Published in final edited form as:

Future Neurol. 2013 September 1; 8(5): 529–554.

***In vivo* astrocytic Ca²⁺ signaling in health and brain disorders**

Shinghua Ding

Dalton Cardiovascular Research Center, Department of Biological Engineering, University of Missouri, Columbia, MO 65211, USA dings@missouri.edu

Abstract

Astrocytes are the predominant glial cell type in the CNS. Although astrocytes are electrically nonexcitable, their excitability is manifested by their Ca²⁺ signaling, which serves as a mediator of neuron–glia bidirectional interactions via tripartite synapses. Studies from *in vivo* two-photon imaging indicate that in healthy animals, the properties of spontaneous astrocytic Ca²⁺ signaling are affected by animal species, age, wakefulness and the location of astrocytes in the brain. Intercellular Ca²⁺ waves in astrocytes can be evoked by a variety of stimulations. In animal models of some brain disorders, astrocytes can exhibit enhanced Ca²⁺ excitability featured as regenerative intercellular Ca²⁺ waves. This review first briefly summarizes the astrocytic Ca²⁺ signaling pathway and the procedure of *in vivo* two-photon Ca²⁺ imaging of astrocytes. It subsequently summarizes *in vivo* astrocytic Ca²⁺ signaling in health and brain disorders from experimental studies of animal models, and discusses the possible mechanisms and therapeutic implications underlying the enhanced Ca²⁺ excitability in astrocytes in brain disorders. Finally, this review summarizes molecular genetic approaches used to selectively manipulate astrocyte function *in vivo* and their applications to study the role of astrocytes in synaptic plasticity and brain disorders.

Keywords

Alzheimer's disease; G-protein coupled receptors; *in vivo* imaging; intercellular Ca²⁺ waves; IP₃ receptor; molecular genetics; photothrombosis; spontaneous Ca²⁺ signaling; status epilepticus; traumatic brain injury; two-photon microscopy

Background

Astrocytes in the CNS

Astrocytes are the predominant glial cell type and outnumber neurons in the CNS [1–3]. There are two major types of astrocytes: protoplasmic astrocytes in gray matter and fibrous astrocytes in white matter (**Figure 1A**). Protoplasmic astrocytes are highly branched with fine processes and their endfeet wrap around blood vessels. Fibrous astrocytes have thicker and less-branched processes. It is well known that astrocytes provide nutritional and structural support for neurons, and act as a K⁺ sink to maintain extracellular K⁺ homeostasis

Financial & competing interests disclosure

The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

[4]. In addition to these passive roles, it is also established that they can remove glutamate from the synaptic cleft by their glutamate transporters to avoid glutamate toxicity [5,6]. Astrocytes express the unique protein marker, glial fibrillary acidic protein (GFAP); however, expression levels in astrocytes are different in different regions. For example, under normal conditions in mice, protoplasmic astrocytes in the cortex express much lower levels of GFAP than protoplasmic astrocytes in the hippocampus (**Figure 1A**); however, the densities of astrocytes in these two regions are similar (**Figure 1B & C**). The heterogeneity of astrocytes in different regions might indicate different properties and functions of astrocytes (see the 'Spontaneous astrocytic Ca^{2+} signaling *in vivo*' section for the discussion of astrocytic Ca^{2+} signaling in the cortex and hippocampus).

Ca^{2+} signaling pathway in astrocytes

The discovery that astrocytes could mediate Ca^{2+} signaling (i.e., transient Ca^{2+} increase) suggests that they can play more active roles in the CNS than previously delineated [7]. Astrocytes express a variety of G-protein coupled receptors (GPCRs), for example, GABA, ATP, serotonin, norepinephrine and dopamine can all mediate astrocytic Ca^{2+} signaling and waves *in vivo* by the activation of metabotropic glutamate receptors (mGluRs) [8–10], P2Y receptors [10–14], GABA_B receptors (GABA_BRs) [11,15], noradrenergic receptors [16] and dopamine receptors [17,18]. mGluRs have been classified into three groups [19–21]. Group I includes mGluR1 and mGluR5, which are coupled to the phospholipase C/inositol 1,4,5-trisphosphate (IP_3) pathway to mobilize Ca^{2+} from internal stores. Group II (mGluR2 and mGluR3) and III (mGluR4–8) are negatively coupled to adenylyl cyclase. Cortical and hippocampal astrocytes predominantly express mGluR5 and mGluR3 [22]. GABA_BRs were also reported to be expressed in the cortical and hippocampal astrocytes [15,23–25]. GABA_BRs can mediate Ca^{2+} elevations in astrocytes in response to interneuron activation in brain slices [15,26]. Similarly, the GABA_BR agonist, baclofen, can stimulate Ca^{2+} elevations in astrocytes in brain slices [15] and *in vivo* [11]. Although the mechanism has not been fully studied, it is likely that GABA_BR-stimulated Ca^{2+} signaling in astrocytes is mediated through the release from an internal Ca^{2+} store [27]. ATP has been extensively used to stimulate Ca^{2+} release *in vivo* through P2Y receptors [8,10–14].

Following GPCR stimulation, astrocytes generally mediate Ca^{2+} signaling through coupling with Gq/11 to activate phospholipase C to liberate IP_3 that activates IP_3 receptors (IP_3Rs) to release Ca^{2+} from the internal store (i.e., phospholipase C/ IP_3R pathway) (**Figure 2A**) [1,3,28]. Among the three types of IP_3R ($\text{IP}_3\text{R1}$ –3), $\text{IP}_3\text{R2}$ is primarily expressed and is the predominant IP_3R in astrocytes in the rodent brain [29–31]. $\text{IP}_3\text{R2}$ knockout (KO) mice do not exhibit GPCR agonist-evoked Ca^{2+} increase in astrocytes in brain slice preparations nor *in vivo* [14,28], thus providing compelling evidence that $\text{IP}_3\text{R2}$ is a key mediator of intracellular Ca^{2+} release in astrocytes. Other proteins that affect Ca^{2+} homeostasis in astrocytes include $\text{Na}^+/\text{Ca}^{2+}$ exchanger [32], plasma membrane Ca^{2+} ATPase [32], store operated channels [33], P2X purinoceptors [34], transient receptor potential A [35] and C channels [33,36].

Astrocyte & tripartite synapse

Studies with cultured astrocytes [37], as well as with brain slice preparations [8,9,38,39], have linked the increase in astrocytic Ca^{2+} to the release of chemical transmitters. Thus, Ca^{2+} signaling in astrocytes serves as a mediator of bidirectional interactions between neurons and astrocytes, which are the integral part of a tripartite synapse. A tripartite synapse is composed of the presynaptic nerve terminal, the postsynaptic nerve terminal and astrocytic processes (**Figure 2B**) [3]. Thus, Ca^{2+} signaling is now considered to be a primary form of cellular excitability in astrocytes that can be determined by fluorescent imaging using different Ca^{2+} indicators. The process of Ca^{2+} -dependent release of chemical transmitters is called gliotransmission, which is predominantly mediated by mGluRs [9,38,40,41]. Glutamate [8,9,38,39,42,43], ATP [44–46] and D-serine [47–51] can all be released from astrocytes. Thus astrocytes can send signal back to neurons through Ca^{2+} -dependent gliotransmission to modulate neuronal activity characterized as slow inward currents [8,9,38,39] and synaptic plasticity in brain slice and *in vivo* [45,51–53].

In vivo astrocytic Ca^{2+} signaling in health & brain disorders

In the past decade, the application of two-photon (2-P) laser-scanning fluorescent microscopy in *in vivo* imaging opened a door for us to understand the behavior of astrocytic Ca^{2+} signaling in live animals. Recently studies revealed that astrocytes exhibit enhanced Ca^{2+} signaling in several brain disorders, ranging from precipitating invasive brain injury, such as stroke, status epilepsy and traumatic brain injury (TBI), to neurodegenerative diseases such as Alzheimer disease (AD). This part will first introduce the methods for *in vivo* 2-P imaging, and then will summarize the current studies on *in vivo* Ca^{2+} signaling in astrocytes in health and brain disorders. Data from the literature regarding Ca^{2+} signaling in cultured astrocytes will not be discussed as the morphology and gene profiles of cultured astrocytes do not resemble those of *in vivo* astrocytes.

Methods for *in vivo* astrocytic Ca^{2+} imaging using 2-P microscopy

Although astrocytic Ca^{2+} signaling has been extensively studied in cultured astrocytes, the development of 2-P fluorescent microscopy and astrocyte-specific dyes make it possible to study the Ca^{2+} signaling in astrocytes in live animals [54–58]. On the other hand, astrocyte-expressing green fluorescent protein and E-green fluorescent protein mice provide tools for the structural study of astrocytes *in vivo* [59–61]. Owing to the use of the long wavelength infrared femto-second pulse laser as an excitation light source, a distinct advantage of 2-P fluorescent microscopy is the reduction of laser scattering in brain tissue; therefore, astrocytes or neurons in the deep region of the brain can be imaged. The principle of 2-P excitation has been described in details elsewhere [56,62]. A concise introduction of the surgery procedures and dye loading in the mouse brain for Ca^{2+} imaging is given in this section. For more information, readers can consult detailed reviews by Ding and Tian *et al.* [63,64]. Briefly, a cranial window in the cortex must be prepared for dye loading. Mice can be anesthetized using urethane, ketamine/xylazine or isoflurane, held in a custom-made immobilization device. A circular cranial window (2.0 mm diameter) is drilled in the skull overlying the cortical region of interest for dye loading (**Figure 3A**). A metal frame for attaching the skull to the microscope platform needs to be glued to the skull using

cyanoacrylate, and the dura should be carefully removed. Organic acetoxymethyl ester form of Ca^{2+} indicators, such as fluo-4, Oregon Green® BAPTA-1 (Invitrogen, CA, USA), Rhod-2 or x-Rhod-1 is mixed with pluronic (20% pluronic plus 80% dimethyl sulfoxide) and artificial cerebrospinal fluid (ACSF). The mixture is applied to the surface of the cortex through the cranial window. After approximately 60 min, excess dye is removed by irrigation with ACSF. This protocol leads to the selective labeling of astrocytes with the Ca^{2+} indicator [8,11,13,55,63]. The selectivity of labeling can be confirmed by labeling of astrocytes with sulforhodamine 101 (SR101), an astrocyte selective dye [57]. To label astrocytes with SR101, 100 μl of 100 μM SR101 dissolved in ACSF can be applied on the surface of cranial window for 1–5 min before being washed away with ACSF. After 45–60 min, astrocytes can be brightly and selectively labeled with SR101 (**Figure 3B & C**). To reduce movement artifacts, a glass cover slip is glued over the cranial window on the metal frame, and the gap between glass and cranial window is filled with 2% agarose premelted in ACSF solution. Using two wide-field photonmultiplier detectors, we confirmed the colabeling of astrocytes with fluo-4 and SR101. In addition to surface labeling, pressure injection of acetoxymethyl dye can label both neurons and astrocytes in the brain (**Figure 3C**) [8,58].

