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Review Article

Astrocytic Ca^{2+} Signaling and its Role in Modulating Cerebral Blood Flow

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Abstract

Astrocytes are a predominant glial cell type in the CNS and an integral part of a synapse and vasculature unit in CNS. *In vitro* and *in vivo* studies have revealed that astrocytes play a variety of roles in physiology and pathology. In particular, recent studies indicate that astrocytic Ca^{2+} signaling is involved in the regulation of functional hyperemia. This article reviews the astrocytic Ca^{2+} signaling pathway and recent advances regarding the role of Ca^{2+} signaling in the regulation of cerebral blood flow. The article discusses the discrepancies from different studies (especially *in vivo* studies) and the potential role of IP_3R-mediated Ca^{2+} signaling pathway, and suggests that the involvement of astrocytic Ca^{2+} in functional hyperemia can be affected by tissue metabolism, animal species, age, brain region and wakefulness of animals. Thus the precise mechanisms by which astrocytic Ca^{2+} regulates cerebral blood flow can only be elucidated in a defined preparation.

Abbreviations

GAFP: glial fibrillary acidic protein; CNS: central nervous system; 2-P: two-photon; L1: layer 1; L2/3: layer 2/3; GPCRs: G-protein coupled receptors; mGluR5: metabotropic glutamate receptors; PLC/IP_3: phospholipase-C/inositol 1,4,5-triphosphate; TRP: transient receptor potential; NMDARs: GECIs: genetically encoded Ca^{2+} indicators; CBF: cerebral blood flow; PLA2: phospholipase A2; BG cells: Bergmann glial cells; Kir channel: inward rectifier K^+ channel; COX1: cyclooxygenase; fMRI: Functional magnetic resonance imaging.

Introduction

Astrocytes are predominant glial cell type in the central nervous system (CNS) [1-3]. Protoplasmic astrocytes in grey matter and fibrous astrocytes in white matter are the major type of astrocytes which are morphologically different. Protoplasmic astrocytes are complex (sponge like) and highly branched with numerous fine processes and their endfeet wrap around blood vessels, while fibrous astrocytes are less complex and have thicker and less branched processes. Under normal conditions, protoplasmic astrocytes occupy distinct non-overlapping domains *in vivo*, and their processes completely wrap or ensheath synapses as well as blood vessels [4-6]. Studies using immunofluorescence labeling of neuronal somata in mouse brains revealed that a single astrocyte enwraps on average four neuronal somata with an upper limit of eight. Halassa et al. [5] determined from single-neuron dye-fills that one astrocyte contacts 300–600 neuronal dendrites. The processes from an individual astrocyte envelope approximately 140,000 synapses from multiple neurons [6]. Thus astrocytes can be stimulated by synaptic activities. On the other hand, it has been known for a long time that astrocytes and blood vessel have intimate anatomic relationship. Recent studies using different approaches including fluorescence imaging and electron microscopy revealed that the cerebral vascular surface is almost completely covered by astrocytic endfeet [7-10]. *In vivo* imaging using 2-P fluorescence microscopy also indicate that astrocyte endfeet wrap around the blood vessels in the mouse brain (Figure 1). Importantly, the endfeet of perivascular astrocytes express high levels of aquaporin and connexin 43 [7]. The spatial occupation and the intimate contact with both synapses and blood vessels render astrocytes as ideally situated to relay neuronal signals to blood vessels and regulate cerebral blood flow. This article reviews the astrocytic Ca^{2+} signaling pathway and recent advances regarding the role of Ca^{2+} signaling in the regulation of cerebral blood flow (CBF). The various aspects of neurovascular coupling and the signaling pathways have been reviewed previously [11,12]. Analysis of data from different studies (especially from those *in vivo* studies) suggests that the involvement of astrocytic Ca^{2+} in functional hyperemia can be affected by tissue metabolism, animal species, age, brain region and wakefulness of animals. Thus the precise mechanisms by which astrocytic Ca^{2+} regulates cerebral blood flow can only be elucidated in a defined preparation.

