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Astrocytic Calcium Signaling: Mechanism and Implications for Functional Brain Imaging

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Abstract

Astrocytes are electrically non-excitabile cells that, on a slow time scale of seconds, integrate synaptic transmission by dynamic increases in cytosolic Ca^{2+} . A number of groups have recently shown that astrocytic Ca^{2+} signaling regulates vascular tones and that astrocytes play a central role in functional hyperemia by Ca^{2+} -dependent release of Prostaglandin E2 (PGE_2). Astrocytes are, however, not simple detectors of excitatory transmission, since a number of neuromodulator and hormones trigger elevations in astrocytic Ca^{2+} independently of synaptic transmission. Furthermore, astrocytes exhibit ex vivo intrinsic Ca^{2+} excitability, or spontaneous increases in Ca^{2+} that are not triggered by receptor activation. The notion that astrocytes can regulate vascular tone independently of synaptic transmission challenges the notion that changes in the blood oxygenation level dependent (BOLD) signal is directly proportional to neuronal activity and may thus require a reevaluation of the large body of data accumulated using functional magnetic resonance imaging (fMRI).

Keywords

Photolysis; 2-photon imaging; functional brain imaging

Until about two decades ago, astrocytes were regarded as non-excitabile supporting cells in the brain, lacking membrane properties required to generate action potentials. The view of astrocytes as simple supporting cells was questioned in the early 1990s, with the demonstration that glutamate can trigger Ca^{2+} oscillations among cultured glial cells often resulting in propagating intercellular Ca^{2+} waves (1). The notion that astrocytes can actively propagate Ca^{2+} waves among each other led to the studies of Ca^{2+} signaling between astrocytes and neurons in mixed cultures. These studies revealed that astrocytic Ca^{2+} waves modulate neuronal cytosolic Ca^{2+} , large spike-like increases in neuronal Ca^{2+} levels, suggesting that astrocytes participate more directly in neurotransmission than previously recognized (2, 3). Bidirectional communication between neurons and astrocytes was later demonstrated in acute brain slices (4–7) and the isolated retina preparation (8). Although bidirectional communication has been studied for more than a decade, it was first recently demonstrated that astrocytes are activated during sensory stimulation in vivo (9). This was an important step, because activation of astrocytes in previous ex vivo experiments often required excessive stimulation, including high intensity electrical stimulation (10), mechanical distortion (11), traumatic injury (12), agonist application (13) and laser light (14). Understanding the role of astrocytic Ca^{2+} signaling in the intact brain has direct implication for functional brain imaging, since new lines of evidence suggest that astrocytes play a key role in functional hyperemia (reviewed in (15)). The basis of blood oxygen level dependent (BOLD) functional magnetic resonance imaging (fMRI), and positron emission tomography (PET) functional imaging, as well as intrinsic signal optical imaging, is that neural activity is coupled to increases in local blood flow (16). However, the mechanisms linking neuronal activity to increases in local perfusion remain poorly understood. Since the first description by Golgi at the end of 1800s that astrocytic endfeet contact arterioles and

capillaries in the brain, it has been speculated that astrocytes have the potential to modulate the cerebral blood flow. Indeed, several in vitro studies highlighted the importance of neuron-to-glia signaling in vessel diameter (10, 17–20). Although different mechanisms have been proposed for the function of astrocytes in control of microcirculation, a common conclusion is that elevations in astrocytic cytosolic Ca^{2+} levels are necessary and sufficient to cause changes in the diameter of arterioles. Yet, an essential question to be addressed is whether these astrocytic Ca^{2+} transients occur in vivo during physiological neural activation.

We have, in recent work, addressed the signaling pathway from neurons to astrocytes in live adult mice using 2-photon laser scanning microscopy. We analyzed changes in neuronal local field potentials (LFPs) and astrocytic Ca^{2+} signaling evoked by whisker stimulation in barrel cortex layer 2 of adult mice. We found that whisker stimulation promptly evoked astrocytic Ca^{2+} responses in vivo. The slow onset (~3 s) and long time course (~20 s) of the astrocytic Ca^{2+} signaling compared with the properties of LFPs suggested that temporal summation of electrical events with a long time constant determined the development of the astrocytic Ca^{2+} elevation. We found that the changes of astrocytic Ca^{2+} signaling were a direct function of the summated LFPs amplitude, which was commonly used to predict the amplitude of increases in cerebral blood flow changes during sensory stimulation (21, 22). Our recent observation on astrocytic Ca^{2+} signaling will here be discussed in the context of functional brain imaging.