The animals labeled by organic Ca^{2+} dye can only be imaged once. Recently developed genetically encoded Ca^{2+} indicators (GECIs) including fluorescence resonance energy transfer-based GECIs, such as YC3.6, and single-fluorophore GECIs, such as GCaMP3, provide an alternative way to label astrocytes *in vivo*. The GECIs can be expressed in astrocytes using viral transduction, *in utero* electroporation (IUE) or transgenic mice [65–68]. Astrocyte-specific labeling of GECIs can be achieved using GFAP promoter in the viral vectors (see ‘Molecular genetic approaches to selectively express transgenes to modulate GPCRs-mediated Ca^{2+} signaling pathway in astrocytes’ for details) [69,70]. Transgenic mice expressing floxed GCaMP3 can cross with Cre mice with specific promoters for neuronal and astrocytic Ca^{2+} imaging [66]. For astrocytic-specific expression of GCaMP3, GFAP-Cre mice are commercially available. These mice can be used to generate different disease models or cross with other transgenic disease mice to directly image astrocytic Ca^{2+} signaling under normal and pathological conditions. Since GECIs can be expressed in astrocytes for prolonged times, it is feasible for long-term and repeated *in vivo* Ca^{2+} imaging in astrocytes to track the change of Ca^{2+} signaling over time during the course of disease progress in animal models.

***In vivo* astrocytic Ca^{2+} signaling in health Spontaneous astrocytic Ca^{2+} signaling in vivo**

Spontaneous Ca^{2+} signaling of astrocytes in the cortex and hippocampus in rodents has been observed *in vivo* using 2-P microscopy. By carefully examining data from published studies, it was found that the properties of spontaneous astrocytic Ca^{2+} signaling were affected when different preparations were used. The properties of spontaneous astrocytic Ca^{2+} signaling include the frequency, duration and amplitude of spontaneous Ca^{2+} signals and the degree of synchrony among the Ca^{2+} signals. It should be noted that the amplitudes are difficult to compare among the studies owing to the use of different Ca^{2+} indicators, which have different dynamic ranges and sensitivities to Ca^{2+} concentration. These properties are also affected by animal species (e.g., mice, rats or other rodents), animal ages (e.g., young or

adult animals), locations of astrocytes (e.g., in the cortex, hippocampus or other regions), anesthesia (e.g., isoflurane, ketamine/xylazine or urethane) and wakefulness of animals (i.e., anesthetized or awake animals).

The author and others found that the cortical astrocytes in adult mice (more than 2 months old) usually exhibit low-frequency spontaneous Ca^{2+} oscillations in the cell body and processes when anesthetized with urethane [8,11] and ketamine/xylazine [13] and isoflurane (**Figure 4A & B**) [12]. The duration of the Ca^{2+} signal in the cell body and processes is short (approximately between 10 and 25 s). Importantly, Ca^{2+} signals in the cell bodies of different astrocytes and in the different processes are independent. In summary, spontaneous astrocytic Ca^{2+} signaling in anesthetized adult mice do not exhibit intercellular wave-like behavior, and Ca^{2+} signals in the processes are limited within micro-domains of the processes (**Figure 4C**). Antagonists of metabotropic receptors for glutamate, ATP and acetylcholine, as well as suppression of action potentials by tetrodotoxin (TTX) did not have a significant effect on the spontaneous somatic Ca^{2+} activity in cortical astrocytes [71], suggesting that spontaneous astrocytic Ca^{2+} transients occur intrinsically and are not driven by neural activity.

In young rats (postnatal day 12–42), anesthetized with urethane and ketamine/xylazine, cortical astrocytes exhibit Ca^{2+} signaling with relative high frequency but with similar duration as compared with, cortical astrocytes in mice [55,57,71]. Interestingly, Takata *et al.* found that astrocytes in cortical layer 1 exhibited distinct Ca^{2+} dynamics *in vivo* from astrocytes in cortical layer 2/3 in rats anaesthetized with urethane [71]. Astrocytes in layer 1 showed nearly double the Ca^{2+} activity of astrocytes in layer 2/3. Furthermore, Ca^{2+} fluctuations of processes within an astrocyte were independent in layer 1, while those in layer 2/3 were more synchronous [71]. On the other hand, in urethane anesthetized young mice (postnatal day 9–25), hippocampal astrocytes in the network exhibited synchronized Ca^{2+} oscillations and intercellular waves [72,73]. Almost all astrocytes participated in regenerative waves that propagated from cell to cell referred to as ‘glissandi’, which are dependent on neuronal activity (TTX sensitive) and accompanied by a reduction in infraslow (i.e., low-frequency), as well as high-frequency fluctuations of local field potentials and blood flow [72]. Kuga *et al.* claimed that glissandi were also observed in the neocortex, albeit, with lower frequency [72]. Such a difference in Ca^{2+} activities from the cortical astrocytes in mice might reflect the functional heterogeneity between the cortex and the hippocampus, which are possibly resulted from the different neuronal activities, and micro environment and/or different properties of astrocytes *per se*. The fact that GFAP levels are higher in hippocampal astrocytes than in cortical astrocytes provides evidence for the latter reason (**Figure 1A**). On the other hand, since the mice used in their study were very young (postnatal day 9–25), whether astrocytes in adult mice exhibit similar or the same behavior of spontaneous Ca^{2+} signaling must still be determined. A study from brain slice imaging also indicated that astrocytes in the neocortex and hippocampus have different properties of intercellular Ca^{2+} waves [74]. Ca^{2+} waves in cortical astrocytes are dependent on gap junctions, while Ca^{2+} waves in hippocampal astrocytes are dependent on purinergic signaling.

More recently, 2-P microscopy was used to image neuronal and astrocytic Ca^{2+} signaling in awake mice [12,75,76]. Cortical astrocytes exhibit much higher frequency of spontaneous Ca^{2+} signaling in the cell body and processes in awake mice than in mice anesthetized by isoflurane, ketamine and urethane [12]. In addition, somatic Ca^{2+} signals in astrocytes become highly correlated in awake mice. These Ca^{2+} signals are $\text{IP}_3\text{R2}$ dependent, but resistant to a local blockade of glutamatergic or purinergic signaling. It appears that synchronized cortical Ca^{2+} signaling is a sensitive hallmark of the wakefulness of an animal. Similarly, spontaneous Ca^{2+} bursts were also observed in cerebellar Bergmann glial (BG) cells in resting awake mice [75]. Bursts appeared as radially expanding waves involving up to approximately 40 BG cells. Further study showed that bursts were not eliminated by TTX or isoflurane anesthesia, but were virtually eliminated by pyridoxal phosphate-6-azophenyl-2', 4'-disulfonic acid, suggesting that the phenomena might involve ATP-triggered release of Ca^{2+} from intracellular stores.

In summary, the properties of spontaneous Ca^{2+} signaling in astrocytes could be affected by age, animal species, anesthesia and the location of astrocytes in the brain. It should be pointed out that the 2-P laser can stimulate Ca^{2+} increase in astrocytes [13,72]. During the imaging experiment, the laser power should be set high enough to obtain sufficient fluorescence but low enough to avoid such laser-induced artificial fluorescent signals presumably resulting from phototoxicity. In addition to these factors, subtle difference in imaging protocol (e.g., surgery and temperature, among others) might also affect the properties of Ca^{2+} signaling.

Evoked astrocytic Ca^{2+} signaling in vivo

Astrocytic Ca^{2+} signals can be evoked by a variety of approaches including direct GPCR activation by agonists, sensory stimulations, mechanical stimulations, and photolysis of caged compounds such as caged glutamate and IP_3 [8,10,13,77,78]. Pharmacological approaches provide a tool to study the function and mechanisms underlying the Ca^{2+} activity in astrocytes *in vivo*. ATP is a powerful agonist that can stimulate regenerative intercellular Ca^{2+} waves in a large number of astrocytes [10–14]. Regenerative intercellular Ca^{2+} waves with high frequency can be elicited if ATP is continuously present in the cortex (**Figure 4D & E**) [8,11,63], while (RS)-2-chloro-5-hydroxyphenylglycine and (RS)3,5-dihydroxyphenylglycine (DHPG) stimulate intercellular Ca^{2+} waves with low frequency [8]. The basic feature of an intercellular Ca^{2+} wave is the highly synchronized Ca^{2+} increase in the cell bodies in the astrocytic network, which propagates to the processes (**Figure 4F**). A recent study suggested that mGluR5 is developmentally regulated with lower expression levels in adult mice than in neonatal mice [10]; therefore, DHPG stimulation induces low frequency and small magnitude Ca^{2+} increases in adult mice [8,10].