Astrocytes and astrocytic Ca^{2+} signaling in the CNS

It is well known that astrocytes act as a K^+ sink to maintain

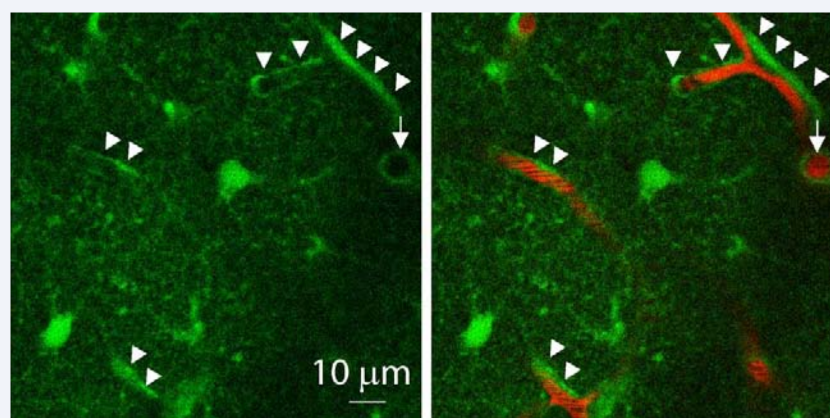

Figure 1 ***In vivo*** **two-photon (2-P) imaging of astrocytes and blood vessels in the cortex of a mouse brain.** A single optical section image of EGFP-expressing astrocytes (Green, left panel) from Glt1-EGFP mice and blood vessels labeled by Rhodamine-Dextran (Red, right panel of the merged image). The arrow heads indicate astrocyte endfeet surrounding blood vessels. The arrows indicates a penetrating arteriole. The image is used through the courtesy of N. Zhang, University of Missouri.

extracellular K^+ homeostasis [13] and remove glutamate from the synaptic cleft by their glutamate transporters to avoid glutamate toxicity [14-16]. Astrocytes also provide nutritional and structural support for neurons. Some astrocytes express the glial fibrillary acidic protein (GFAP), which is used as a specific marker to distinguish them from other cell types; however, its expression levels are different in astrocytes in different regions. For example, under normal conditions, cortical astrocytes express much lower levels of GFAP than do protoplasmic astrocytes in the hippocampus of a mouse brain, although the densities of astrocytes in these two regions are similar [17,18]. It is also clear now that not all astrocytes express GFAP and vice versa, not all cells that express GFAP are astrocytes [19]. It has been recently shown that spinal cord astrocytes express region specific genes that are functionally different, e.g., a recent study revealed that ventral astrocytes in mouse spinal cord express Semaphorin 3a that is important for sensorimotor circuit organization [20]. Electrophysiological recordings also showed that astrocytes even in the same region have differential patterns of current-voltage relationship known as outward rectifying astrocytes and variably rectifying astrocytes [21]. Similarly, in vivo intracellular recording of astrocytic membrane potential revealed a significant variation in fluctuations depending on the local field potential state and cell body location [22]. Astrocytes also exhibit regional heterogeneity in spontaneous Ca^{2+} signaling. Using two photon (2-P) laser-scanning fluorescence microscopy, Takata and Hirase [23] used an anesthetized adult rat to show how the astrocytes in the cortical layer 1 (L1) exhibited distinct Ca^{2+} dynamics *in vivo* when compared to astrocytes in the cortical layer 2/3 (L2/3). They found that astrocytes in L1 had nearly doubled the Ca^{2+} activity of astrocytes in L2/3 [23]. Furthermore, Ca^{2+} fluctuations in the processes within an astrocyte were independent in L1, while those in L2/3 were more synchronous [23]. In urethane, anesthetized young mice (P9-25), hippocampal astrocytes exhibited synchronized Ca^{2+} oscillations and intercellular waves [24,25]. Furthermore, subcellular Ca^{2+} analysis revealed a complex dynamics in different domains within an astrocyte [26,27]. The difference in astrocytic Ca^{2+} activities in different brain regions and different microdomains within an astrocyte reflects a functional heterogeneity, which may be the result of different neuronal activities or synaptic integrations, microenvironment and/or different properties of astrocytes *per se*. These findings demonstrate that astrocytes in the CNS are heterogeneous in morphology, molecular expression, and function.

