in vivo (discussed below), summarized in Figure 4, have been extensively discussed in recent reviews (Attwell et al., 2010; Iadecola and Nedergaard, 2007; Koehler et al., 2009). Briefly, several brain slice studies showed that stimulation of cortical astrocytes, either directly or through nearby neurons, triggers an intraastrocytic calcium surge and a subsequent dilation or constriction of neighboring arterioles. Vasodilation was triggered by activation of astrocytic metabotropic glutamate receptors (mGluR) and either cyclooxygenase products (Filosa et al., 2004; Zonta et al., 2003) or combined activation of different potassium channels on astrocytes and smooth muscle cells (Filosa et al., 2006). Vasoconstriction, in turn, was mediated by the conversion of astrocytic arachidonic acid derivates to 20-hydroxyeicosatetraenoic acid (20-HETE) in smooth muscle cells by the cytochrome P450 system (Mulligan and MacVicar, 2004). In other studies, vasoconstriction and vasodilation were both observed, depending on the level of nitric oxide (Metea and Newman, 2006) or oxygen in the tissue (Gordon et al., 2008). Finally, the level of the astrocytic calcium elevation itself has been suggested to determine the polarity of the arteriolar response (Girouard et al., 2010).

Studying vascular regulation in slices has significant advantages, including the exquisite control over cellular elements. However, an inherent and critical limitation of studies in brain slices is that blood vessels in these preparations lack perfusion and, therefore, are maximally dilated, because myogenic tone induced by intraluminal pressure is missing (Iadecola and Nedergaard, 2007). In most studies, slices were pretreated with vasoconstrictive agents to compensate for the loss in tone (Filosa et al., 2004, 2006; Metea and Newman, 2006; Zonta et al., 2003). Preconstriction of vessels in slices, as well as large changes in the oxygen tension, can result in the conversion of arteriolar constriction into dilation (Gordon et al., 2008; Mulligan and MacVicar, 2004). This conversion has been suggested to underlie competing roles of astrocytes during different states of brain activation, but it is difficult to decide what is more physiological or at least less artificial—preconstriction of vessels by pharmacologically blocking the production of important signaling molecules such as NO (Zonta et al., 2003), leaving vessels untreated and, thus, maximally dilated (Mulligan and MacVicar, 2004) (Figure 3A), or inducing variations of tissue oxygen tension (Gordon et al., 2008) that are larger than those measured in the intact brain during physiological activation (Ances et al., 2001; Offenhauser et al., 2005). Another important point to consider is how slice stimulation protocols relate to typical physiological sensory stimulation (Anderson and Nedergaard, 2003). It is also difficult to speculate whether the very slow time scale at which vessel tone changed in some studies (Gordon et al., 2008; Zonta et al., 2003) (Figure 3A) is an effect of slice temperature, maximally dilated vessels, or lack of perfusion.

In the following paragraphs, we will discuss how astrocytes might mediate functional hyperemia in vivo (also summarized in Figure 4). As outlined below, there are several open questions regarding how astrocytes are activated by glutamate, how quickly and by what pathways they respond, and by what mechanisms they might ultimately regulate functional hyperemia.

How and at What Time Scale Do Astrocytes Respond to Synaptic Activity in Vivo?

Takano et al. (2006) were the first to show that astrocytic calcium elevations induce vasodilation of cortical penetrating arterioles (Figure 5A). However, whether the time course of sensory-evoked astrocytic calcium changes is actually compatible with the onset and temporal sequence of functional hyperemia has remained an open question. Following initial demonstrations of spontaneous calcium dynamics in astrocytes in the intact brain (Hirase et al., 2004; Nimmerjahn et al., 2004), it was shown that sensory stimulation of whiskers (Wang et al., 2006) or direct cortical electrical stimulation (Takano et al., 2006) elicited calcium transients in layer II astrocytes in mouse somatosensory cortex. Astrocytic responses peaked at stimulation frequencies at which local synaptic input was highest (measured by summed local field potential) and were much smaller at weaker synaptic activation (Wang et al., 2006). The latency of onset of these calcium changes was in the order of 1–6 s—i.e., later than the onset of functional hyperemia, which typically occurs at about 1 s after stimulus onset (Tian et al., 2010). In another study in ferret visual cortex, astrocytes responded at a delay of 3–4 s (Figure 5B), and, similar to somatosensory cortex, were sharply tuned to maximal synaptic input (Schummers et al., 2008). In olfactory glomeruli, astrocytic calcium elevations in response to odor stimulation commenced about 1–2 s after stimulus onset (Petzold et al., 2008), although the precise stimulus onset is more difficult to determine here because of variations in the flow of odorants to the nose as well as breathing and sniffing rates of the animals. In the cortex, a subset of astrocytes showed rapid responses more compatible with the onset of functional