2. Astrocytes Can Sense and Modulate Synaptic Activity

2.1. Astrocytes Increase Ca^{2+} in Response to Receptors Activation

Astrocytes make contact with most synapses in the CNS and respond to synaptic activity with increases in cytosolic Ca^{2+} ex vivo (4, 23). Although astrocytes are electrically non-excitable, they express receptors for most neurotransmitters (24), including glutamate (1), GABA (4), norepinephrine (25) and acetylcholine (26). Retinal Müller cells and astrocytes also respond to synaptic release of ATP by activation of purinergic receptors (14). The types of neurotransmitter receptors found on astrocytes in situ and in vivo are mainly metabotropic receptors, which link to the second messenger system including, activation of phospholipase C, adenylyl cyclase and production of IP₃, Ca^{2+} and cAMP (27,28) (**Fig. 5.1**).

Several lines of evidence indicate that metabotropic glutamate receptors (mGluR) play a key role in the mobilization of intracellular Ca^{2+} stores in response to synaptic release of glutamate (5,6). In culture, astrocytes respond to glutamate with oscillatory increases in Ca^{2+} and intercellular Ca^{2+} waves propagation (1). Glutamate released during neuronal activity may reach astrocytic receptors through synaptic “spillover” (29, 30) or ectopic release (31). In addition, neurotransmitters may directly activate astrocytes through volume transmission (32).

2.2. Modulatory Role of Gliotransmitters Released by Astrocytes

Although we know that astrocytes sense the neurotransmitters in extracellular space, we know very little about the consequences of astrocytic receptors activation. In vitro preparations have shown that astrocytic Ca^{2+} signaling is associated with release of gliotransmitters. Several of the best studied gliotransmitters include glutamate, ATP, D-serine, TNF- α , and arachidonic acid metabolites (3,10,33–35) (**Fig. 5.1**). Thus, by sensing the extracellular neurotransmitter concentration and increasing intracellular Ca^{2+} , astrocytes may actively participate in the information processing in the normal brain.

3. Interface Between Astrocytic Endfeet and the Vasculatures

Astrocytes send processes to contact not only synapses but also the vasculature forming specialized endfoot structures in the astrocyte-vasculature interface (AVI). A variety of channels and receptors required for interaction between astrocytes and vasculatures are clustered in the astrocytic endfoot processes. For instance, purinergic receptors P2Y₂ and P2Y₄ as well as gap junctions composed of Cx43 have been shown to be preferentially expressed in the astrocytic endfeet (36). Accordingly, direct application of ATP, electrical stimulation and uncaging evoked astrocytic Ca²⁺ waves can propagate along the AVI (17,36). Increases of astrocytic Ca²⁺ in the endfeet can activate Ca²⁺ – sensitive K⁺ channels, which is also abundantly expressed in the endfeet (37), and subsequently dilate intracerebral arterioles by increasing extracellular K⁺ concentration in the AVI (19,38) (Fig. 5.1).

4. In Vivo Imaging of Astrocytic Ca²⁺ Signaling by 2-Photon Laser Scanning Microscopy (2PLSM)

Due to the technical challenges, imaging astrocytic Ca²⁺ signaling in vivo was only possible after the introduction of 2PLSM (39) and the in vivo Ca²⁺ indicator loading techniques (40,41).

The invention of 2PLSM by Webb's group in 1990 (39) has tremendously improved our understanding of neurobiological phenomena in both normal and diseased brain. With two-photon excitation (2PE), it is possible to image deep brain tissue in the live animals with single cell spatial resolution (42). We have used 2PLSM because 2PE provides several key advantages compared with single photon excitation (1PE). First, because of the localized excitation in 2PE, all emission fluorescent photons contribute to the useful signals. In contrast, a pinhole has to be used to block the emission light from the unfocused plan with 1PE. Therefore, 2PE has much higher efficiency than 1PE. Second, since only the fluorophores in the focused point are activated by 2PE, whereas the entire light pathway is activated during 1PE, phototoxicity and photobleaching are greatly reduced in 2PE. This is especially important for time-lapse imaging of live tissue. Moreover, the excitation wavelength used in 2PE (740~900 nm) penetrates tissue better than 1PE (350~550 nm), which is essential for in vivo imaging of live animals.