It was discovered more than two decades ago that cultured astrocytes could mediate Ca^{2+} signaling (i.e., transient Ca^{2+} increase) [28,29], suggesting that astrocytes can play more active roles in the CNS than previously found. More recent studies using 2-P microscopy have found that astrocytes can mediate Ca^{2+} signaling and intercellular waves *in vivo* by the activation of a variety of G-protein coupled receptors (GPCRs) including Group I metabotropic glutamate receptors (mGluRs) [30-32], P2Y receptors [32-36], $GABA_B$ receptors ($GABA_BRs$) [33;37], α1-adrenergic receptors [38-40], cholinergic receptors [41;42], and dopamine receptors [43;44]. GPCR-mediated Ca^{2+} signaling is now considered a primary form of Ca^{2+} signaling pathways in astrocytes as shown by Ca^{2+} imaging. GPCR stimulation activates phospholipase-C/inositol 1,4,5-triphosphate (PLC/IP_3) pathway to release Ca^{2+} from the ER through the activation of IP_3Rs [1,3,45] for Ca^{2+} signaling pathways (for review see Verkhratsky et al. [46] and Ding [47]). Among the three subtypes of IP_3R (IP_3R1-3), IP_3R2 seems to be predominant in stimulating astrocytes in the rodent brain [48-50]. IP_3R2 knock-out (IP_3R2 KO) mice did not exhibit GPCR agonists-evoked Ca^{2+} release in astrocytes in brain slice preparation and in studies of live mice, demonstrating that IP_3R2 is a key mediator of intracellular Ca^{2+} release in astrocytes [36;45]. In addition to GPCR stimulations, astrocytic Ca^{2+} signals can also be evoked by sensory stimulations, mechanical stimulations, and photolysis of caged compounds [30,32,35,51,52]. Sensory stimulations, including whisker deflection [35], locomotion [53,54], limb stimulation [38;55;56], light illumination of visual cosrtex [42;57], and odor stimulation of olfactory glomeruli [8] can all induce Ca^{2+} elevation and intercellular waves in astrocytes *in vivo* in anesthetized animals. A number of plasma membrane proteins also control Ca^{2+} homeostasis through regulating Ca^{2+} influx from the extracellular side. Those proteins include the Na^+/Ca^{2+} exchanger [58-60], plasma membrane Ca^{2+} ATPase [58], store

operated channels [61], P2X purinoceptors [62,63], transient receptor potential A (TRPA) [64] and C (TRPC) channels [61,65-67], and N-methyl-D-aspartate (NMDA) receptors [63].

Astrocytic Ca^{2+} signaling has been extensively studied in cultured astrocytes as well as in live animals using fluorescence imaging. Both synthetic organic Ca^{2+} probes and genetically encoded Ca^{2+} indicators (GECIs) can be used to label astrocytes. The acetoxymethyl (AM) ester form of synthetic organic Ca^{2+} indicators such as fluo-4, Oregon green BAPTA-1 (OGB), Rhod-2, or x-Rhod-1 have been largely used for *in vitro* and *in vivo* Ca^{2+} imaging due to their high sensitivity and speed [23,30,32-35,41,51,68-72]. The selective labeling of astrocytes *in vivo* by synthetic organic Ca^{2+} indicators can be confirmed by an astrocyte selective dye sulforhodamine 101 (SR101) [70]. Recently developed GECIs including FRET-based GECIs such as YC3.6 and intensity-based single-fluorophore GECIs such as GCaMP provided an alternative way to image Ca^{2+} signaling in astrocytes *in vivo*. The major advantage for using GECIs is that they can be selectively expressed in astrocytes using an astrocyte-specific promoter. They can be expressed in astrocytes using viral transduction, *in utero* electroporation or transgenic mice [40,73-78]. Astrocyte-specific expression of GECIs using adeno-associated viral vectors can be achieved by astrocyte-specific promoters [18,76,79]. The most recently developed GCaMP5 and GCaMP6 yielded a cytosolic Ca^{2+} increase in a neuron after triggering a single action potential and sensory stimulation [75,80]. Transgenic mice expressing floxed GCaMP3 and GCaMP5G have been crossed with Cre mice having neuron- and astrocyte-specific promoters for neuronal and astrocytic Ca^{2+} imaging [40,74,77]. For astrocytic specific expression of GCaMP3 and GCaMP5G, GFAP-Cre mice are commercially available [81,82]. Although Ca^{2+} signals in the processes of astrocytes can be readily observed using bulky loading of organic synthetic Ca^{2+} indicators [30,33-35,38], GECIs especially GCaMP can reveal more detailed features of Ca^{2+} signaling in microdomains than organic synthetic Ca^{2+} indicators [40,76-78]. Since GECIs can be expressed in astrocytes for prolonged times, another advantage for using GECIs is that it is feasible to conduct long-term and repeated *in vivo* Ca^{2+} imaging in astrocytes. Usually, for *in vivo* imaging, an open skull or thin skull cranial window in the cortex must be prepared for optical access and/or loading organic Ca^{2+} indicators [68,83-86]. It is also worth mentioning that GECIs are also advantageous over synthetic organic Ca^{2+} indicators when they are used for mitochondrial Ca^{2+} uptake as GECIs can be selectively targeted in mitochondrial matrix, overcoming the partial localization of synthetic organic Ca^{2+} indicators in the cytosol [87,88].