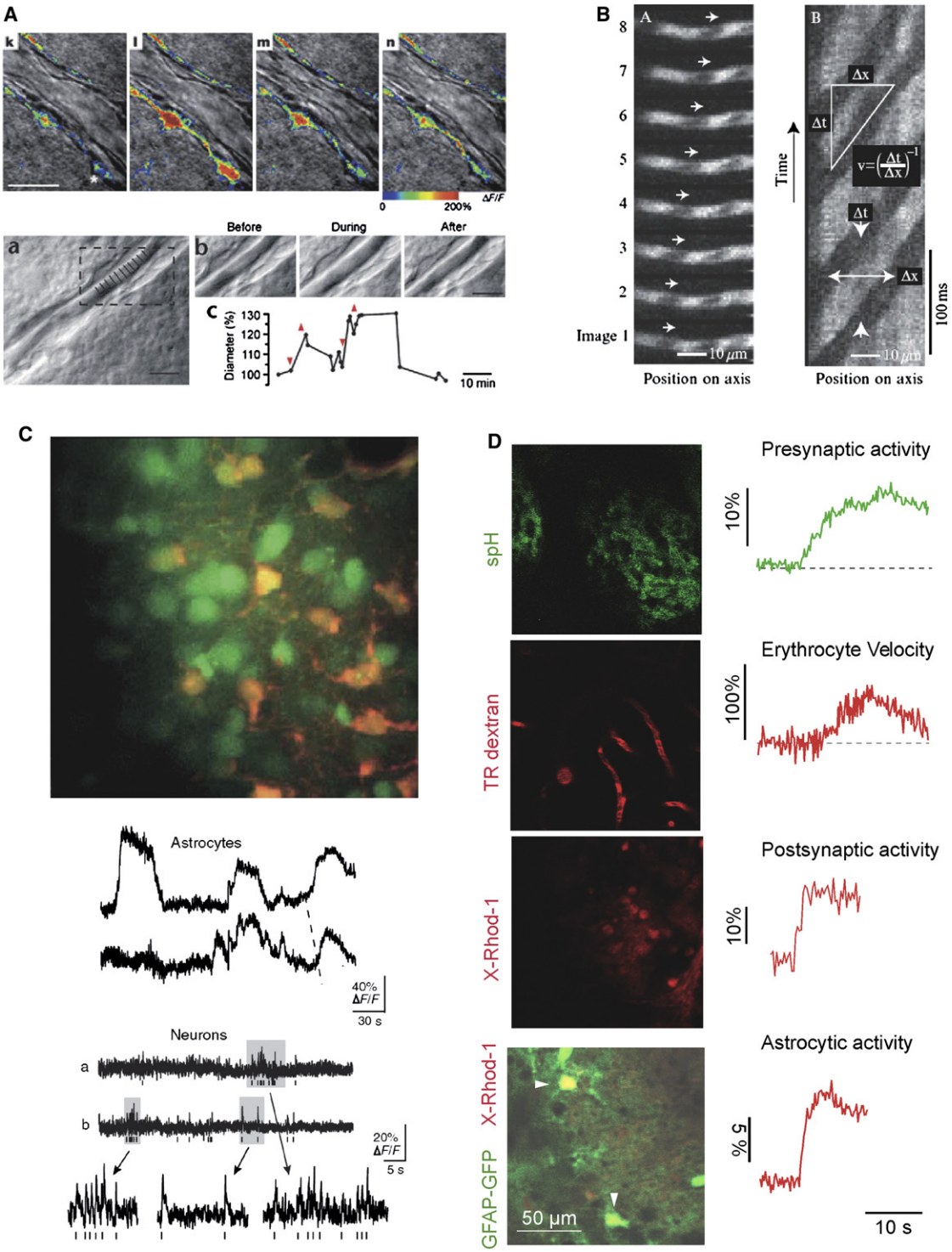

Figure 3. Cellular-Level Measurements of the Elements in Neurovascular Coupling

(A) Vessel diameter can be directly measured with calcium dynamics in ex vivo preparations, such as the acute brain slice preparation. Upper panel: calcium uncaging in astrocytes (labeled with the calcium indicator Rhod-2) is associated with subsequent arteriolar vasoconstriction (scale bar, 20 μm). Lower panel: afferent electrical stimulation induces arteriolar vasodilation (scale bars, 10 μm). Down and up triangles in the graph indicate the duration of stimulation. Note the relatively slow kinetics of the vessel response compared to in vivo conditions.

(B) Blood flow can be measured in vivo by high-speed imaging of red blood cell (RBC) movement in capillaries using multiphoton microscopy. In this example, fluorescent dyes in the plasma allow erythrocytes to be seen as dark shadows. By performing rapid line scans along the capillary axis, passing RBCs appear as dark bands in the fluorescent plasma. CBF parameters can then be calculated from these bands (the time between two bands, Δt, is inversely proportional to