Although blind intracellular recordings (43) have been widely used for loading Ca²⁺ indicators into neuronal structures under 2PLSM, delivering Ca²⁺ indicators into single astrocyte through recording pipette in live animals is still challenging. However, recent studies demonstrated that astrocyte population can be labeled by either local dye injection (40) or pial surface dye application (41). Together with the discovery of specific astrocyte fluorescent indicator sulforhodamine 101 (44), these pioneer studies enable, for the first time, the observation of astrocytic Ca²⁺ activity in response to physiological stimulation in the live animals (9) (Fig. 5.2).

We found that astrocytic Ca²⁺ signals can be clearly detected from pial surface to ~300 μm deep in adult mouse primary somatosensory cortex (barrel cortex) following surface loading with fluorescent Ca²⁺ indicators. This penetration depth correlates with the layer I and part of the layer 2/3 of mouse barrel cortex.

4.1. Animal Preparation

Adult FVB or C57BL/6 mice (~8 weeks old) were used for in vivo imaging primarily because application of fluorescent Ca²⁺ indicators on mouse pial surface produced satisfactory astrocyte loading in vivo. Same surface loading procedure only works on

immature rats up to ~3 weeks old (unpublished observations). Animals were anesthetized with intraperitoneal injection of ketamine (0.12 mg/g) and xylazine (0.01 mg/g). Depth of anesthesia was monitored by field potential recording and hind limb pinch withdrawal reflexes and kept constant at stage III/3 (45) with supplemental doses of anesthetics. Tracheotomy was performed and the mice were intubated and artificially ventilated with a small animal ventilator (SAAR-830, CWE). The respiration rate was set at ~100/min; inspiration time, 0.3 s; and the tidal volume, ~0.2 ml. Lung pressure was carefully monitored and maintained at lower than 10 cm H₂O to prevent lung injury. Blood gas was tested by taking arterial blood through a femoral artery catheter and pCO₂, pO₂ and pH were analyzed in microsamples (Rapidlab 248, Bayer, sample size 40 µl). Blood samples were taken every 1–2 h and totally 3–4 times from each animal. Experiments were completed only if physiological variables remained within the normal limits. The normal limits for pCO₂ were set at 30–45 mm Hg; pO₂, 80–115 mm Hg; and pH, 7.35–7.45. To image astrocytic Ca²⁺ signals in mouse barrel cortex, a cranial window (2–3 mm in diameter) was prepared centered at 0.5 mm posterior to the bregma and 3.5 mm lateral from midline. To stabilize the brain during imaging, a custom-made metal plate was glued to the skull with dental acrylic cement. Dura matter was removed for surface loading of fluorescent indicators. Body temperature was monitored by a rectal probe and maintained at 37°C by a heating blanket (BS4, Harvard Apparatus).

4.2. Ca²⁺ Indicators Loading

The major difference comparing surface bulk loading and local dye injection is that the former loading procedure only labels astrocyte structures due to the uptake by astrocyte limiting membranes that completely cover the pial surface (41), whereas the latter protocol loads both astrocytes and neurons (40,46). For the surface bulk loading, Fluo-4 AM (0.5–1 mM) was dissolved in dimethylsulphoxide (DMSO) with 20% pluronic acid and mixed in artificial cerebrospinal fluid (aCSF) containing 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose and 26 mM NaHCO₃ (pH 7.4), gassed with 95% O₂ and 5% CO₂ at 37°C (47). aCSF containing the dye (10–15 µl) was dropped on the pial surface, then the cranial window was covered by a small piece of parafilm to prevent the dye from drying. After a 45-min incubation, the exposed brain was washed for 15 min with aCSF without dye. In selected experiments, the exposed cortex was also loaded with the astrocyte-specific fluorescent indicator sulforhodamine 101 (SR101, 10 µM) (44) for 10 min after fluo-4 AM loading (**Fig. 5.2**). To minimize brain pulsation, 0.9% NaCl containing 1% agarose (37°C) was poured on the cranial window and the glass coverslip was glued to the metal plate with dental acrylic cement. A small opening between coverglass and metal plate was left for inserting recording electrode into the cortex (**Fig. 5.2**).