Sensory stimulations, including whisker deflection [13], locomotion [75,76], limb stimulation [16,79,80], light illumination of the visual cortex [52,81] and odor stimulation of olfactory glomeruli [82], can also induce intercellular Ca^{2+} waves in astrocytes *in vivo* in anesthetized animals. The odor-evoked Ca^{2+} increase in astrocytes is correlated with an increase in functional hyperemia and involves mGluR5 [82]. Ca^{2+} increase in astrocytes is correlated with the strength of whisker stimulation [13], locomotor behavior and neuronal

activity [75,80], and is mediated by mGluR5 stimulation as the magnitude of Ca^{2+} increase is largely diminished by TTX and by group I mGluRs antagonists 2-methyl-6-(phenylethynyl)pyridine (MPEP) and LY367385 [13].

Ca^{2+} signals of BG cells were associated with motor behavior and neuronal activity in awake and behaving mice [75]. Interestingly, BG cells exhibit three different forms of Ca^{2+} excitation: flares, bursts and sparkles. The specific animal behaviors dictate Ca^{2+} excitability. Bursts and sparkles are ongoing in awake mice at rest, whereas flares are initiated during locomotor behavior. Locomotor performance initiates synchronized Ca^{2+} signals arising in networks of BG cells extending across several hundred microns or more. Ca^{2+} signals could be abolished by anesthesia or be blockaded by either neural activity or glutamatergic transmission. The macroscopic flare events are correlated with blood perfusion levels, suggesting that they are sufficient to potentially regulate blood flow.

Impact of astrocytic Ca^{2+} signaling on neuronal function & plasticity in vivo

One of the fundamental questions is whether and how astrocytic Ca^{2+} signaling affects neuronal function and plasticity *in vivo*. Takata *et al.* reported that combined stimulation of mouse whisker and the nucleus basalis of Meynert, the principle source of cholinergic innervation to the cortex, leads to enhanced whisker-evoked local field potential [51]. This plasticity is dependent on Ca^{2+} increase in astrocytes and Ca^{2+} dependent D-serine release from astrocytes as $\text{IP}_3\text{R2}$ KO mice abolish the plasticity. A similar study was performed in visual cortex; paired electrical stimulation of the nucleus basalis and visual stimulation induced significant potentiation of visual responses in excitatory neurons of the primary visual cortex in mice [52]. Using *in vivo* 2-P Ca^{2+} imaging, *ex vivo* Ca^{2+} imaging and whole-cell recordings they further show that this pairing-induced potentiation is mediated by direct cholinergic activation of primary visual cortex astrocytes via muscarinic acetylcholine receptors. The potentiation is absent in conditional $\text{IP}_3\text{R2}$ KO mice. By removing the cortical tissue, astrocyte-dependent plasticity was studied in hippocampal CA3–CA1 synapses *in vivo* [53]. Results show that cholinergic-induced long-term potentiation requires astrocyte Ca^{2+} elevation and Ca^{2+} -dependent glutamate release that acts on mGluR5 in astrocytes. Thus, in addition to a wealth of information from *in vitro* studies, astrocytes are actively involved in synaptic plasticity through Ca^{2+} signaling in *in vivo* setting. However, in brain slice preparations, studies using $\text{IP}_3\text{R2}$ KO mice did not draw any positive conclusions that astrocytic Ca^{2+} signaling modulates short- and long-term plasticity in the hippocampus through gliotransmission [28,83,84]. The discrepancy might be due to differences in the properties of brain regions and preparations or in the types of plasticity studied.

Modulation of astrocytic Ca^{2+} signaling on cerebral blood flow in vivo

Another important effect of astrocytic Ca^{2+} is the modulation of cerebral blood flow. In brain slice preparations, Ca^{2+} elevation in astrocytes by neuronal afferent stimulation and photolysis of caged Ca^{2+} induced vasodilation [50,85], while other studies suggested that the polarity of astrocytic Ca^{2+} -dependent regulation of blood flow was dictated by tissue metabolism of brain and retina slices [86,87]. These studies suggest complex mechanisms of blood flow regulation by astrocytic Ca^{2+} signals [50,85–88]. In *in vivo* studies, photolysis of

caged Ca^{2+} induced rapid dilation of the artery in the cortex [77]. Motor behavior induced astrocytic Ca^{2+} flares, which were correlated with blood flow increase, were attenuated by TTX [75]. Odor stimulation induced Ca^{2+} transients in astrocyte endfeet and an associated dilation of upstream arterioles. Ca^{2+} elevations in astrocytes and functional hyperemia depended on mGluR5 in astrocytes and COX activation [82]. Electrical stimulation of the fore-paw induced vasodilation and astrocytic Ca^{2+} increase in the forepaw region of the primary somatosensory cortex in isoflurane-anesthetized mice [14]. However, the stimulus-induced vaso-dilation is independent of IP_3R -mediated Ca^{2+} increase since $\text{IP}_3\text{R2}$ KO mice exhibit normal functional hyperemia. In addition, it was observed that the onset of vasodilation precedes astrocytic Ca^{2+} increase. The results suggest that functional hyperemia induced by forepaw stimulation is mainly neuronal dependent, and astrocyte Ca^{2+} increase may not be sufficient to exert an additional effect on the onset of vasodilation. Similarly, inhibition of group I mGluRs does not affect transient hemodynamic responses following a brief whisker stimulation in rats under isoflurane anesthesia [89]. The results suggest that group I mGluRs, regardless of whether they are expressed in neurons and/or astrocytes, do not play a role in early hemodynamic responses following sensory stimulation in rats.

While aforementioned studies suggest that Ca^{2+} increase in astrocytes is correlated with vasodilation, it was reported that spreading depression induced vasoconstriction in the cortex *in vivo* was associated with Ca^{2+} waves in astrocytes and neurons, and the constriction of intracortical arterioles [90]. The vasoconstriction was correlated with fast astrocytic Ca^{2+} waves and was inhibited when Ca^{2+} increase was abolished by thapsigargin, suggesting that astrocytic Ca^{2+} waves are instrumental in mediating spreading depression induced vasoconstriction. Thus, astrocytic Ca^{2+} signals can differentially modulate blood flow *in vivo* under different physiological and pathological conditions.

***In vivo* astrocytic Ca^{2+} signaling in brain disorders**

***In vivo* astrocytic Ca^{2+} signaling in cerebral stroke & hypoxia**—Stroke is the third leading cause of human death and has a major impact on public health. Cerebral ischemia accounts for approximately 80% of all human strokes. It causes primary neuronal death in the ischemic core, and leads to delayed neuronal death and neuronal degeneration in the penumbra. Cerebral ischemia begins with the mechanical occlusion of blood vessels by thrombus or embolus that cuts off blood flow. The degree to which blood flow is reduced directly determines the damage to the brain. Owing to the loss of glucose and oxygen, synthesis of ATP through glycolysis and oxidative phosphorylation is impaired in the ischemic region. Thus, ischemia results in energy depletion, which leads to membrane potential depolarization and consequently induces the release of glutamate and other neurotransmitters from presynaptic terminals into extracellular space. The energy depletion further contributes to the malfunction of astrocytic and neuronal glutamate transporters, which are critical in clearing the glutamate released into the synaptic cleft. A variety of potential mechanisms that lead to neuronal death and brain damage following ischemia have been proposed from experimental studies of stroke. These mechanisms include glutamate and Ca^{2+} toxicity, oxidative stress, acidosis, inflammation and mitochondrial dysfunction. Glutamate excitotoxicity, largely resulting from influx and intracellular overloading of Ca^{2+}

through over-stimulation of glutamate receptors (especially NMDA receptors), is the primary mediator of neuronal death [91]. The excessive accumulation of glutamate in the extracellular space leads to the overstimulation of ionotropic receptors and mGluRs, especially NMDA receptors, and results in pathological overloading of intracellular Ca^{2+} in neurons after a short latency period [92,93].

In general, astrocytes are more resistant to ischemia than neurons [94] largely because they can use glycogen stores as an energy source [95–98]. After the onset of ischemia, astrocytes undergo numerous pathological processes [99–101], including rapid swelling, to which a number of factors contribute [102]. Meanwhile, glutamate transporters in astrocytes, which normally function to remove glutamate from the synaptic cleft to avoid glutamate toxicity, are impeded owing to energy depletion. This further leads to extracellular glutamate elevations and aggravates glutamate excitotoxicity through the reversal of glutamate transportation. Astrocytes are known to become reactive following ischemia, as characterized by an excessive expression of GFAP (i.e., astrogliosis) [99,103], and eventually form a glial scar around the area of the injury [104]. Although neuronal Ca^{2+} excitotoxicity is widely acknowledged [105], whether or how astrocytic Ca^{2+} is altered after ischemia has not been known until very recently. Using a photothrombosis (PT)-induced ischemia model, which has been well established in the author's laboratory [11,106–108], Ding and colleagues studied *in vivo* astrocytic Ca^{2+} signaling in urethane-anesthetized adult mice using 2-P microscopy [11]. The PT model was used as there are a few advantages. First, the surgical procedure of PT is relatively easy and non invasive. Second, when using this model, ischemia can be induced using light illumination through a microscope objective, and most importantly, 2-P *in vivo* Ca^{2+} imaging can be performed conveniently after ischemia, which is not feasible when using middle cerebral artery occlusion (MCAo) model. Third, using the PT model makes it easy to locate the ischemic core and penumbral region [11,109–112]. In the study, we found that astrocytes exhibit enhanced Ca^{2+} signaling characterized as intercellular Ca^{2+} waves starting approximately 20 min after PT (**Figure 5**). Both the amplitude and the frequency of the astrocytic Ca^{2+} signal were significantly increased as compared with relative quiescent Ca^{2+} signaling prior to ischemia. An important feature of the Ca^{2+} signals was that most of them initiated and returned to the basal level at the same time among astrocytes in the imaging field, in other words, they exhibited a high degree of synchrony (**Figure 5D**). This can be further analyzed by calculating cross correlation coefficient between the adjacent astrocytes. There is a large cross-correlation coefficient with near zero lag time between adjacent astrocytes under ischemic conditions, while there is a very small cross-correlation coefficient between astrocytes under normal conditions [11]. To determine the nature of astrocytic Ca^{2+} signals in ischemia, antagonists for GPCRs including mGluR5, GABA_B R and P2Y receptors were applied after emergence of Ca^{2+} signals. MPEP, an antagonist of mGluR5, significantly reduced Ca^{2+} signals (~62%), while CGP54626, the antagonist of GABA_B R, also significantly reduced Ca^{2+} signals (~55%). When MPEP and CGP54626 were coadministered, the astrocytic Ca^{2+} signals were further reduced. However, suramin, a nonspecific inhibitor for P2Y receptors, did not reduce the PT-induced Ca^{2+} signal. Pyridoxal phosphate-6-azophenyl-2', 4'-disulfonic acid, a general P2 receptor antagonist, did not attenuate the Ca^{2+} signal either. Similarly, inhibition of adenosine receptor A1 by its