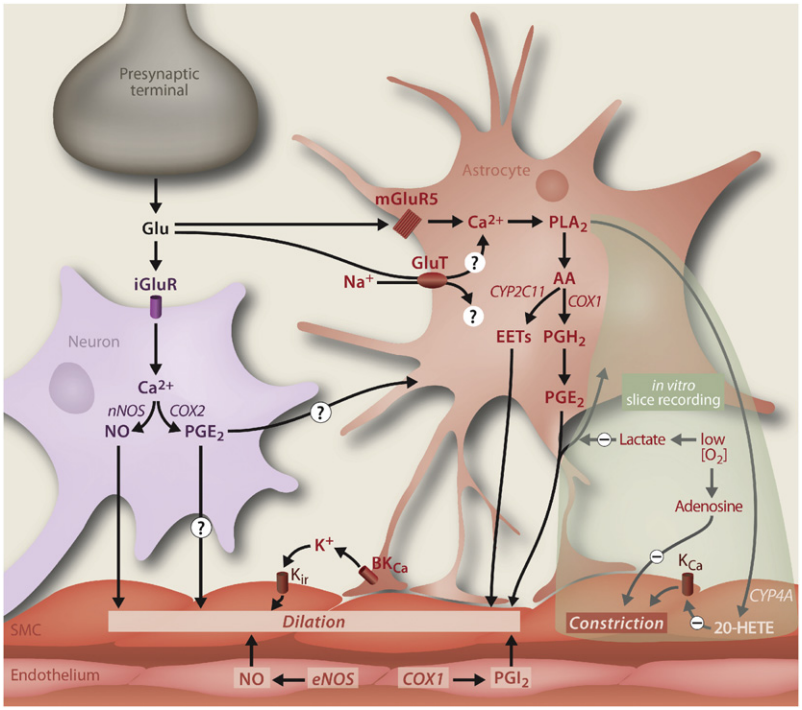

Figure 4. Postulated Signaling Pathways from Synapse to Blood Vessel

Molecular pathways that have been identified in in vivo and in vitro studies are displayed. Pathways that have only been identified in vitro, but await characterization in vivo, have been highlighted to the right. Question marks indicate unknown molecular mediators. In summary, presynaptic terminals release glutamate (Glu), which acts on postsynaptic neurons and astrocytes. In postsynaptic neurons, glutamate activates ionotropic receptors (iGluR), which trigger calcium elevations that activate neuronal NO synthase (nNOS) and cyclooxygenase 2 (COX2), which in turn leads to production of PGE_2. It is not known whether PGE_2 dilates blood vessels by acting on smooth muscle cells or on astrocytes. In astrocytes, glutamate activates metabotropic glutamate receptors-5 (mGluR5), which triggers calcium elevations that—by phospholipase A_2 (PLA_2)-mediated synthesis of arachidonic acid (AA)—lead to production of PGE_2 through COX1 and epoxyeicosatrienoic acids (EETs) through cytochrome P450 2C11 epoxygenase (CYP2C11). Moreover, calcium induces potassium release from astrocytic endfeet through large conductance calcium-dependent potassium channels (BK_{Ca}), which in turn triggers vasodilation through hyperpolarization of smooth muscle cells via inward-rectifier potassium channels (K_{ir}). Glutamate is also taken up into astrocytes through glutamate/Na^+-cotransport, but the pathways that eventually lead to vasodilation remain unknown. In addition to these neurovascular pathways, endothelial cells can also trigger vasodilation via endothelial NO synthase (eNOS) and PGI_l production by COX1. Slice studies have suggested that astrocytic calcium can by unknown mechanisms also lead to vasoconstriction through the production of 20-hydroxyeicosatetraenoic acid (20-HETE) mediated by cytochrome P450 4A (CYP4A) ω-hydroxylase. In slices, low $[O_2]$ may inhibit vasoconstriction through increased production of lactate (which may inhibit astrocytic PGE_2 reuptake) and adenosine.

hyperemia, following brief mechanical limb stimulation (Winship et al., 2007). Another study found astrocytic calcium elevations in somatosensory cortex in awake mice, which appeared 1–2 s after the onset of voluntary running (Dombeck et al., 2007). However, in both studies, but in contrast to other studies (Schummers et al., 2008) (Figure 5B), the onset and kinetics of calcium responses in neurons and neuropil, which were simultaneously labeled with the same calcium indicator, were similar to the astrocytic response (Dombeck et al., 2007; Winship et al., 2007), indicating that they might have been included in the axial depth of the optical plane and may have contributed to the imaging signal. In yet another study in awake behaving mice, the onset of calcium "flares," which were abundant in awake mice but absent in anesthetized animals, in cerebellar Bergmann glia, closely matched the onset of functional hyperemia (Nimmerjahn et al., 2009) (Figure 5C). However, CBF was measured in separate animals by laser-Doppler flowmetry in a much larger tissue volume than the calcium measurements, making it difficult to accurately relate the onset of functional hyperemia with astrocytic calcium. In summary, calcium elevations in different systems and after different stimulation paradigms typically occur in areas of maximal synaptic activity and often start somewhat later than functional hyperemia.

Given that astrocytic responses in vivo are typically detected after the onset of functional hyperemia, how can they be responsible for the vascular changes? One can envision three possible scenarios. First, calcium elevations in astrocytes in all of these studies were monitored using synthetic dyes, loaded into cells using the membrane permeant AM ester form, and by identifying astrocytes using either genetic markers (Zhuo et al., 1997) or sulforhodamine 101 (Nimmerjahn et al., 2004). However, the dye is taken up by all cells, and even when using counterstains (Figures 3C and 5C), signal separation can become difficult (Göbel and Helmchen, 2007; Grewe and Helmchen, 2009). In addition, the time course of calcium responses in neurons and astrocytes is influenced by the properties of the indicator as well as the

RBC flux; the distance between bands, Δx, is inversely proportional to RBC linear density; and the slope of the bands, Δt/Δx, is inversely proportional to RBC velocity).

(C) Calcium signals in neurons and astrocytes can be imaged using multiphoton microscopy and selective labeling of astrocytes using the dye sulforhodamine-101 (SR101). In this example from Nimmerjahn et al. (2004), astrocytes are visualized as yellow regions (overlap of red SR101 and green calcium indicator). Spontaneous calcium responses were measured in both cell types, and had significantly different temporal dynamics. Evoked responses in both cell types can also be imaged under various conditions.

(D) In the olfactory bulb, several different cellular elements can be imaged simultaneously or separately under very similar conditions. Presynaptic activity can be estimated by fluorescence imaging, of nerve terminals expressing synaptopHluorin (spH), postsynaptic and astrocytic activity (identified by GFP expression) using calcium imaging, and blood flow by measuring erythrocyte velocity.

The following images are reproduced with permission: (A) from Mulligan and MacVicar (2004) (upper row) and Zonta et al. (2003) (lower row), (B) from Helmchen and Kleinfeld (2008), (C) from Nimmerjahn et al. (2004), and (D) from Petzold et al. (2008).