Serial images from pial surface to barrel cortex layer 2/3 (~300 µm deep) revealed that fluo-4 AM signals strictly co-localized with SR101 staining (**Fig. 5.2**). Astrocytes appear as bright fluorescent cells with multiple processes. Astrocyte end-feet are clearly labeled which outline the vasculatures. In contrast, neuronal cell bodies appear as dark, round shaped areas which can be detected at ~90 µm deep in the barrel cortex, owing to their lack of uptake of both fluo-4 AM and SR101 (**Fig. 5.2**).

4.3. Two-Photon Imaging

A custom-built microscope attached to Tsunami/Millinium laser (10W, SpectraPhysics) and a scanning box (FV300, Olympus) using Fluoview software and a 20X (0.9 NA, Olympus) objective was used for in vivo imaging. Excitation wavelength was in the range of 820–840 nm for both fluo-4 AM and SR101 imaging simultaneously. Two-channel detection of emission wavelength was achieved by using a 565 nm dichroic mirror (Chroma) and two

external photomultiplier tubes. A 525/40 bandpass filter (Chroma) was used to detect fluo-4 AM emission light, and a 620/60 bandpass filter (Chroma) was used to detect SR101 signals (**Fig. 5.2**). For image collection, ~1 s was needed to record a single frame of image at 512×512 pixels resolution. Time-lapse images of astrocytic Ca^{2+} signaling were collected every 1–3 s. Normally, a sampling interval of 3 s was found sufficient to detect whisker stimulation evoked astrocytic Ca^{2+} elevation, and this low sampling rate was used to avoid the photodamage. The two-photon laser power was carefully adjusted according to the depth of imaging in the brain, since the photodamage induced by high power laser can cause astrocytic Ca^{2+} oscillation in vivo (9). The average laser power that was used for detecting astrocytic Ca^{2+} signaling in barrel cortex layer 2 was less than 30 mW under the objective.

4.4. Whisker Stimulation

Whiskers were stimulated by air puffs (48). Briefly, whiskers on one side of the snout were trimmed to a 10-mm length. Air puffs consisted of pulses of compressed air, which were generated from a high pressure nitrogen tank and controlled by picospritzer (General Valve) at a pressure of 10 psi and delivered by a thick wall plastic tubing (4.5 mm outer-diameter, 2.5 mm inner-diameter). The tube was placed parallel to the left side of the mouse snout and 10–20 mm away from the C6 whisker. The direction of air flow was from rostral to caudal along the whisker row. This protocol will stimulate most of the vibrissae on one side of the whisker pad (at least three rows of whiskers were consistently activated by air puffs) (**Fig. 5.2**). The frequency of air puffs was set to 1, 3, 5, 7 or 10 Hz and controlled by Master-8 (A.M.P.I). Pulse width in all experiments was 10 ms. The duration of stimulation was adjusted from 1 to 60 s in different experiments. Single whisker stimulation was not attempted at this point, but in future studies, it will be intriguing to deflect a principle whisker using mechanic stimulation (49) and image the astrocyte Ca^{2+} responses in its related barrel column and the nearby columns.

4.5. Electrophysiological Recording

LFP recordings were obtained from layer 2 of barrel cortex (100–150 μm below the pial surface) by a patch pipette (TW100F-4, WPI; outer diameter, 1.0 mm; inner diameter, 0.75 mm; tip diameter, ~3 μm ; tip resistance 3–5 M Ω), containing 0.2% Texas red–dextran (77,000 molecular weight, Sigma) in aCSF. LFP signals were amplified using Axopatch 700B and pCLAMP 8.2 program with DigiData 1332A interface (Axon Instruments). The signals were bandpass filtered at (1–100 Hz) and digitized at 10 kHz.