antagonist, 8-cyclopentyltheophylline, did not inhibit PT-induced astrocytic Ca^{2+} signals. The pharmacological study suggests that glutamate and GABA are likely to be released into the extracellular space following PT to stimulate intercellular waves among astrocytes. It is surprising that neither the P2Y nor the A1 receptor contributes to the enhanced Ca^{2+} signaling after PT, presumably owing to the rapid degradation of ATP and adenosine by enzymes such as ectonucleotidases after ischemia [113].

In addition to the acute changes in astrocytic Ca^{2+} signaling, the responses of astrocytes are also altered in the chronic phase after ischemia. The functional recovery starts 2 weeks after ischemia in both ipsilateral and contralateral hemispheres. Winship *et al.* studied the neuronal and astrocytic Ca^{2+} signaling and functional rewiring in somatosensory neurons in the ipsi lateral hemisphere after stroke [111]. Astrocytic Ca^{2+} responses were examined in anesthetized mice to study whether the prevalence of these responses was altered in peri-infarct cortex 0.5, 1 and 2 months after PT. Increased astrocyte responses to both preferred and nonpreferred limbs after stroke were observed. Univariate analysis revealed that there was a significant increase in the prevalence of responses to preferred limb stimulation in the primary somatosensory cortex. The selectivity of astrocyte responses between regions of overlap between contralateral hindlimb and contralateral forelimb, evoked intrinsic optical signals and regions without intrinsic optical signals overlap were also significantly different. These results suggest that mechanisms for rapid neuron–astrocyte communication are preserved, or even enhanced, after stroke.

To study the role of astrocytes in the functional recovery in the contralateral hemisphere, Takatsuru *et al.* performed *in vivo* Ca^{2+} imaging to examine the neuronal and astrocytic Ca^{2+} responses in the region contralateral to the site of stroke at different times following PT [114]. The results showed the number of astrocytes with a response to limb stimulation was small in the sham group, but in the stroke group the number was significantly increased in the contralateral somatosensory cortex responding to ipsilateral limb stimulation at the first and second week after infarction. A significantly larger number of astrocytes responded only to the single-limb stimulation in the sham group compared with stroke groups, but a smaller number of astrocytes responded to multiple-limb stimulation in the sham group compared with the stroke groups. Interestingly, unlike neurons, astrocytes showed no preference in response to contralateral or ipsi-lateral limb stimulation [114]. The peak amplitude of Ca^{2+} response was also increased in the stroke groups. *In vivo* microdialysis showed a large increase in glutamate concentration 2 weeks after the stroke compared with the sham and 1-week groups; however, glutamine concentration was much higher in the contralateral side in the 1-week group than in the sham and 2-week groups. Further study suggested that astrocytic glutamate transporter, GLT-1, may contribute to the uptake of glutamate in 1 week. Together, these findings demonstrate that activated astrocytes increase the uptake of glutamate by glutamate transporters during the first week, and indicate that astrocytes may play an important role in functional recovery and cortical remodeling in the area contralateral to ischemic lesion in the postischemic period.

Hypoxia is a pathological condition in which the body or a region of the body has a reduction in oxygen supply to a level insufficient to maintain cellular function [115]. The brain is the most hypoxia-sensitive organ owing to its need for high oxygen supply. Hypoxia

does not cause severe brain injury provided the system circulation is preserved, but it can cause malfunction of neurons and astrocytes. BG cells are a special type of astrocytes in the cerebellum and they display radially transglial Ca^{2+} waves, which are mediated by P2Y receptors [116]. A recent study shows that ATP-induced Ca^{2+} increase is correlated with the reduction of local tissue O_2 tension in the cerebellum [117]. Furthermore, the incidence and frequency of spontaneous Ca^{2+} waves in BG cells were significantly higher in aging mice than those in adult mice. The aging mice had lower basal O_2 tension than adult mice, but they also had comparable blood oxygen saturation. This suggests that aging-increased incidence and frequency of Ca^{2+} wave are correlated with decreased tissue O_2 tension. They further studied whether the increase in frequency of Ca^{2+} waves could be mimicked by hypoxia. Indeed, acute hypoxia of the reduction of O_2 tension by 20% for as short as 20 min could increase spontaneous Ca^{2+} waves in BG. The study suggests that the cortical O_2 tension may play a role in inducing glial Ca^{2+} waves in aging mice, and ATP levels in brain tissue might be higher in the aging brain. Whether the increased Ca^{2+} waves in BG are beneficial or detrimental to neurons is unclear and the mechanisms of hypoxia-induced increase in Ca^{2+} signal in BG cells need to be further studied.

All the aforementioned studies on astrocytic Ca^{2+} signaling in ischemia used the PT model. Therefore, the infarction was much smaller than that from results using MCAo model. Given the different severities of brain injury, it is likely that the responses of astrocytes and neurons to ischemic insult will differ between the two models. Due to the complicated surgical procedure, it is difficult to perform *in vivo* imaging on mice using MCAo model to study astrocyte and neuronal Ca^{2+} signaling before, during and after ischemia. Nevertheless, it is still feasible to study neuronal structural changes owing to its low time resolution [118].

The nature of increased Ca^{2+} waves in ischemia and hypoxia might be different. Ischemia can cause immediate cell death in the brain, and the release of a large amount of glutamate or GABA; hence, mGluR5 and GABA_BR can play a major role in astrocytic Ca^{2+} signaling after ischemia. Compared with ischemia, hypoxia does not cause immediate neuronal death. The study from BG cells suggests that hypoxia can release ATP to induce Ca^{2+} hyperexcitability in astrocytes. On the other hand, glial Ca^{2+} can cause a reduction of O_2 tension in the brain, which means that the glial Ca^{2+} waves are closely related to brain energy metabolism in hypoxia. In summary, elucidation of the mechanisms of Ca^{2+} signaling in astrocytes may provide insights for potential therapeutic targets in stroke.

In vivo astrocytic Ca^{2+} signaling in epilepsy

Epilepsy is a common chronic neurological disorder characterized by recurrent seizures. The exact cause of seizure cannot be identified, but factors that are associated with epileptic seizure include brain trauma, strokes and brain tumor [119]. It is well known that seizures occur when a large population of neurons are overexcited. Status epilepticus (SE) is a condition, in which prolonged seizures develop into nonstop seizures. It is a medical emergency that needs immediate attention. SE is another precipitating brain injury that stimulates neuronal death lasting for days after the episode, the production of new neurons, axon collateral sprouting, synaptogenesis and reactive astrogliosis [120–129]. SE eventually leads to the delayed death of neurons, cognitive decline and the generation of the epileptic

brain in which spontaneous recurrent seizure is developed [130]. Astrocytes exhibit a variety of alterations in epilepsy. Astrocytes become reactive in hippocampus after temporal lobe epilepsy in human and animal models [131,132]. The expression of inwardly rectifying K^+ channels in astrocytes is reduced in epileptic hippocampus, thus causing impaired K^+ buffering and enhanced seizure susceptibility [133,134]. Another important change after epilepsy is the reduced expression levels of glutamine synthetase. Glutamine synthetase is exclusively expressed in astrocytes and converts glutamate to glutamine [135]. Glutamine is released from astrocytes and then uptaken by neurons to synthesize glutamate and GABA, the process is the so-called glutamate–glutamine cycle. The downregulation of glutamine synthetase activity may contribute to elevated extracellular glutamate levels at seizure foci in humans [136]. Astrocytes become reactive to generate gliosis and their K^+ buffering capacity is diminished around seizure focus. The expression of many other important molecules and receptors are either down- or up-regulated [136,137]. However, the functional alterations of astrocytes during the latent period between the SE and the spontaneous seizures are poorly understood *in vivo*.