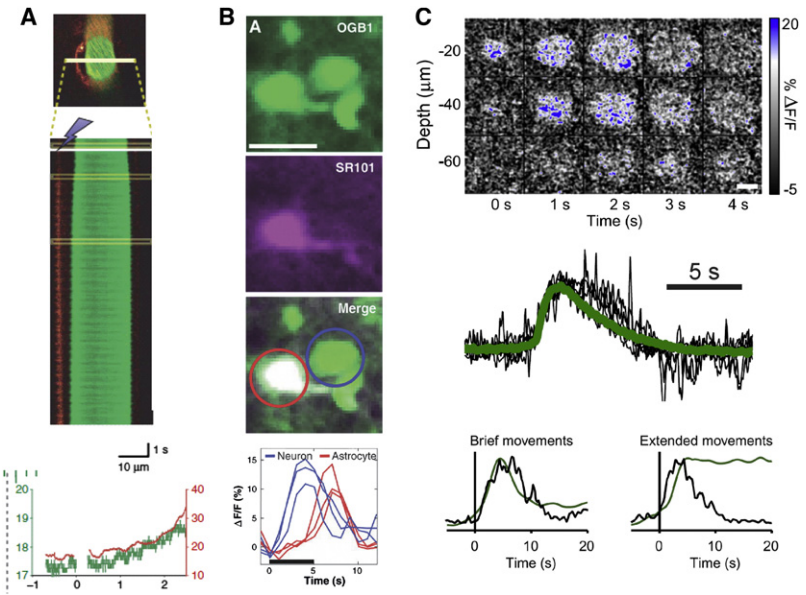

Figure 5. Relation between Astrocyte Activity and Blood Flow

(A) Increasing calcium concentration in astrocytic endfeet surrounding a blood vessel causes vasodilation. In this example, calcium increases were induced by uncaging, and measurements of calcium levels and vessel diameter done using multiphoton microscopy (in the line scan mode). Calcium was measured using Rhod-2, and vessels were visualized using a dextran-coupled dye. Although the overall time course is gradual, the vessel diameter tracks the rise in calcium concentration in the endfoot.

(B) Astrocytic responses following sensory stimulation occur several seconds after the stimulus and have a time course distinct from neuronal responses. Images show astrocytes and neurons in ferret visual cortex labeled with the calcium indicator Oregon Green BAPTA-1 (OGB1, green). The astrocyte was identified by costaining with sulforhodamine 101 (SR101, purple). Lower image: time course of neuronal (blue) and astrocytic (red) calcium responses to visual stimulation (the thick bar represents stimulation time). Scale bar, 25 μm.

(C) Calcium waves can occur in astrocytes vivo. In this example, radially expanding calcium waves, termed "Ca^{2+} bursts," were measured using OGB1 in cerebellar Bergmann glia in awake mice. Fifteen frames are illustrated (five time points and three different depths). The time courses of these calcium signals are shown in the middle panel (black, individual traces; green, mean time course). In the bottom panel, the time course of large-scale concerted glial calcium signals, termed "Ca^{2+} flares," is shown (black) along with average blood flow changes (green), both averaged by triggering on movement onset (vertical line; note, however, that these measurements were taken in separate groups). Two different types of movement were considered: brief movement and extended movements. The onset of blood flow changes tracks glial calcium well for both cases. However, for extended movements, blood flow stays high even though glial calcium levels return to baseline.

The following images are reproduced with permission: (A) from Takano et al. (2006), (B) from Schummers et al. (2008), and (C) from Nimmerjahn et al. (2009).

endogenous calcium buffer capacity (Helmchen et al., 1996; Neher and Augustine, 1992), although onset kinetics are probably not affected significantly.

A second scenario is that calcium changes in astrocytes do occur earlier than functional hyperemia but that they are not picked up by the indicator. This may be either because the affinity of typical bulk-loaded indicators is too low to detect very subtle calcium changes or because the indicators tend to accumulate in somata and larger processes, leaving out the extensive network of smaller astrocytic processes and their even finer ramifications. There is clear evidence for differences in calcium signals recorded in astrocyte somata and fine processes (Reeves et al., 2011). Perhaps progress can be made if astrocytes can be selectively labeled with calcium indicators, especially with genetically encoded indicators such as GCaMPs (Nakai et al., 2001; Shigetomi et al., 2010; Tian et al., 2009), troponin-based probes (Mank et al., 2006, 2008), and chameleons (Atkin et al., 2009; Miyawaki et al., 1997; Truong et al., 2007).

A third scenario is that calcium changes in astrocytes indeed appear later than functional hyperemia. For example, it is possible that nonastrocytic mechanisms—e.g., neuronal NO or dedicated interneurons—trigger the initial rise of functional hyperemia, but that astrocytic pathways are necessary to maintain the response. Moreover, signaling steps between astrocytic activation and calcium increase, such as diacylglycerol production, may also be vasoactive. It is also feasible that calcium represents just one of many different vasoactive astrocytic messengers, such as sodium (Bernardinelli et al., 2004), protons (Amato et al., 1994; Chesler and Kraig, 1987), cAMP (Moldrich et al., 2002), ATP (Cotrina et al., 2000; Pascual et al., 2005), or lactate (Gordon et al., 2008). Future experiments may benefit from monitoring changes in these parameters within astrocytes in vivo.

In addition to monitoring calcium rises, it is also important to be able to perturb calcium levels within astrocytes at will. One method that has been used successfully is optical uncaging—caged calcium loaded into astrocytes can be released by ultraviolet light, a method that has been used extensively in vitro (Filosa et al., 2006; Gordon et al., 2008; Metea and Newman, 2006; Zonta et al., 2003) (Figure 3A). In vivo uncaging was shown to lead to vessel dilation (Takano et al., 2006) (Figure 5A), but additional experiments with more controlled and spatially confined changes in calcium may offer more refined information.

How Are Astrocytes Activated by Neuronal Activity?