5. Properties of Astrocytic Ca^{2+} Signaling Evoked by Whisker Stimulation

Astrocytes in barrel cortex layer 2/3 of adult mouse were labeled with Ca^{2+} indicator fluo-4 AM. Recording electrode was inserted into the field of imaging to detect LFP activity (**Fig. 5.3A**). Although increased neuronal activity in layer 2/3 of barrel cortex can be detected as early as 10–12 ms after whisker deflection (50), increases in astrocyte somatic Ca^{2+} were delayed by ~3 s after the onset of whisker stimulation (9). The onset of the increase in astrocytic Ca^{2+} was defined as the earliest time point at which the changes of somatic fluorescent signal reached 3 standard deviation of baseline fluorescent activity. Notably, 1-s stimulation failed to evoke astrocytic Ca^{2+} signaling, whereas 3-s stimulation was sufficient to induce astrocytic Ca^{2+} elevation (asterisk) (**Fig. 5.3B**).

In addition, the amplitude of astrocytic Ca^{2+} increases was similar in response to 10-s and 30-s stimulation, while the duration of astrocytic Ca^{2+} elevation was longer during 30-s stimulation. This evidence indicates that the amplitude of astrocytic Ca^{2+} elevation was mainly determined by the neuronal activity during the first ~10 s of stimulation (**Fig. 5.3B**). Most astrocytes showed a single increase in Ca^{2+} in response to 1 min of whisker

stimulation (72 cells from 13 mice), whereas others showed a second Ca^{2+} increase (11 cells from 13 mice).

In addition to the effects of the duration of stimulation on astrocytic Ca^{2+} elevations, astrocytic Ca^{2+} responses are also a function of the frequency of stimulation. During 1 Hz whisker stimulation, only 3 of 37 astrocytes tested in 9 mice showed an increase in Ca^{2+} (9). Rapid sensory adaptation, or a decrease in LFP amplitude within 1 s, occurred at 10 Hz stimulation. Accordingly, 10 Hz whisker stimulation did not trigger astrocytic Ca^{2+} responses (9). Additional experiments using 3 and 7 Hz whisker stimulation confirmed that astrocytic Ca^{2+} responses peaked at 5 Hz and decreased at both lower and higher frequencies (**Fig. 5.3C**). Since astrocytic Ca^{2+} signaling was delayed by ~3 s compared with neuronal field potential signals, there must be an accumulating effect of neuronal activity on the astrocytic Ca^{2+} elevations. To test the idea that astrocytic Ca^{2+} increases are a function of the intensity of local synaptic input, we calculated the summed LFP amplitude during the first 9 s of stimulation. As an index of total neuronal activity within a given time window, the sum of LFPs has also been widely used to positively correlate neuronal activity with cerebral blood flow (CBF) (21). Notably, the summed LFP amplitudes peaked at 5 Hz during the first 9 s of stimulation (**Fig. 5.3D**) and had a strong correlation with astrocyte somatic Ca^{2+} signaling ($R^2 = 0.68$, $P < 0.001$; **Fig. 5.3E**)

6. Implications for BOLD Signaling

6.1. Astrocytic Ca^{2+} Elevations Contribute to Cerebral Blood Flow Regulation

The basis for a positive BOLD signal is the increase in blood flow and volume that occur during neural activity. As discussed above, new development has shown that astrocytes can mediate functional hyperemia by release of vasoactive agents, indicating that astrocytic Ca^{2+} signaling may be both necessary and sufficient for changes in the BOLD signal (10, 17–19, 51). The initial studies on astrocytic control of the microvasculature were performed in slice preparations. One line of work showed that astrocytic Ca^{2+} increases are linked to activation of Ca^{2+} - sensitive phospholipase A2 (PLA2), which, in turn, stimulate the production of arachidonic acid (AA). Several AA metabolites are powerful vasoactive compounds. For example, the products of cyclooxygenase (COX) induced vessel dilation in both brain slice and in vivo preparations (10). Furthermore, the cytochrome P450 epoxygenase pathway produces epoxyeicosatrienoic acids (EETs), which mediated vessel dilation in retina (18), whereas astrocytic Ca^{2+} signaling triggered vessel dilation through activation of Ca^{2+} - sensitive K^+ channels in the astrocyte endfeet in cortical slices (19). In addition, two lines of work have suggested that astrocytic Ca^{2+} signaling in both retina and acute hippocampal slices is linked to production of the vasoconstrictor 20-hydroxyeicosatetraenoic acid, 20-HETE (17,18) (**Fig. 5.1**). However, a major drawback of studying blood flow regulation in ex vivo preparation is the loss of pressure-induced vasculature tone. Our in vivo experiments, employing targeted photolysis of caged Ca^{2+} in astrocytic endfeet, indicated that the primary mechanism by which astrocytes mediated vessel dilation is through release of PGE2 in a COX-1-dependent pathway (51) (**Fig. 5.4**).