In brain slice model, chemically-induced epileptiform activity evokes intense neuronal discharges, as well as stimulating Ca^{2+} oscillations and waves in the astrocytic network [78,138]. The increase in astrocytic Ca^{2+} hyperexcitability outlasts the period of epileptiform activity [9]. Whether and how the excitability of the astrocytes is altered *in vivo* after SE is less studied. Using pilocarpine-induced SE model and 2-P *in vivo* imaging, Ding and colleagues studied whether astrocytes exhibited enhanced Ca^{2+} excitability and whether the Ca^{2+} excitability resulted from the stimulation of mGluR5 after SE [8]. This hypothesis was directly tested by using 2-P imaging of astrocytic Ca^{2+} to ask whether seizures *per se* persistently modify the excitability of astrocytes. Layer 2/3 astrocytes, selectively loaded with the Ca^{2+} indicator fluo-4 in the barrel cortex, were imaged either prior to or 2 or more days after pilocarpine-induced SE. Astrocytes in normal mice have a very low frequency of Ca^{2+} oscillation. A significant increase in astrocytic Ca^{2+} oscillation was observed 3 days following SE in mice that had entered SE compared with the control mice which either received a saline injection or subthreshold injection of pilocarpine (**Figure 6A & B**). This prolonged enhancement of Ca^{2+} excitability was detected at day 2 and 3 following SE, then returned to control levels thereafter [8].

To further investigate the molecular nature of Ca^{2+} signaling in astrocytes *in vivo* after SE, we used the glutamate antagonist MPEP [8] since mGluR5 contributes to astrocytic Ca^{2+} oscillations in brain slices and *in vivo* [8,10,13,38,40,43,85]. Under normal conditions, enhanced Ca^{2+} signaling within astrocytes was induced when mGluR5 agonists (either DHPG [25 μ M], a class I mGluR agonist or (RS)-2-chloro-5-hydroxyphenylglycine [1 mM], a selective mGluR5 agonist) were applied to the cortex of control mice. This provided evidence that mGluR5 has the capability of stimulating astrocytic Ca^{2+} oscillations *in vivo* [8]. Ca^{2+} oscillations, which initiate within an astrocytic process, propagate through the cell and couple to adjacent astrocytes in the form of a Ca^{2+} wave. (RS)-2-chloro-5-hydroxyphenylglycine-induced Ca^{2+} signals are due to the selective activation of mGluR5 as they are prevented by the mGluR5 antagonist MPEP. Since activation of mGluR5 does stimulate astrocytic Ca^{2+} oscillations, experiments were performed to determine whether

mGluR5 is responsible for the enhanced astrocytic Ca^{2+} oscillations after SE. mGluR5 antagonist MPEP (1 mg/kg weight) injected through the tail vein, which crosses the blood–brain barrier, significantly reduced the astrocytic Ca^{2+} oscillations 3 days following SE. These results demonstrate the importance of mGluR5 for the activation of Ca^{2+} oscillations in astrocytes following SE. These results also indicate that elevated extracellular glutamate levels may last several days *in vivo*. The time course of enhanced Ca^{2+} signaling after SE provides information for therapeutic intervention. Given the nature of tripartite synapse, such an enhanced Ca^{2+} signaling may impose a long-term effect on neuronal hyperexcitability and excitotoxicity through gliotransmitter release. This was confirmed by our experiment that administration of mGluR5 antagonist MPEP can significantly reduce neuronal death in hippocampus [8]. A recent study indicates that astrocytes in adult mice express reduced levels of mGluR5 as compared with the astrocytes from neonatal mice [10]; however, the enhanced Ca^{2+} signaling in astrocytes in adult mice can be inhibited by the antagonist of mGluR5, suggesting that mGluR5 expression levels are increased following SE. This hypothesis is supported by the results that mGluR5 is upregulated in a rat model of temporal lobe epilepsy [139]. The Ca^{2+} oscillation in astrocytes may not be restricted to result from the neuronal activity, but it might have a causative link to the generation of focal ictal discharge. From brain slice model, a feedback signal, for instance Ca^{2+} -induced release of glutamate and/or D-serine, may result in recruitment of a large population of neurons to fire in a synchronized manner [140,141].

Direct evidence of the effect of astrocytic Ca^{2+} on paroxysmal depolarization shifts, abnormal prolonged depolarizations with repetitive spiking that are reflected as interictal discharges in the electroencephalogram, was presented by Tian *et al.* [78]. They found that epileptogenic agents including TTX, 4-aminopyridine, bicuculline and penicillin evoked Ca^{2+} oscillations, which precede paroxysmal depolarization shifts. Further experiments showed that photolysis of caged Ca^{2+} nitrophenyl-EGTA (NP-EGTA) in astrocytes induced Ca^{2+} increase within astrocytes and generated negative depolarization shifts in brain slice preparation, suggesting Ca^{2+} increase in astrocytes is correlated and sufficient to generate local depolarization shifts (**Figure 6C**). Microdialysis experiments found that epileptogenic agents can increase the glutamate concentration, while antiepileptic agents can reduce astrocytic Ca^{2+} increase induced by 4-aminopyridine; thus, glutamate released by astrocytes can trigger paroxysmal depolarization shifts in several models of experimental seizure. Hence, these studies from experimental models indicate that astrocytic Ca^{2+} plays a critical role in neuronal excitotoxicity in epilepsy.

In vivo astrocytic Ca^{2+} signaling in TBI

TBI is caused by mechanical force applied to the cranium and brain. Within hours after traumatic impact, irreversible acute neuronal and astrocytic damage associated with dendritic beading and spine loss, and astrocytic swelling takes place [142]. Robust responses are elicited in the injured brain including the activation of astrocytes and increased intracranial pressure due to cerebral edema in the following days [143,144]. A recent study by Choo *et al.* showed that a micromechanical impact (micro-TBI model) of exposed cortex to a depth of 150–200 μm , immediately elicits Ca^{2+} waves in astrocytes *in vivo* [145]. The Ca^{2+} increase can be inhibited by apyrase that hydrolyzes ATP to AMP and inorganic

phosphate, but not by flufenamic acid, a gap junction inhibitor. The results demonstrated that mechanical impact to astrocytes *in vivo* triggers ATP-mediated Ca^{2+} waves, which propagate beyond the initial epicentre of mechanical trauma. Combined with an *in vitro* model of neurotrauma, it was found that mechanical stretch triggers inter cellular Ca^{2+} waves and ATP release in cultured astrocytes. Furthermore, the P2Y1 receptor antagonist, MRS2179, could reduce neuronal loss in the CA3 subregion in the hippocampus and ameliorate cognitive deficit after cortical brain impact in mice [145]. This study shows that mechanically triggered signaling through astrocytes significantly contributes to the pathology of TBI through gliotransmission. In contrast to the results of this study, Watts *et al.* showed that P2Y1 receptor stimulation using its agonist 2MeSADP significantly reduced cerebral edema and reactive astrogliosis, suggesting a neuro-protective effect of P2Y1 agonism [143]. To support the role of astrocytic Ca^{2+} in neuroprotection, they used $\text{IP}_3\text{R}2$ KO mice (in which $\text{IP}_3\text{R}2$ has been knocked out selectively from the astrocytes) and found that 2MeSADP did not affect cerebral edema nor reactive astrogliosis. Their hypothesis is that P2Y1R stimulation can increase intracellular ATP levels, which in turn allows cells to better maintain ion homeostasis. The discrepancy between the two studies might be due to the severity of brain injury in the models they used.

In vivo astrocytic Ca^{2+} signaling in AD

AD is a fatal neurodegenerative disease associated with neuronal dysfunction, synaptic loss, and progressive cognitive decline. Abnormal cerebral accumulation of extracellular amyloid- β ($\text{A}\beta$) and the formation of senile $\text{A}\beta$ plaques are the hallmark of AD (**Figure 7A**). In transgenic animal AD models, it is well known that AD is associated with reactive astrogliosis, and astrocytes in the vicinity of $\text{A}\beta$ plaques become hypertrophic [146]. Studies using animal models have demonstrated that there is a Ca^{2+} overload in neurites in the cortex with $\text{A}\beta$ plaque, thus altering Ca^{2+} homeostasis in neurons [147]. On the other hand, time lapse *in vivo* imaging showed that 50% of neurons become either hyperactive or silent in Ca^{2+} transients in the cortex compared with wild-type (WT) mice of the same age [148]. Further analysis revealed that the hyper active neurons are usually located within 60 μm of the plaque border. In hippocampal CA1 region, there is a marked increase in the fraction of silent and hyperactive neurons in plaque-bearing mice *in vivo*; interestingly, an increase in hyperactive CA1 neurons was observed in young mice without plaque formation [149].

Astrocytes also exhibit altered Ca^{2+} signaling in *in vitro* and *in vivo* AD models. In neuronal–glial co-culture, application of $\text{A}\beta$ triggers Ca^{2+} elevation in astrocytes that is initiated from a focal rise [150]. The Ca^{2+} elevation can be maintained for a long period as long as $\text{A}\beta$ is continuously present, suggesting a possible sustained effect of astrocytes on neurons through gliotransmission. To study Ca^{2+} signaling *in vivo* in the AD mouse model, Kuchibhotla *et al.* used $\text{A}\beta$ precursor protein/presenilin mice that express mutant human $\text{A}\beta$ precursor protein and mutant presenilin in neurons, but not in astrocytes [151]. The mice had increased $\text{A}\beta$ production and plaque deposition beginning at approximately 4.5 months of age. Using multiphoton fluorescent lifetime imaging microscopy, they found the resting astrocytic Ca^{2+} in mice with plaques is higher than in WT mice; nevertheless, the resting Ca^{2+} concentration is independent of the proximity of astrocytes to the plaques. Remarkably, the frequency of spontaneous astrocytic Ca^{2+} activity in mice with cortical $\text{A}\beta$

deposition is much higher than in younger mice before developing senile plaques or WT mice. The increased spontaneous Ca^{2+} activity is also independent of the plaque proximity. Using TTX to block neuronal activity, their data also suggest that the increased Ca^{2+} activity is a direct consequence of A β interacting with the astrocytic network since astrocytes do not produce Ab. The most surprising result was that astrocytes in the mice with cortical plaques exhibit intercellular waves with synchronized Ca^{2+} oscillations (**Figure 7B & C**). Interestingly, the Ca^{2+} wave initiated from astrocytes near the senile plaque and propagated to the astrocytes distant from the plaque. The magnitude of the Ca^{2+} change during intercellular waves is higher than the spontaneous Ca^{2+} transients. The property of the waves is similar to the waves induced by ATP stimulation [11,12]. Kuchibhotla *et al.* further found that these Ca^{2+} oscillations are not mediated by neuronal activity from the study using TTX [151]. Although the exact factors remain to be determined, it is likely that senile A β plaques-induced release of proinflammatory factors from microglia or astrocytes *per se* modulate intra- and intercellular Ca^{2+} signaling events in the AD model.