Neuron-derived substances, such as glutamate, GABA, noradrenaline, acetylcholine, dopamine, ATP, and nitric oxide, trigger propagating astrocytic calcium elevations in culture (Haydon and Carmignoto, 2006; Volterra and Meldolesi, 2005). So far, only glutamate has been shown to be relevant for astrocytic activation following sensory stimulation in vivo. In cortex, local inhibition of the metabotropic glutamate receptors mGluR1 and mGluR5, respectively, decreased astrocytic calcium elevations evoked by whisker stimulation by about 40%–50% each (Wang et al., 2006). Similarly, in olfactory glomeruli, inhibition of mGluR5 decreased the rise of astrocytic calcium (as well as functional hyperemia) following odor stimulation (Petzold et al., 2008). Neither presynaptic (Petzold et al., 2008) nor postsynaptic neuronal activity (Wang et al., 2006) was altered by the pharmacological treatment, indicating that the effect was mainly attributable to astrocytic but not neuronal mGluRs. As an additional

pathway, inhibition of glutamate uptake into astrocytes driven by the glutamate/sodium cotransporter decreased functional hyperemia in the mouse olfactory bulb (Petzold et al., 2008) and in the ferret visual cortex (Schummers et al., 2008).

As in any complex signaling system, many uncertainties remain. For example, some studies have suggested that mGluR5 might be predominantly expressed in neurons in the mature brain (Barres, 2008; D'Antoni et al., 2008). Moreover, pharmacological blockade of glutamate uptake increases the concentration and half-life of glutamate at the synaptic space, although the effect of astrocytic glutamate transport inhibition on functional hyperemia remained in the olfactory bulb when all neuronal targets of glutamate as well as astrocytic mGluRs were blocked (Petzold et al., 2008). It is currently unclear to what extent glutamate uptake-mediated functional hyperemia depends on a rise in astrocytic calcium. Astrocytic calcium responses remained unaltered after relatively specific blockade of astrocytic transporters in the olfactory bulb (Petzold et al., 2008). In contrast, injections of high concentrations of the unspecific neuronal and astrocytic uptake inhibitor TBOA decreased astrocytic calcium elevations in ferret visual cortex (Schummers et al., 2008). The mechanisms responsible for this decrease remain to be determined, especially since neuronal calcium responses increased simultaneously because TBOA elevated synaptic glutamate and this should theoretically increase astrocytic calcium. Additional nonpharmacological studies will be necessary to clearly delineate the role of astrocytic glutamate uptake and mGluRs for functional hyperemia. This could be achieved by using genetically engineered mice in which metabotropic receptor pathways are knocked in or out specifically in astrocytes (Fiacco et al., 2007; Petravicz et al., 2008). Better temporal and spatial resolution may be achieved by the use of optically activated G protein-coupled receptors, referred to as OptoXRs (Airan et al., 2009). These chimeric receptors have opsin domains that can be activated by light, and intracellular domains—e.g., the signaling domain of mGluR5—that allow them to signal like native receptors. In addition, specific manipulation of neurons using optogenetic probes such as channelrhodopsins (Boyden et al., 2005; Miesenböck, 2009; Nagel et al., 2003) could reveal the role of pre- and postsynaptic activation (see below), and the contribution of specific interneurons. Astrocytes could also be activated directly, bypassing neurons, using channelrhodopsins (Gourine et al., 2010; Gradinaru et al., 2009). It remains to be established, however, that activation of channelrhodopsin-2 (ChR2) in astrocytes can cause significant depolarization (because of the low electrical impedance) and that these depolarizations have a signaling role. Finally, to test the roles of glutamate transporters, gene-targeted mice lacking specific transporters in astrocytes can be used (Colin et al., 2009).

What Is the Role of Postsynaptic Activity for Astrocyte-Mediated Functional Hyperemia?

All astrocytic pathways identified so far require the direct action of glutamate on astrocytes (Petzold et al., 2008; Schummers et al., 2008; Takano et al., 2006; Wang et al., 2006). In contrast, when the activity of postsynaptic neuronal NMDA and AMPA receptors was blocked locally, no changes were seen in functional hyperemia (Chaigneau et al., 2007; Petzold et al., 2008) or intrinsic optical signals (Gurden et al., 2006) in the olfactory bulb. Moreover, no effect on astrocytic calcium transients evoked by sensory stimulation was observed in somatosensory cortex in vivo after blockade of postsynaptic NMDA and AMPA receptors (Wang et al., 2006).

These results indicate that astrocytes mainly detect presynaptically released glutamate, and that local postsynaptic neuronal activity plays only a minor role in the vasoactive actions of astrocytes. Accordingly, presynaptic activity, when measured simultaneously with CBF using a fluorescent marker for glutamate release, correlates strongly with functional hyperemia in olfactory glomeruli (Petzold et al., 2008) (Figure 3D). In contrast, earlier studies have shown that postsynaptic neuronal activity triggered by ionotropic glutamate receptor activation represents an important pathway in functional hyperemia in the neocortex and cerebellum (Gsell et al., 2006; Lauritzen, 2005; Yang and Iadecola, 1996). In addition, recent studies may indicate that the neuronal stimulus strength might influence which mechanism—presynaptic/astrocytic activity or postsynaptic/neuronal activation—prevails in the control of functional hyperemia. While sparse activation of single glomeruli in the olfactory bulb using low odorant concentrations revealed a contribution of presynaptic glutamate release and astrocytic activity to the local glomerular CBF response (Petzold et al., 2008), another study reported that clustered activation of many glomeruli, i.e. a stronger and more widespread stimulus, triggered CBF responses that were attenuated by global, but not local, postsynaptic blockade (Chaigneau et al., 2007).

It is possible that the contribution of presynaptic activity may have been underestimated in studies focusing on postsynaptic activity because of the lack of direct markers of presynaptic release in these systems, and because classical electrophysiological indicators such as the local field potential mainly report postsynaptic activity (Aroniadou-Anderjaska et al., 1997). Moreover, topical application of postsynaptic blockers will not only decrease the activity of principal neurons, but also presynaptic glutamate release from local excitatory neurons, which are normally recruited by recurrent activity. Notably, thalamocortical synapses contribute to only a small fraction of the total number of excitatory synapses in many sensory cortical areas (Douglas and Martin, 2007; Peters and Payne, 1993; White, 1989). Therefore, an experimental perturbation of postsynaptic activity will probably also alter presynaptic release, which is usually very difficult to measure concomitantly.