Interestingly, several lines of work have documented that the changes in blood flow and BOLD signal detected during brain activation have a stronger correlation with synaptic inputs compared with spiking activity (52, 53). Indeed, local spiking activity does not appear to play a central role in functional hyperemia (53). Thus, activity-dependent vascular signals reflect excitatory synaptic activity or synaptic release of glutamate (54, 55). Similarly, astrocytes respond to the synaptic inputs, or glutamate released from synaptic terminal during sensory stimulation, and local spiking activity has no effect upon astrocytic Ca^{2+} elevation (9). Thus, astrocytic Ca^{2+} signaling may represent a signaling pathway that locally integrates synaptic inputs and controls the microvasculature. Astrocytes detect presynaptic

neurotransmitters release and display increases in intracellular Ca^{2+} . In turn, astrocytic Ca^{2+} signaling activates PLA2 resulting in release of vasoactive COX-1 metabolic products. Other pathways, such as NO, adenosine and pH may in parallel contribute to functional hyperemia. Several of these pathways may be similar to glutamatergic pathways which require astrocytes as a necessary intermediary linking synaptic activity to vasodilatation.

6.2. Astrocytic Ca^{2+} Signaling May Increase Oxidative Metabolism

Glutamate transporters (GluTs), which are mainly expressed in the perisynaptic processes of astrocytes, play an important role in removing the glutamate released during synaptic transmission preventing the extracellular glutamate concentration from reaching excitotoxic concentrations (56). Glutamate uptake is an energy requiring process and stimulates the glycolytic metabolism in cultured astrocytes (57, 58). The main energy consuming processes associated with glutamate transport into astrocytes include extrusion of Na^+ and conversion of glutamate to glutamine (59). In the model proposed by Pellerin and Magistretti, astrocytes utilize ATP produced from glycolytic glucose metabolism and lactate is transported to neurons for oxidative metabolism (57, 58). Because astrocytes express the full machinery for oxidative phosphorylation (60), it is somewhat surprising that glutamate uptake increases glycolysis rather than oxidative metabolism (57). A possible explanation is that the perisynaptic processes of astrocytes are essentially devoid of most organelles (Fig. 5.1). The fine processes of astrocyte covering synapses have a small diameter ($\sim 0.3 \mu\text{m}$) and are almost devoid of mitochondria (61). Therefore, the glycolytic metabolism of glucose, most likely occur in these fine structures of astrocytes which are heavily engaged in glutamate uptake (59, 62). Does oxidative metabolism in the astrocytic cell bodies contribute to increased ATP production during periods of intense glutamate uptake? Astrocytes contain a large number of mitochondria and the mitochondria volume fraction in the somatic areas of neurons and astrocytes are directly comparable (60,61).

It is in this regard important to note that mGluRs are expressed in the astrocyte perisynaptic processes in both rodent and primate (63, 64). Ex vivo experiments have shown that electrical stimulation of Schaffer collaterals elicits mGluRs-dependent Ca^{2+} elevation in hippocampal astrocytes (5, 6). Depending on the intensity of electrical stimulation, astrocytic Ca^{2+} signaling can be detected either in the perisynaptic microdomains or propagate intracellularly into the soma. Furthermore, activation of astrocytic mGluRs contributes to increases of astrocytic Ca^{2+} signaling during sensory stimulation in vivo (9). Astrocytic Ca^{2+} increases are first initiated in the perisynaptic processes and propagate within a time frame of 0.5–2 s to the soma triggering a 50–300% fold increase in cytosolic Ca^{2+} (9). Although little is known about the role of Ca^{2+} signaling as a modulator of glycolytic metabolism, it has been established that elevations in mitochondria Ca^{2+} are strong stimulator of oxidative-phosphorylation and accelerate ATP production (65). Therefore, we proposed that astrocytic Ca^{2+} signaling initiated in the perisynaptic processes following propagation into mitochondria rich somatic regions may stimulate the oxidative-phosphorylation. ATP produced by mitochondria in the soma may in turn diffuse back into the perisynaptic processes and supplement ATP produced by local glycolysis during periods of intense glutamate uptake. Thus, a possible function of astrocytic Ca^{2+} signaling evoked during synaptic activity may be to link energy metabolism in processes and cell body by activating oxidative-phosphorylation in mitochondria located distant to the perisynaptic processes.