The role of astrocytic Ca^{2+} signaling in the regulation of CBF

CBF is regulated by cerebrovascular autoregulation and functional hyperemia. The latter refers to matched delivery of blood flow to the brain regions with different activity levels. Given that Ca^{2+} signaling is the primary form of astrocytic excitability, the role of astrocytic Ca^{2+} signaling in regulating CBF has been studied using *in vitro* and *in vivo* preparations.

Using brain slices, Stobart et al. [89] and Zonta et al.[90] found that Ca^{2+} elevation in astrocytes induced by neuronal afferent stimulation and photolysis of caged Ca^{2+} induced vasodilation. Studies from brain and retina slices suggest that the polarity of astrocytic Ca^{2+}-dependent regulation of blood flow is dictated by tissue metabolism [91-93]. Brain slice study has also shown that the levels of astrocytic Ca^{2+} increase determine vessel dilation or constriction regardless of the mechanism by which astrocytic endfeet Ca^{2+} is elevated [94]. The modest increases in Ca^{2+} induced dilation, whereas larger increases lead to constriction. These studies suggest complex mechanisms of blood flow regulation by astrocytic Ca^{2+} signals [11]. Different pathways and vasoactive mediators derived from astrocytes are involved in vessel dilation and constriction. Synaptically released glutamate activates metabotropic glutamate receptors (mGluRs) and stimulates astrocytic Ca^{2+} elevation that activates downstream pathways through phospholipase A2 (PLA2). PLA2 mediates arachidonic acid generation and subsequently three types of metabolite:1) Prostaglandins (PG) by cyclooxygenase (COX1) [51,90,91] and 2) exoxyeicosatrienoic acids (EETs) by cytochrome P450 (CYP-450) epoxygenase [93] in astrocytes dilate vessels, and 3) 20-hydroxyeicosatetraeonic acid (20-HETE) by CYP-450 ω-hydroxylase in smooth muscle, which constricts vessels [91,93]. Nitric oxide (NO) can regulate vascular tone by inhibiting the synthesis of the vasoconstricting 20-HETE as well as the vasodilating EETs [95]. Oxygen levels affect the vasoactive mediators [91,92]. When oxygen levels are lowered and astrocyte Ca^{2+} concentration is increased, glycolysis is dominated in astrocytic energy metabolism and lactate is accumulated in extracellular space. Extracellular lactate attenuates transporter-mediated uptake of extracellular prostaglandin E_2, leading to accumulation and subsequent vasodilation [91]. These studies indicate that cellular energy metabolism regulate astrocytic Ca^{2+} mediated vascular changes. Astrocytic K^+ also plays a role in vascular tone. Using rat brain slice preparations, it was reported that neuronal activity-induced astrocytic Ca^{2+} signals opened large-conductance Ca^{2+}-sensitive K^+ (BK) channels in astrocytic endfeet [96]. BK channels in turn activated inward rectifier K^+ (Kir) channels in smooth muscle cells and cause vasodilation. However, further study showed that a high concentration of astrocytic Ca^{2+} (induced by either uncaging or electric field stimulations) and perivascular K^+ caused vessel constrictions, while low astrocytic Ca^{2+} perivascular K^+ caused vasodilation [94]. A study from retinal slice preparation provided contradictive evidence for the role of astrocytic K^+ in functional hyperemia. Depolarization-induced release of astrocytic K^+ from endfeet did not cause vasodilation in arterioles [97]. Furthermore, the magnitude of light-evoked vasodilations was identical in Kir4.1 knock-out and wild-type animals, indicating that astrocytic K^+ in the retina does not contribute to neurovascular coupling. Thus the role of astrocytic K^+ in regulating functional hyperemia is not defined.