In vivo astrocytic Ca^{2+} signaling in other brain disorders

Astrocytes are involved in many other neuronal disorders including Parkinson's disease, Huntington's disease (HD), fragile X syndrome (FXS), amyotrophic lateral sclerosis (ALS), and so on. However, there is no report whether astrocytes also exhibit Ca^{2+} hyperexcitability *in vivo* in the animal models of these neurodegenerative diseases.

Parkinson's disease is characterized by the extensive loss of dopamine neurons in the substantia nigra in the midbrain. Overexpression or deletion of astrocyte-specific proteins provides neuroprotection against toxicity in animal models. Ablation of S100B, a calcium-binding protein found in, and secreted by, astrocytes, protects against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced excitotoxicity [152]. Overexpression of Nrf2 in astrocytes delayed the onset and extended the life span of α -synuclein mutant mice [153]. It was reported that in brain slice preparation, astrocytes in the substantia nigra displayed spontaneous Ca^{2+} transients with 2.6% of astrocytes having synchronous activities in physiological conditions [154]. However, it is unknown whether substantia nigra astrocytes exhibit enhanced Ca^{2+} excitability *in vivo* under physiological conditions or in Parkinson's disease model since it is difficult to image this region in live animals using 2-P microscopy.

HD is another neurodegenerative disorder that is characterized by motor, cognitive and psychiatric symptoms. HD is caused by an expansion of the polyglutamine repeat (>37Q) in huntingtin, and the age of onset is inversely correlated with the length of the polyglutamine repeat [155]. The most characteristic structural feature of this disease is neurodegeneration accompanied by astrogliosis in the striatum [156]. In addition to the increased GFAP expression, astrocytes were also observed with decreased cholesterol levels [157]. Expression of poly glutamine in astrocytes causes age-dependent neurological symptoms and impairs glutamate transport [158,159], suggesting a noncell-autonomous neurotoxicity in HD. Although many studies demonstrated that astrocytes play an important role in neurodegeneration in HD, it remains to be determined whether astrocytic Ca^{2+} signaling is altered during the disease progression.

FXS is a common form of inherited mental retardation, and it typically results from the transcriptional silencing of the *FMR1* gene and loss of the encoded protein FMRP. Accumulating evidence indicates the abnormal dysfunction of astrocytes. Astrocytes affect neuronal dendrite development and synaptic function in FXS [160,161]. In *Fmr1* KO (*Fmr1*^{-/-}) mice, astrocytic glutamate transporter GLT1 and glutamate uptake are significantly reduced in the cortex [162]. In addition, the protein expression and the DHPG-dependent Ca²⁺ responses of astrocytic mGluR5 receptors are also selectively reduced in *Fmr1*^{-/-} astrocytes and brain slices, attenuating neuron-dependent GLT1 expression. These data suggest that astrocytes may exhibit reduced Ca²⁺ signaling in *Fmr1*^{-/-} mice, but neuronal excitability may be enhanced due to the reduction of GLT1.

ALS is an adult neurodegenerative disease characterized by motor neuron degeneration in the cortex, brainstem and spinal cord, eventually resulting in paralysis and death [163]. ALS is linked to the mutations in the gene encoding the antioxidant enzyme SOD1. It is known that *SOD1* is expressed in motor neurons as well as in astrocytes [164,165]. Astrocytes expressing ALS-linked mutant *SOD1* release soluble neurotoxic factors that can selectively kill motor neurons [164]. It remains unknown whether astrocytic Ca²⁺ signaling plays a role in this process, but it is feasible to perform *in vivo* imaging on astrocytes in the affected regions.

The mechanisms of astrocytic Ca²⁺ signaling in brain disorders

An earlier study from cultured cells and brain slices provided evidence that Ca²⁺ waves in astrocytes were mediated through the diffusion of IP₃ via gap junctions [166] and released extracellular ATP [44,167]. As discussed before, astrocytes in the brain usually exhibit low frequency and asynchronous Ca²⁺ signaling in the somata and processes in anesthetized adult mice under normal conditions. However, in a few experimental disease models discussed, cortical astrocytes exhibit network-wide elevation in resting Ca²⁺ and increased frequency of Ca²⁺ activity featured with synchronized intercellular Ca²⁺ waves under anesthesia. Such dramatic changes may represent the micro-environmental changes in brain tissue that stimulate astrocytes with elevated resting Ca²⁺ levels and enhanced frequency of Ca²⁺ transients. Thus, astrocytes exhibit distinct properties of Ca²⁺ signaling in health versus diseases. Although the patterns of Ca²⁺ responses in the discussed disease models are similar, the mediators of the synchronized Ca²⁺ waves might be different. In precipitating invasive brain injury such as stroke, epilepsy and TBI, the large amounts of glutamate and other released neurotransmitters could stimulate GPCR in astrocytes and induce enhanced Ca²⁺ hyperexcitability in astrocytes. This was supported by results whereby the mGluR5 antagonist was shown to inhibit Ca²⁺ excitability and reduce neuronal deaths after SE [8] and PT [11], and the P2Y1R antagonist was shown to reduce neuronal loss in CA3 subregion in the hippocampus after TBI [145]. In a less severe condition such as hypoxia, ATP released from the brain tissue may induce astrocytic Ca²⁺ waves [117]. In chronic neurodegenerative diseases, the pro inflammatory factors released from microglia and astrocytes might be potential candidates. In AD mice, Aβ plaques or plaque-induced or -associated bioactive species may induce intercellular Ca²⁺ waves in astrocytes [151]; on the other hand, the modification of the proteins that maintain Ca²⁺ homeostasis might change

the resting Ca^{2+} levels in astrocytes. Candidate proteins for this change include $\text{Na}^+/\text{Ca}^{2+}$ exchanger, P2X receptors and transient receptor potential channels [32,35,36].

Although enhanced astrocytic Ca^{2+} excitability is found in different disease models, the observation of this phenomenon can be dependent on various factors. For example, the time from disease onset and the distance from the disease core will likely affect the magnitude and frequency of Ca^{2+} increase. The enhanced intercellular Ca^{2+} waves could cause the release of vast amounts of gliotransmitter in disease; hence, Ca^{2+} -dependent gliotransmission might impose a detrimental effect on neuronal excitotoxicity, and inhibition of astrocytic Ca^{2+} signals could represent a potential target for therapeutic intervention in the treatment of brain disorders.

Molecular genetic approaches to selectively express transgenes to modulate GPCR-mediated Ca^{2+} signaling pathway in astrocytes

It is known that many of the same GPCRs are expressed in both neurons and astrocytes, therefore, the actions of their antagonists to stimulate astrocytic Ca^{2+} elevation are not astrocyte-specific. To further advance our understanding of the properties of astrocytic Ca^{2+} signaling, neuron–glia interactions, and the role of astrocytic Ca^{2+} signaling in neuronal disorders, it is necessary to develop cell-type-specific approaches that can modulate astrocytic Ca^{2+} signaling *in vivo*. Given the importance of Ca^{2+} signaling in astrocytes, molecular genetic manipulations that specifically perturb (disturb) astrocytic Ca^{2+} signaling pathway will shed light on the potential role of astrocytes in health and disease. In this article, a few approaches that are currently used to introduce and delete genes in astrocytes *in vivo* are summarized, and their advantages and disadvantages are discussed.

Viral transduction

Viral transduction has been used to transduce genes of interest, for instance, transgenes, into cells *in vitro* and *in vivo*. As some types of viruses can deliver genes to the nondividing cells including neurons and astrocytes in adult brains, this viral approach is becoming more and more widely used to introduce/delete transgenes in the nervous system [168]. Stereotaxic injection of virus provides a flexible approach for delivering genes to specific brain regions [169]. Currently, a few types of recombinant viral vectors including recombinant adeno-associated virus (rAAV), retroviruses and lentivirus are used to deliver genes into the CNS. The following represents a brief discussion of their application in transducing genes in the CNS and their advantages and disadvantages.

Recombinant adeno-associated virus—As rAAV vectors are nonpathogenic and very effective for gene delivery in nondividing cells, and can also express a transferred gene for the life of the animal, they are widely used for gene transduction in the CNS [170]. Combining a cell-type-specific promoter with specific serotype, rAAV vectors has been used increasingly to selectively transduce transgenes into specific types of cells *in vivo* [168,171–173]. Most serotypes of rAAV vectors reported have preferential neuronal tropism *in vivo*. Serotype 2/5 rAAV (rAAV2/5) could introduce reporter genes into both neurons and glial cells in the brain [171,174]. Using an astrocyte-specific promoter, rAAV2/5 can

selectively transduce genes into astrocytes [69,175]. Ding and colleagues reported that transfection of p130PH, a pleckstrin homology domain of phospholipase C-like protein 130, in cultured astrocytes can effectively inhibit ATP-stimulated Ca^{2+} elevation [69]. p130PH acts as a mobile cytosolic IP_3 'sponge' and thus can inhibit Ca^{2+} release from internal stores by reducing the concentration of free IP_3 to open IP_3R [176]. Using a novel astrocyte-specific promoter gfaABC1D, rAAV2/5 can exclusively transduce this gene in astrocytes in adult mice after cortical injection (**Figure 8**). Furthermore, *in vivo* imaging using 2-P microscopy showed that the expression of p130PH could effectively inhibit ATP-stimulated Ca^{2+} increase in astrocytes in the mouse cortex. Although rAAV vectors have aforementioned advantages, vector genomes have been limited to 5 kb in length. Thus, it is not feasible to efficiently express large protein molecule using rAAV transduction.