6.3. Energy Consumption by Astrocytes During Neural Activation

Several reviews have proposed that astrocytes only account for 5% of the total energy budget consumed in rodents and about 6% in humans (66). These estimations were based on calculation of known properties of channels and synapses in neurons and astrocytes (66). In

contrast, studies using in vivo nuclear magnetic resonance spectroscopy indicate that astrocytes contribute to 15% to 30% of total oxidative metabolism in the brain (67, 68). The prior calculations may neglect many of the energy requiring functions of protoplasmic astrocytes (66). In addition to glutamate uptake, multiple ATP consuming processes are activated in astrocytes during neural activation. For instance, restoring astrocytic Ca^{2+} concentrations following mGluRs mediated Ca^{2+} mobilization requires activation of both plasma membrane Ca^{2+} -ATPase and ER Ca^{2+} -ATPase (69). Furthermore, astrocytes buffer at least, in part, extracellular K^{+} by active uptake of K^{+} through Na^{+} - K^{+} -ATPase. Astrocytes are the principle cell type responsible for water homeostasis and clearance of tissue swelling during episodes of intense synaptic activity likely requires increased metabolism (70). Moreover, Ca^{2+} dependent activation of PLA2 results in release of arachidonic acid, which in turn requires new lipid synthesis. In addition to this energy requiring supportive function of astrocytes, astrocytic Ca^{2+} signaling likely evokes downstream effects that increase energy demands of both neurons and other non-neuronal cells. Intercellular astrocytic Ca^{2+} signaling is mediated by ATP release from astrocytes (71). ATP is, in addition to its function as an energy metabolite, an important gliotransmitter that triggers Ca^{2+} signaling in surrounding astrocytes (33), neurons (72), microglia (73) and smooth muscle cells (19). Normalization of Ca^{2+} in all of these cell types occurs through activation of Ca^{2+} -ATPases. Conversely, hydrolysis of extracellular ATP results in accumulation of adenosine, which reduces release of glutamate and thereby dampens energy demands. The notion that astrocytic Ca^{2+} signaling is an energy requiring process initiated during synaptic activity finds support in the observation that inhibition of postsynaptic activity by application of CNQX/APV and TBOA failed to block changes in NADH signaling evoked during intense electrical stimulation in brain slice preparation (74). Glycolytic metabolism was shown not to play an essential role in the CNQX/APV resistant NADH changes (74), indicating that astrocytic Ca^{2+} increases most significantly affect oxidative- phosphorylation.

Of interest is the observation that the various tasks of astrocytes may preferentially activate oxidative or glycolytic metabolism. In a recent study using 2-photon NADH imaging, we found that cortical spreading depression was associated with a sharp decrease in NADH signaling reflecting increased oxidation in perivascular endfeet. The decrease in NADH occurred during the repolarization phase during which plasma membrane pumps are engaged in normalization of ion gradients (75).

7. Concluding Remarks

Deciphering the metabolic responses of astrocytes in response to neural activation is likely to add a new dimension to our understanding of BOLD signal changes during fMRI. A question for future research is whether astrocytic Ca^{2+} signaling is necessary and sufficient for increases in local blood flow similar to what have been demonstrated in mice using targeted photolysis of caged Ca^{2+} (51).

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Fig. 5.1.

Proposed mechanisms that link synaptic activity, cerebral vasculature regulation and astrocytic energy metabolism with astrocytic Ca²⁺ signaling. Synaptic released neurotransmitters activate metabotropic receptors in astrocytic perisynaptic processes, causing elevation of intracellular Ca²⁺ through a phospholipase C-dependent pathway. Increases in astrocytic Ca²⁺ are associated with release of gliotransmitters which feed back to the synaptic activity. Astrocytic Ca²⁺ signaling may also propagate to the astrocytic endfoot, releasing vasoactive agents through phospholipase A₂(PLA₂)-dependent pathway as well as activation of Ca²⁺-sensitive K⁺ channels. Furthermore, Ca²⁺ is a well established stimulator of oxidative-phosphorylation which accelerates ATP production from mitochondria (Mit). Due to the small diameter of perisynaptic processes and the absence of mitochondria in these structures, ATP can also be generated through glycolysis and glycogenolysis pathways particularly in astrocyte perisynaptic processes. The major energy consuming processes in astrocyte include converting glutamate (Glu) to glutamine (Gln) after taken up by glutamate transporters, uptake of K⁺ from extracellular space through Na⁺-K⁺ ATPase and restoring astrocytic cytosolic Ca²⁺ concentration by activating both plasma membrane and endoplasmic reticulum (ER) Ca²⁺-ATPase.