While brain slice preparations provide ready access for pharmacological manipulations, Ca^{2+} imaging and patch clamp recording, a major drawback in using brain slice preparations is that arterioles are usually preconstricted and lack spontaneous tone. *In vivo* approach using 2-P microscopy to image cellular Ca^{2+} signals and blood flow through a cranial window provides a unique tool to study the mechanism of neurovascular coupling in the intact brain. Different studies indicate that Ca^{2+} evaluation

in astrocytes is involved in functional hyperemia. Takano et al. reported that photolysis of caged Ca^{2+} in the endfeet of astrocytes induced rapid dilation of the arteries, but not of the veins and capillaries in the mouse cortex [51]. Similar phenomenon was observed following neuronal stimulation. Electrical stimulation evoked an increase in local neuronal activity and a widespread increase in astrocytic Ca^{2+} and was associated with vasodilation. Combined with pharmacological manipulation, they further showed that astrocyte-mediated vasodilation is partially dependent on COX-1 activity. In cerebellum, Ca^{2+} signals of Bergmann glial (BG) cells are associated with motor behavior and neuronal activity [53]. In awake and behaving mice, BG cells have exhibited three different forms of Ca^{2+} excitation: flares, bursts, and sparkles. Bursts and sparkles were ongoing in awake mice at rest, whereas flares were initiated during locomotor behavior. Locomotor performance initiated synchronized Ca^{2+} signals in the network of BG cells. Motor behavior induced astrocytic Ca^{2+} flares were correlated with blood flow increase, which can be attenuated by tetrodotoxin through the inhibition of neuronal activity. Thus, the specific animal behavior noted in the experiments dealing with awake and behaving mice dictated Ca^{2+} excitability in BG cells and vascular response. Odor stimulation also induced Ca^{2+} transients in astrocyte endfeet and an associated dilation of upstream arterioles; furthermore, Ca^{2+} elevations in astrocytes and functional hyperemia depended on mGluR5 in astrocytes and cyclooxygenase activation [8]. Electrical stimulation of a forepaw of isoflurane-anesthetized mice induced vasodilation and an astrocytic Ca^{2+} increase in the forepaw region of their primary somatosensory cortex [36]. However, a recent study showed that IP_3R2 KO mice exhibited normal functional hyperemia in the same tests [36], indicating that the stimulus-induced vasodilation is independent of IP_3R-mediated Ca^{2+} increase since IP_3R2 KO mice lack cytosolic Ca^{2+} elevation in astrocytes. In addition, it was observed that the onset of vasodilation preceded astrocytic Ca^{2+} increase. Consistent with this study, Takata reported that IP_3R2 knockout mice showed similar changes in CBF with WT mice after a brief electrical stimulation of the nucleus basalis of Meynert (NBM), the primary source of cholinergic projection to the cerebral cortex [98]. Moreover, whisker stimulation resulted in similar degrees of CBF increase in IP_3R2 KO mice and WT mice. *In vivo* electrophysiological recording on dentate gyrus and fMRI showed that WT and IP_3R2 KO mice exhibited no difference in electrical responses and BOLD signals after electrical stimulation of perforant pathway [99]. Using *in vivo* 2-P imaging on awake mice and astrocyte-specific expression of GCaMP6s, a very recent study from Bonder and McCarthy further confirmed that IP_3R2-mediated Ca^{2+} signaling is not critically involved in meditating functional hyperemia [100]. These results from *in vivo* indicate that neural activity-driven CBF modulation could occur without large cytosolic increases of Ca^{2+} in astrocytes. Similarly, inhibition of group I mGluRs did not affect transient hemodynamic responses following a brief whisker stimulation in rats under isoflurane anesthesia [101]. This study suggests that group I mGluRs, whether they are expressed in neurons and/or astrocytes, do not play a role in early hemodynamic responses following sensory stimulation in rats, which is contradictive to other *in vitro* and *in vivo* studies [8,51,90] and raises the question whether neurovascular coupling involves signaling from neurons to glial cells to blood vessels.