Retroviruses—Recombinant retroviruses were also used for gene transduction and cell labeling. Kanemaru *et al.* used retroviral vectors to introduce IP_3 5-phosphatase (5ppase) in cultured astrocytes [177]. Since 5ppase specifically hydrolyzes IP_3 , overexpression of 5ppase can effectively inhibit IP_3R -mediated Ca^{2+} signaling in astrocytes. In their study, they showed that inhibition of spontaneous Ca^{2+} signaling in astrocytes by IP_3 5ppase reduced the growth of dendrites and axons of hippocampal neurons co-cultured with infected astrocytes. As retroviruses can only transduce dividing cells, it is only suitable for introducing genes into cultured astrocytes, not in nondividing astrocytes in adult animals.

Lentivirus—Another viral approach is to use lentiviruses to introduce genes into astrocytes. Lentiviral vectors are able to produce functional vector particles with a genome size much larger than rAAV vectors (up to 16 kb [178]). Thus, it is advantageous to use lentiviruses when large protein molecules need to be expressed *in vivo*. In a study of the tropism of pseudotyped lentiviral vectors, Cannon *et al.* found that isogenic lentiviral vectors pseudotyped with envelope glycoproteins derived from lymphocytic choriomeningitis virus and Moloney murine leukemia virus exclusively transduced astrocytes in rats, while isogenic lentiviral vectors pseudotyped with vesicular stomatitis virus and Mokola virus transduced midbrain neurons [179]. Their data suggest that pseudotyped lentiviral vectors will be useful for neuronal and astrocytic-targeting gene transduction. Using cell-type-specific promoters, lentiviral vectors can transduce genes into astrocytes and neurons [180,181]. Using astrocyte glutamate transporter GLAST (EAAT₁) promoter, lentivirus pseudotyped with Mokola virus can target gene expression in astrocytes *in vivo* [181].

It should be mentioned that other viruses also infect astrocytes with high efficiency. Those viruses include adenovirus [182,183], baculovirus [184] and Semliki Forest virus [185]. The viral approach provides an effective tool to achieve gene overexpression and silencing *in vivo*. While viral transduction has advantages, such as being quick and convenient to introduce and knockdown the genes of interest, local injection itself may cause brain tissue damage and astrogliosis [69,186]. Astrogliosis after viral injection can also cause inhibitory neuronal deficit due to the downregulation of glutamine synthetase [186]. These detrimental effects might be reduced over time after viral injection as the brain can recover from injection-induced injury and inflammation. Another disadvantage of viral transduction by

local injection is that the number of infected cells is relatively small and transgene expression is usually limited to around the injection site. To increase the number of transduced cells *in vivo*, a new technology, for instance, recombinant ‘self-complementary AAV (scAAV) vectors’ that package a double-stranded genome, has emerged. Several scAAV vectors that can efficiently cross the blood–brain barrier have been identified using facial vein injection in neonatal mice, and can transduce neurons and astrocytes in CNS [187]. Intravenous administration of serotype 2/9 scAAV (scAAV2/9) can robustly transduce neurons in neonatal mice and astrocytes of entire CNS in adult mice by passing the blood–brain barrier [188,189]. Importantly, intravenous administration of scAAV does not cause gliosis [187]. By optimizing the combination of serotype and cell-type-specific promoter, scAAV vectors hold bright promise for manipulating gene expression in neurons, as well as in astrocytes, thus providing a powerful tool to study gliotransmission, neuron–glia interactions and therapeutic effect of transgenes in neurodegenerative diseases.

Genetically modified mice

The second molecular genetic approach is to generate genetically modified mice using genetic engineering techniques. KO and transgenic mice were used to delete or overexpress genes of interest *in vivo* to study the effect on astrocyte on synaptic transmission. Fiacco *et al.* utilized the Tet-Off® system (Clontech Laboratories Inc., CA, USA) to generate MrgA1-green fluorescent protein mice that specifically express the Gq-coupled receptor, MrgA1, in astrocytes [83]. MrgA1 is normally expressed in dorsal root ganglion nociceptive sensory terminals in the spinal cord where it is activated by the neuropeptide Phe–Leu–Arg–Phe amide resulting in Ca^{2+} release from internal stores. In the cortex and hippocampus of MrgA1 mice, Phe–Leu–Arg–Phe amide can selectively stimulate Ca^{2+} mobilization in a large population of astrocytes. In brain slice preparations, stimulation with Phe–Leu–Arg–Phe amide generated sustained, robust and widespread Ca^{2+} signals in astrocytes. Thus, this line of transgenic mice provides a novel tool to study astrocyte–neuronal interactions in the brain [83,190,191]. To study Ca^{2+} signaling in atrial myocytes, Li *et al.* generated type II IP_3R ($\text{IP}_3\text{R2}$) KO mice [192]. Since astrocytes predominantly express $\text{IP}_3\text{R2}$, this line of KO mouse provides another important tool to study astrocyte–neuronal interactions [12,28,51]. In a study of the effect of astrocytic Ca^{2+} signaling on synaptic plasticity, it was found that selective stimulation of astrocytic Ca^{2+} in MrgA1⁺ mice and disruption of astrocytic Ca^{2+} in $\text{IP}_3\text{R2}$ KO mice does not affect excitatory synaptic activity nor hippocampal short- and long-term plasticity in brain slice preparations [83,84]; therefore, it remains controversial whether astrocytic Ca^{2+} signaling plays a role in modulating synaptic activity and plasticity. As discussed in ‘Impact of astrocytic Ca^{2+} signaling on neuronal function and plasticity *in vivo*’, in *in vivo* studies, astrocytic Ca^{2+} is indeed involved in synaptic plasticity [51–53]. On the other hand, $\text{IP}_3\text{R2}$ KO mice were used to study the brain protection after stroke. Interestingly, opposing results were obtained from different laboratories. Using a PT model of a single vessel ischemia (i.e., a tiny infarct area), $\text{IP}_3\text{R2}$ KO mice have larger infarction than WT mice [193], while $\text{IP}_3\text{R2}$ KO mice have smaller infarction than WT after MCAo [194]. The author's own results using the astrocytic-selective Ca^{2+} chelator BAPTA acetoxymethyl and antagonists of mGluR1 and mGluR5 suggest that inhibition of astrocytic Ca^{2+} has a beneficial effect on brain protection [11,108]. These results are not necessarily

contradictory and may reflect multiple Ca^{2+} targets within the astrocytes as well as differential magnitudes of Ca^{2+} responses related to either receptor type or location.

Tet-Off inducible transgenic mice that selectively express dominant-negative SNARE in astrocytes were generated (dn-SNARE mice) to study astrocytic purinergic signaling on neuronal plasticity [45]. By releasing ATP, astrocytes suppress synaptic transmission and long-term potentiation. The study suggests that astrocytic purinergic signaling can coordinate synaptic network. SNARE-dependent gliotransmission also plays a role in modulation of sleep homeostasis, ischemia-induced brain damage and depressive-like behaviors [195–197]. Thus from the studies of dn-SNARE mice, SNARE-dependent exocytosis of gliotransmitter has a variety of impacts on neuropathology.

Although transgenic and KO mouse models are particularly useful for studies of neuronal function and plasticity, functional and developmental compensation may eliminate the phenotypic effect [198]. Thus, inducible gene deletion and knockin mice including Tet-Off and tamoxifen-inducible CreERT2/loxP systems may be better choices to avoid compensatory effect [199]. In addition, it is time consuming to generate KO and transgenic mice. In summary, transgenic mice that can manipulate astrocytic Ca^{2+} signaling pathways may provide good tools for studying astrocytic function and neuron–glia interactions in health and brain disorders.

In utero electroporation

By applying an external electrical field, electroporation can significantly increase electrical conductivity and permeability of the cell plasma membrane; thus it has been used to transfect DNA in cells. Since the first publications of IUE in 2001 [200–202], it has become an increasingly powerful tool for gain- and loss-of-function studies in embryonic cerebral cortex. IUE can efficiently transfect and manipulate cerebral cortical neural-precursor cells of the rodent parietal cortex and their progeny *in vivo* [203]. By positioning the positive and negative electrodes, IUE can direct the location of transfection. In addition, using DNA plasmid with cell-type-specific promoters, preferential expression of transgenes in different subsets of progenitor cells can be achieved. For astrocyte-specific transfection using IUE, the time of electroporation is critical owing to the temporal order of cell generation. It was reported that more astrocytes were transfected in the cortex when IUE was administered at embryonic day 18 than when IUE was administered at day 16 [203]. Astrocyte-specific expression of the transgene can also be achieved using DNA plasmid with astrocyte-specific promoters in IUE. This strategy was used to study astrocyte cortical heterogeneity [204]. GCaMP3 can be robustly expressed in cortical neurons for Ca^{2+} imaging using IUE [65]. IUE combined with an astrocyte-specific promoter should be able to express GECIs or other proteins that perturb Ca^{2+} signaling pathway in astrocytes. In comparison with other *in vivo* DNA transfer methods, IUE has a few advantages [200]: it is quick and easy; highly efficient; delivers localized and unidirectional gene transfer; and achieves transfection of multiple genes into individual cells. However, IUE also has potential limitations for transgene expression. One limitation is that the transgenes introduced by IUE are often lost from cells following successive cell divisions. The other limitation is the relative small

number of transfected cells. In combination with molecular biology, transfection reagents and physical methods, these limitations can be overcome.