Overall, the results available today indicate that postsynaptic neuronal activity may predominate in the control of CBF when stimulation intensity is high or if widespread activation or co-activation of distant areas occur, while presynaptic/astrocytic activity may predominantly regulate CBF during mild or local sensory stimulation. Such a shift may be optimal for matching the CBF response to metabolic needs—for example, a quantitative analysis of glomerular metabolic demands in the olfactory glomerulus (Nawroth et al., 2007) showed that postsynaptic receptor activation contributes to less than 0.3% of the total energy budget during low activation but increases exponentially to one-third with stronger activation patterns comparable to those used by Chaigneau et al. (2007). In future studies, these

computational predictions could be tested experimentally by harnessing optogenetics to express light-activated proteins in neurons, allowing the experimenter to excite neurons more specifically than feasible with physiological stimuli. Such exogenous activation of neurons with spatiotemporal precision could yield answers to questions such as: (1) how much activity is necessary to cause hemodynamic changes, (2) how local (nonlocal) is the hemodynamic change when neuronal activity is focused to a small volume, (3) is postsynaptic activity dispensable for neurovascular coupling—this can be addressed by expressing optical inhibitors (Han and Boyden, 2007; Zhang et al., 2007) in postsynaptic neurons. In structures such as the olfactory bulb with clear morphological boundaries (such as the glomeruli), optical stimulation of individual elements using light can be used to great advantage (Dhawale et al., 2010). Recent studies have demonstrated the feasibility of using optogenetic activation to study the origins of vasoactive signals (Desai et al., 2011; Lee et al., 2010).

What Intraastrocytic Pathways Are Involved?

Although many questions regarding the activation of astrocytes by glutamate remain unanswered, the picture is clearer for the effects downstream of mGluR5 activation. Photolysis of "caged" calcium in perivascular astrocytic endfeet triggered vasodilation of cortical penetrating arterioles in anesthetized mice (Takano et al., 2006) (Figure 5A). This dilation was strongly reduced by inhibition of cyclooxygenase-1 (COX-1), which is expressed in perivascular astrocytes (Takano et al., 2006) and microglia (Capone et al., 2010), resulting in the synthesis of vasoactive prostaglandins (Koehler et al., 2009), but not by inhibition of COX-2 (Takano et al., 2006), which is expressed in neurons (Wang et al., 2005). Similarly, in olfactory glomeruli, COX-1 is expressed by glomerular astrocytes, and its inhibition reduced functional hyperemia, probably downstream of mGluR5 activation (Petzold et al., 2008). A strong reduction in functional hyperemia was recently found in human subjects carrying a COX-1 genotype that results in lower enzymatic function (Hahn et al., 2011). In contrast, a role for COX-2 in neurovascular coupling was supported by studies in somatosensory cortex (Niwa et al., 2000a; Stefanovic et al., 2006), and functional hyperemia was not attenuated in COX-1 null mice (Niwa et al., 2001a). It is currently unclear whether glial COX-1 and neuronal COX-2 may be activated at different kinetics, in different regions, or following different stimulus paradigms. Moreover, the selectivity of some COX inhibitors is lower in vivo than in vitro (Brenneis et al., 2006), and some COX inhibitors have additional pharmacological effects (Niwa et al., 2001a). To make matters even more complicated, COX-2 and also COX-3 might also be expressed in astrocytes under some conditions (Hirst et al., 1999; Kis et al., 2003), and the expression profile of prostaglandin receptors remains to be characterized in full detail (Andreasson, 2010). Finally, the effect of COX inhibition is regionally heterogeneous (Dahlgren et al., 1984; Niwa et al., 2001a), and the effect of mGluR5 inhibition differs profoundly between different brain regions (Sloan et al., 2010), indicating that some pathways might prevail over others depending on the location, and that lessons learned in one region may not be applicable elsewhere. Interestingly, regional diversity has also been observed for the role of nitric oxide in cortex (Lindauer et al., 1999) versus cerebellum (Akgören et al., 1996; Yang et al., 2000).

The vasoactive pathways downstream of glutamate uptake into astrocytes are largely unknown, but there are several intriguing possibilities. First, the cotransport of sodium and glutamate slightly depolarizes the astrocyte, which could lead to potassium efflux at astrocytic end-feet and subsequent vasodilation (Filosa et al., 2006; Howarth and Attwell, 2006; Paulson and Newman, 1987). However, the current generated by glutamate transport is small compared to that generated by astrocytic K^+ uptake at the synaptic cleft in olfactory glomeruli (De Saint Jan and Westbrook, 2005), and it seems unlikely that the blockade of a comparatively small current would reduce functional hyperemia as much as observed. Second, CBF may increase as a result of metabolic activation induced by glutamate uptake. Sodium/glutamate cotransport consumes energy for the restoration of the ionic gradient by the Na^+-/K^+-ATPase, and for the conversion of glutamate to glutamine. While the contribution of these processes to the brain's energy budget is small (Attwell and Laughlin, 2001), glutamate uptake into astrocytes also directly initiates astrocytic nonoxidative glycolysis and lactate release (Pellerin, 2005). Lactate itself may initiate vasodilation (Gordon et al., 2008), but it is also possible that sodium ions cotransported into astrocytes with glutamate may trigger a vasoactive pathway. Sodium ions shuttled into astrocytes by this cotransport propagate as interastrocytic sodium waves in cell cultures (Bernardinelli et al., 2004), and they have also been shown to couple synaptic activity and astrocytic nonoxidative glucose consumption (Voutsinos-Porche et al., 2003). This stimulation of nonoxidative glycolysis in astrocytes is thought to underlie the disproportionate rise of CBF and glucose compared to a smaller increase in oxygen consumption—the mismatch that forms the basis of functional brain imaging (Magistretti and Pellerin, 1999). Therefore, glutamate transport into astrocytes may simultaneously activate functional hyperemia and nonoxidative glycolysis in astrocytes, and may contribute to the high temporal and spatial correlation of CBF increase and glucose consumption observed in functional brain imaging (Raichle and Mintun, 2006).