Discussion and Conclusions

Several *in vitro* and *in vivo* studies demonstrated that astrocytic Ca^{2+} signals were involved in the regulation of CBF. However, data from different studies were not consistent. The fact that from multiple *in vivo* studies, IP_3R2 KO mice do not show a difference from the wild type mice in CBF after sensory and electrical stimulation suggests that functional hyperemia induced by these stimulations is mainly neuronal dependent, and IP_3R2-mediated Ca^{2+} increase may not be sufficient enough to exert an additional effect on the onset of vasodilation [36,98-100]. One must realize that anesthetized or awake animals might have different responses to functional hyperemia as astrocytes exhibit different Ca^{2+} properties in anesthetized versus awake mice. In awake mice, cortical astrocytes exhibited much higher frequency of spontaneous Ca^{2+} signaling in the cell body and processes than in mice anesthetized by isoflurane, ketamine and urethane [34]. In addition, spontaneous somatic Ca^{2+} signals in astrocytes were expressed as intercellular wave characterized as synchronized Ca^{2+} elevation in the astrocyte network in awake mice. Thus, synchronization of cortical astrocytic Ca^{2+} activity is a hallmark of wakefulness of an animal and the concentration of vasoactive mediators released from astrocytes might also depend on the wakefulness of an animal. The role of astrocytic Ca^{2+} in CBF regulation might also be regional difference while most *in vivo* studies were conducted on the somatosensory cortex. Age of animals could also be a factor. A recent study showed that spontaneous Ca^{2+} wave in BG increased with age [102], thus, the threshold for the glial Ca^{2+} in aged mice to exert an effect on neurovascular couple might be different from young mice. The role of mGluR5 in CBF regulation is also controversial. Inhibition of group I mGluR reduced sensory stimulation-induced astrocytic Ca^{2+} signals in cortex and olfactory glomeruli [8,51,90], suggesting that signaling from neuron to glial cells to blood vessel is involved in neurovascular coupling. However, another study showed that the antagonists of mGluR5 did not affect functional hyperemia, suggesting that neuron-derived glutamate is not involved in hemodynamic response to sensory stimulation [101]. Since young mice express much higher mGluR5 than adult mice [32], the discrepancy could be due to the age difference of animals used. Thus it is unclear to what extent functional hyperemia is dependent on glutamate-stimulated astrocytic Ca^{2+}. On the other hand, functional hyperemia is multifactorial, involving neurons, glial cells, smooth muscle cells and endothelial cells; furthermore, their coordinated actions are required. Thus a unifying mechanism can only be determined using a defined preparation. Tissue metabolism, animal species, age, brain region for study, and wakefulness of animals may all affect the involvement of astrocytic Ca^{2+} signaling in the regulation of CBF. It is also worth pointing out that although KO mouse models are particularly useful for studies astrocytic Ca^{2+} signaling and neuron-glial vasculature coupling, functional and developmental compensation may eliminate the phenotypic effect [100]. As multiple studies with negative results were from the same IP_3R2 KO mice, it is important to develop and use other animal models to further clarify the role and involvement of astrocytic IP_3R2-mediated Ca^{2+} in the regulation of CBF. Inducible IP_3R2 KO mice might be a direction as the target gene of interest can be inactivated in specific cell types at specific time points, thus

reducing or eliminating the phenotypic effect. However, inducible IP_3R2 KO mice are not available for the time being. Another approach is to use viral transduction to disrupt IP_3R-mediated Ca^{2+} signaling in astrocytes by overexpressing IP_3 sponge and IP_3 phosphatase specifically in astrocytes [18,88,103], however, it is expectable that the effect can only be achieved when enough number of astrocytes around the blood vessels is transduced.

While we have discussed the involvement of astrocytic Ca^{2+} in functional hyperemia in healthy animals, several studies showed that astrocytes have increased Ca^{2+} signals in the mouse models of neural diseases (for review see Ding [17]). Using *in vivo* 2-P imaging, Ding et al. [30,33] reported that astrocytes exhibited enhanced Ca^{2+} signaling and intercellular waves *in vivo* after status epilepticus and photothrombosis-induced ischemic stroke. Traumatic brain injury [104] and Alzheimer diseases [105] also dramatically increase astrocytic Ca^{2+} signaling. Therefore, it is important to determine whether and how astrocytic Ca^{2+} plays a role in CBF regulation in neural diseases. Understanding the mechanisms by which enhanced astrocytic Ca^{2+} is induced and the role of astrocytic Ca^{2+} is played in the regulation of CBF should provide therapeutic implications for these and other neural diseases.

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