Fig. 5.2.

Diagrams of experimental setup. **(A)** A cranial window was opened on the primary somatosensory cortex. A metal frame was glued to the skull to prevent the movement of the brain. Whisker stimulation was performed in the contra lateral side of the animal's snout by air puffs which were generated from a high pressure nitrogen tank and control by picospritzer. A plastic tube connected to the picospritzer was placed ~2 cm away from the C6 whisker in a rostral-caudal direction to deliver the high pressure air. Electrical field potential recording and 2-photon Ca^{2+} imaging were performed simultaneously during whisker stimulation. Agarose (1%) was poured onto the pial surface before the coverglass was mounted to the metal frame to minimize the brain pulsation. **(B)** Dual labeling of barrel cortex with Ca^{2+} indicator fluo-4 AM (*top*) and astrocyte specific marker SR101 (*bottom*). Only SR101 positive astrocytes were labeled with fluo-4 AM (*white arrowheads*). Neurons appear as dark, round areas without either fluo-4 AM or SR101 labeling (*gray arrowheads*). The image shown was located 120 μm below the pial surface. Scale bar, 30 μm .

Fig. 5.3.

Whisker stimulation evokes astrocytic Ca^{2+} increases in mouse barrel cortex. **(A)** Two-photon fluorescence image taken from layer 2/3 of mouse barrel cortex ($\sim 150 \mu\text{m}$ below the pial surface), showing that astrocytes were labeled with Ca^{2+} indicator fluo-4 AM (*open arrowheads*). Neuronal cell bodies appear as dark, round area, due to their lack of uptake of fluo-4 AM. The location of recording electrode is indicated by white dashed line. Scale bar, $30 \mu\text{m}$. **(B)** 5 Hz whisker stimulation induced astrocytic Ca^{2+} increases as a function of the stimulation duration. Upper trace, LFP recorded from recording electrode shown in panel A. Lower trace, averaged relative fluorescent change $\Delta F/F$ in the astrocytic cell bodies indicated in panel A. **(C)** Mean increase in fluo-4 emission after 9 s of different frequencies of stimulation. **(D)** Summed LFP amplitude within the first 9 s of whisker stimulation as a function of the frequency of stimulation. Data are presented as mean $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with 5 Hz whisker stimulation induced responses. One-way analysis of variance with Dunnett's test. **(E)** Correlation between the summed LFP amplitude within the first 9 s and the mean increase in fluo-4 emission 9 s after the onset of stimulation ($R^2 = 0.68$, $P < 0.001$).

Fig. 5.4.

Vasodilation triggered by uncaging (**A**) *Upper panel*: Two-photon images of a vascular astrocyte, which displayed Ca^{2+} increases in the endfoot following photolysis of caged Ca^{2+} . Astrocytes were loaded with the Ca^{2+} indicator dye, rhod-2/am and DMNP-EDTA/am, whereas the vasculature was stained with FITC-dextran. Gray arrow indicates the position of photostimulation. Scale bar, 10 μm . (**B**) Time-course tracings show that photostimulation caused a rapid Ca^{2+} increase and arterial vasodilation. Arrows indicate time of photolysis stimulation. Dark arrowhead indicates the peak of Ca^{2+} elevation, and light arrowhead indicates peak dilation. (**C**) Summary histograms of maximum arterial cross-section area (*left panel*) and maximum Ca^{2+} increase (*right panel*) without photostimulation, with photostimulation but omitting DMNP-EDTA/am loading, and with DMNP-EDTA loading and photolysis. Maximum increase within 1 min after photostimulation was measured relative to baseline before stimulation. Mean $\pm$ s.e.m. * $P < 0.01$ compared to no-stimulation, Tukey-Kramer.