Advances in imaging and cellular manipulation may be harnessed to overcome the uncertainties regarding the role of astrocytic molecular agents in functional hyperemia. Optical imaging during physiological activity can, in principle, be extended to any small molecule for which there is an appropriate fluorescent indicator (Zhang et al., 2002). Genetic manipulations may also be valuable, particularly if the perturbations can be performed in a cell-type-specific and temporally precise manner (Kennedy et al., 2010). Methods to stimulate or downregulate the expression of genes, such as those for glutamate transporters, specifically in mature astrocytes are increasingly becoming available (Colin et al., 2009). Pharmacological methods with increased specificity may also be useful, as will methods that marry chemical approaches with genetic methods ("chemical genetics") (Knight and Shokat, 2005). For example, mutations in transporters that confer susceptibility to blockade by exogenous small molecules that have no effects on native proteins could allow acute and reversible inhibition of transporters in astrocytes.

What Is the Spatial Range of Astrocytic Input and Output in Relation to Functional Hyperemia?

It is worth considering some of the physical and chemical constraints for functional hyperemia. First and foremost, since neurovascular coupling is spatially confined, the molecular signals need to be generated and communicated locally. Therefore, it is important to determine the range of integration and range of influence of astrocytes, especially since astrocytes are extensively coupled through gap junctions (Haydon, 2001). Second, even if the vasoactive signals are generated locally, they may spread far if they diffuse rapidly and have a long lifetime—these parameters need to be measured for molecules such as NO and ions such as potassium. Third, affecting blood vessels in one place may affect the perfusion nonlocally because of vascular connectivity and passive redistribution of blood (Boas et al., 2008). These considerations have been recognized for some time but are not always attended to in molecular and cellular studies. Finally, the same messenger might have different or even opposing effects on blood flow (Attwell et al., 2010).

Another open issue is related to the spatial "reach" of astrocytes. Cortical astrocytes are organized into nonoverlapping functional domains (Halassa et al., 2007) (Figure 2C). On the input side, a single cortical astrocyte can, in principle, listen to tens of thousands of synapses by virtue of its extensive processes (Haydon, 2001), but it is unclear how many synapses are needed to activate an astrocyte. Recent in vivo experiments in visual cortex indicate that astrocytes respond to visual stimulation with calcium rises with exquisite selectivity, suggesting that their "input" field may be highly selective (Schummers et al., 2008). Selective astrocytic responses were also found in slice experiments in barrel cortex (Schipke et al., 2008). On the other hand, what is the spatial extent of a single astrocyte's output? In theory, the organization of astrocytes into separate domains may contribute to the spatial distribution of the CBF response (Iadecola and Nedergaard, 2007). However, the input and output selectivity may not be limited by the spatial extent of a single astrocyte's processes, since extensive gap junction coupling of astrocytes may extend the range substantially by allowing intercellular transfer of signaling molecules (Haydon, 2001; Scemes and Giaume, 2006). The degree of astrocyte coupling may also be regulated to make network topology modifiable.

The extent of astrocyte coupling in vivo is unclear. One signaling event that has been observed to propagate across astrocytes is a rise in calcium concentration. Calcium waves spreading across multiple astrocytes were imaged more than two decades ago in vitro (Cornell-Bell et al., 1990), but their occurrence in vivo in the mature brain under physiological conditions has remained in question (Wang and Bordey, 2008). Recently, spontaneous and movement-evoked nonsynchronous events (Nimmerjahn et al., 2009) and large coherent transglial calcium waves (Hoogland et al., 2009; Nimmerjahn et al., 2009) were observed in the cerebellum, as well as in cortex (Dombeck et al., 2007). Whether these are all truly glial signals and whether similar transglial calcium waves occur in other brain regions awaits experimental testing. The existing data, however, suggest that glial coupling is not an all-or-none phenomenon, but that it is highly regulated. Of particular interest, Nimmerjahn et al. (2009) found that hemodynamic changes elicited in the cerebellum by motor activity were accompanied by calcium rises in a large number of Bergmann glia (Figure 5C), although the two signals were measured in separate groups (see above). Whether such large-scale coordinated signaling is required for local hemodynamic changes is not clear, but is an important question for future work. Interestingly, it was recently reported that neuronal activity differentially modulated the level of coupling of astrocyte networks in the olfactory bulb (Roux and Giaume, 2009), indicating that the strength and range of astrocytic communication might depend on the ongoing local neuronal activity.

What Is the Influence of the Behavioral State on Astrocytes and Functional Hyperemia?

An important consideration for future experiments is the behavioral state of the animal. For technical reasons, much of the work to date has been conducted in anesthetized animals, where movement is minimized and stimuli can be controlled well. However, anesthetics by their very nature interfere with neuronal signaling, and it is well known that they also affect functional hyperemia (Lindauer et al., 1993, Nakao et al., 2001), although the extent of which remains to be defined (Franceschini et al., 2010). Therefore, the question arises whether astrocytic signaling is also altered by the anesthetic state. A pioneering study by Dombeck et al. (2007) examining calcium responses in the somatosensory cortex of awake, mobile mice noted that astrocyte responses can be coordinated or independent of each other, suggesting specific and variable coupling in astrocyte networks. Recently, Nimmerjahn et al. (2009) found that calcium signals in Bergmann glia in the cerebellar molecular layer had different characteristics in different behavioral states as well as different sensitivity to anesthetics. In the visual cortex, stimulus-evoked astrocytic calcium signals and intrinsic optical signals, which reflect hemodynamics, were reduced by increasing concentration of the volatile anesthetic isoflurane (Schummers et al., 2008). An important direction for future research is the examination of the cellular basis of neurovascular coupling in different waking states—constrained, behaving, and startled—with a particular emphasis on simultaneous imaging of neuronal, astrocytic, and vascular network activity.

Pathophysiological Implications

Neurovascular coupling has been implicated in the pathogenesis of several important neurological diseases. Functional hyperemia is attenuated after experimental and clinical focal ischemia, (Girouard and Iadecola, 2006). It is currently unclear whether this reduction represents a decoupling of functional hyperemia by impaired cerebrovascular reactivity (Kim et al., 2005; Rossini et al., 2004), or whether neurovascular coupling is preserved, but has a reduced amplitude because the underlying neuronal activity is attenuated (Bundo et al., 2002; Weber et al., 2008; Zhang and Murphy, 2007). Moreover, ischemia also reduces the ability of endothelial cells to initiate vasodilation (Kunz et al., 2007). Functional hyperemia is also reduced following global cerebral hypoxia (Schmitz et al., 1998), and in arterial hypertension (Girouard and Iadecola, 2006). In addition, pericyte-mediated contraction of capillaries may also contribute to the perturbation of blood flow after cerebral ischemia (Yemisci et al., 2009).

During migraine aura, as well as after stroke, traumatic brain injury, and subarachnoid hemorrhage, spreading waves of neuronal depolarization occur (Lauritzen et al., 2011). In the healthy brain and during migraine aura, these events are associated with a transient increase in local CBF (Hadjikhani et al., 2001; Lauritzen, 1987), and do not induce overt neuronal injury (Nedergaard and Hansen, 1988). However, during ischemia, as well as after brain injury or hemorrhage, the coupling between these neuronal depolarization waves and CBF is inverted, such that the increased neuronal activity is accompanied by a drop of CBF to ischemic levels, indicating that this inverted neurovascular coupling may contribute to tissue damage (Dohmen et al., 2008; Dreier et al., 2009; Petzold et al., 2003; Shin et al., 2006).

Functional hyperemia is also perturbed in Alzheimer's disease (Iadecola, 2004). In patients, resting CBF is reduced early in the disease (Johnson and Albert, 2000), and functional hyperemia is significantly impaired in animal models and patients (Hock et al., 1996; Nicolakakis et al., 2008; Niwa et al., 2000b; Park et al., 2004; Park et al., 2008; Shin et al., 2007; Smith et al., 1999; Tong et al., 2005). Amyloid-β, the main constituent of amyloid plaques in the brains of patients with Alzheimer's disease, is vasoactive in vitro (Crawford et al., 1998) and in vivo (Niwa et al., 2000b), and soluble amyloid-β contributes to the reduction of functional hyperemia in animal models in vivo (Niwa et al., 2001b; Park et al., 2004), although it has also been suggested that insoluble amyloid plaques and amyloid angiopathy are necessary for this effect (Christie et al., 2001; Hu et al., 2008; Shin et al., 2007). This perturbation of neurovascular coupling, together with nonvascular mechanisms triggering neurodegeneration, may have synergistic detrimental effects on cognition and memory in this disease (Iadecola, 2004).

Interestingly, dysregulation of astrocytic signaling has been reported for all of these aforementioned diseases. Astrocytes swell under ischemia, and because of their proximity to arterioles and capillaries, this edema may contribute to the CBF reduction in the microcirculation after stroke (Frydenlund et al., 2006; Manley et al., 2000). On the other hand, astrocytes are also neuroprotective after ischemia and in other conditions (Barres, 2008; Nedergaard and Dirnagl, 2005). Astrocytes also participate in spreading neocortical depolarizations (Chuquet et al., 2007), but to what extent they also contribute to the CBF response and its inversion under pathological conditions remains unknown. Finally, astrocytic calcium homeostasis is disrupted in animal models of Alzheimer's disease (Kuchibhotla et al., 2009), but it remains to be determined whether these changes also contribute to cerebrovascular dysregulation.

Concluding Remarks

There is substantial evidence now for the role of astrocytes in neurovascular coupling. However, to establish with certainty the exact aspects of functional hyperemia that astrocytes are involved in, the following criteria must be satisfied: (1) astrocytes must be activated in some way by neuronal signals that cause functional hyperemia, (2) removing astrocytic signaling specifically in time and spatial location must perturb or abolish increased blood flow caused by increased neural activity, and (3) specifically activating astrocytic signals in the absence of neuronal activity should lead to functional hyperemia. Of these, the first requirement has significant experimental support, but the last two have not been fully addressed. Significant progress can be anticipated in the coming years in this field. We are particularly optimistic about the use of detailed cellular imaging and cell biological manipulations in vivo. Application of cutting-edge optical imaging methods, including multiphoton microscopy, has allowed a detailed dissection of different cellular components—blood vessels, astrocytes, pericytes, endothelium, and neurons. Since individual cells are elementary units of tissue organization, probing their properties at high resolution allows one to discern individual events that may be smoothed out or buried within population signals. This sort of cell biology in the intact brain will also be aided by unequivocal identification of specific cell types using genetic methods. These novel approaches might be helpful for the interpretation of brain imaging studies and to pinpoint the mechanisms involved in the dysregulation of functional hyperemia in neurological diseases.

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