Although a detailed discussion is beyond the scope of this review, it should be pointed out that the combination of IUE or viral transduction with conditional KO mice will provide a powerful tool for gene expression and deletion in astrocytes. For example, injection of GFAPCre viral vectors or electroporating GFAP-Cre plasmid into floxed GCaMP3 reporter mice [66] should produce selective GCaMP3 expression in astrocytes.

Future perspective

2-P microscopy has changed the way we study Ca^{2+} signaling in astrocytes in the last decade. It has provided an unprecedented opportunity to investigate the function of astrocytes with real-time *in vivo* imaging in living animals. Combined with other technologies including molecular biology and recently developed GECIs, there are a few future directions for *in vivo* Ca^{2+} imaging of astrocytes.

Repeated *in vivo* imaging of astrocytic Ca^{2+} signaling in live animals

Organic Ca^{2+} indicators can be labeled in astrocytes in the brain only when a cranial window is opened and, in addition, it cannot be retained in astrocytes for a prolonged time. However, it is feasible to perform this kind of experiment using GECIs which can be expressed selectively in astrocytes using molecular genetic approaches. Using membrane anchored-GECIs, which can sense local Ca^{2+} signals in the plasma membrane, Ca^{2+} signaling in microdomains can be imaged to study local synaptic functioning in the brain *in vivo*. Combined with mouse disease models, this approach can be used to monitor the real-time disease progress in animals.

Imaging astrocytic Ca^{2+} signaling in awake & behaving animals

Since anesthesia modulates population-wide cellular function and greatly reduces overall brain activity, imaging on awake and/or behaving mice will provide information about astrocytic Ca^{2+} activity that cannot be inferred from anesthetized animals.

Elucidation of the mechanisms underlying *in vivo* astrocytic Ca^{2+} signaling

Since the mediators that cause astrocytic Ca^{2+} hyperexcitability are different in different brain disorders, elucidation of the underlying mechanisms of Ca^{2+} signaling will likely provide useful information for potential therapeutic interventions.

Super-resolution imaging of astrocytic Ca^{2+} signaling in microdomains

Although 2-P microscopy can achieve a resolution of approximately 250 nm using an objective lens with high numerical aperture, it still cannot resolve the fine process of astrocytes. Application of super-resolution microscopy, such as stimulated emission depletion [205] and stochastic optical reconstruction [206] microscopes, which dramatically improve the resolving power, will provide novel insight into astrocytic Ca^{2+} signaling and function *in vivo*.

Acknowledgments

This work was supported by the NIH (R01NS069726) and the American Heart Association National Program Scientist Development Grant (0735133N) to S Ding.

Writing assistance was provided by C Roberts (www.preferredcopyediting.com), funded by NIH grant.

References

Papers of special note have been highlighted as:

■ of interest

■■ of considerable interest

1. Agulhon C, Petravic J, McMullen AB, et al. What is the role of astrocyte calcium in neurophysiology? *Neuron*. 2008; 59(6):932–946. [PubMed: 18817732]
2. Barres BA. The mystery and magic of glia: a perspective on their roles in health and disease. *Neuron*. 2008; 60(3):430–440. [PubMed: 18995817]
3. Haydon PG. Glia: listening and talking to the synapse. *Nat. Rev. Neurosci.* 2001; 2(3):185–193. [PubMed: 11256079]
4. Djukic B, Casper KB, Philpot BD, Chin LS, McCarthy KD. Conditional knock-out of kir4.1 leads to glial membrane depolarization, inhibition of potassium and glutamate uptake, and enhanced short-term synaptic potentiation. *J. Neurosci.* 2007; 27(42):11354–11365. [PubMed: 17942730]
5. Huang YH, Bergles DE, Huang YH, Bergles DE. Glutamate transporters bring competition to the synapse. *Curr. Opin. Neurobiol.* 2004; 14(3):346–352. [PubMed: 15194115]
6. Bergles DE, Diamond JS, Jahr CE, Bergles DE, Diamond JS, Jahr CE. Clearance of glutamate inside the synapse and beyond. *Curr. Opin. Neurobiol.* 1999; 9(3):293–298. [PubMed: 10395570]
7. Cornell-Bell AH, Finkbeiner SM, Cooper MS, Smith SJ. Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science*. 1990; 247(4941):470–473. [PubMed: 1967852]
- 8■■. Ding S, Fellin T, Zhu Y, et al. Enhanced astrocytic Ca^{2+} signals contribute to neuronal excitotoxicity after status epilepticus. *J. Neurosci.* 2007; 27(40):10674–10684. [PubMed: 17913901] [First paper showing enhanced astrocytic Ca^{2+} signaling in vivo and the role of mGluR5 in neuroprotection in epilepsy.]
9. Fellin T, Pascual O, Gobbo S, Pozzan T, Haydon PG, Carmignoto G. Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. *Neuron*. 2004; 43(5):729–743. [PubMed: 15339653]
10. Sun W, McConnell E, Pare JF, et al. Glutamate-dependent neuroglial calcium signaling differs between young and adult brain. *Science*. 2013; 339(6116):197–200. [PubMed: 23307741]
- 11■■. Ding S, Wang T, Cui W, et al. Photothrombosis ischemia stimulates a sustained astrocytic Ca^{2+} signaling in vivo. *Glia*. 2009; 57(7):767–776. [PubMed: 18985731] [First paper to demonstrate that astrocytes exhibit intercellular Ca^{2+} waves in vivo after ischemia.]
12. Thrane AS, Rangroo Thrane V, Zeppenfeld D, et al. General anesthesia selectively disrupts astrocyte calcium signaling in the awake mouse cortex. *Proc. Natl Acad. Sci. USA*. 2012; 109(46):18974–18979. [PubMed: 23112168]
- 13■■. Wang X, Lou N, Xu Q, et al. Astrocytic Ca^{2+} signaling evoked by sensory stimulation in vivo. *Nat. Neurosci.* 2006; 9(6):816–823. [PubMed: 16699507] [First in vivo study on astrocytic Ca^{2+} signaling evoked by sensory stimulation.]
14. Nizar K, Uhlirova H, Tian P, et al. In vivo stimulus-induced vasodilation occurs without IP3 receptor activation and may precede astrocytic calcium increase. *J. Neurosci.* 2013; 33(19):8411–8422. [PubMed: 23658179]
15. Meier SD, Kafitz KW, Rose CR, Meier SD, Kafitz KW, Rose CR. Developmental profile and mechanisms of GABA-induced calcium signaling in hippocampal astrocytes. *Glia*. 2008; 56(10):1127–1137. [PubMed: 18442094]

16. Bekar LK, He W, Nedergaard M. Locus coeruleus α -adrenergic-comediated activation of cortical astrocytes in vivo. *Cerebral Cortex*. 2008; 18(12):2789–2795. [PubMed: 18372288]
17. Haydon PG, Carmignoto G. Astrocyte control of synaptic transmission and neurovascular coupling. *Physiol. Rev.* 2006; 86(3):1009–1031. [PubMed: 16816144]
18. Ni Y, Malarkey EB, Parpura V. Vesicular release of glutamate mediates bidirectional signaling between astrocytes and neurons. *J. Neurochem.* 2007; 103(4):1273–1284. [PubMed: 17727631]
19. Pin JP, Duvoisin R. The metabotropic glutamate receptors: structure and functions. *Neuropharmacology*. 1995; 34(1):1–26. [PubMed: 7623957]
20. Schoepp DD, Jane DE, Monn JA. Pharmacological agents acting at subtypes of metabotropic glutamate receptors. *Neuropharmacology*. 1999; 38(10):1431–1476. [PubMed: 10530808]
21. Niswender CM, Conn PJ. Metabotropic glutamate receptors: physiology, pharmacology, and disease. *Annu. Rev. Pharmacol. Toxicol.* 2010; 50(1):295–322. [PubMed: 20055706]
22. Schools GP, Kimelberg HK. mGluR3 and mGluR5 are the predominant metabotropic glutamate receptor mRNAs expressed in hippocampal astrocytes acutely isolated from young rats. *J. Neurosci. Res.* 1999; 58(4):533–543. [PubMed: 10533045]
23. Nilsson M, Eriksson PS, Ronnback L, Hansson E. GABA induces Ca^{2+} transients in astrocytes. *Neuroscience*. 1993; 54(3):605–614. [PubMed: 8332251]
24. Oka M, Wada M, Wu Q, Yamamoto A, Fujita T. Functional expression of metabotropic GABAB receptors in primary cultures of astrocytes from rat cerebral cortex. *Biochem. Biophys. Res. Commun.* 2006; 341(3):874–881. [PubMed: 16455058]
25. Charles KJ, Deuchars J, Davies CH, et al. GABA B receptor subunit expression in glia. *Mol. Cell. Neurosci.* 2003; 24(1):214–223. [PubMed: 14550781]
26. Kang J, Jiang L, Goldman SA, Nedergaard M. Astrocyte-mediated potentiation of inhibitory synaptic transmission. *Nat. Neurosci.* 1998; 1(8):683–692. [PubMed: 10196584]
27. Doengi M, Hirnet D, Coulon P, Pape HC, Deitmer JW, Lohr C. GABA uptake-dependent Ca^{2+} signaling in developing olfactory bulb astrocytes. *Proc. Natl Acad. Sci. USA*. 2009; 106(41):17570–17575.
28. Petravic J, Fiacco TA, McCarthy KD. Loss of IP_3 receptor-dependent Ca^{2+} increases in hippocampal astrocytes does not affect baseline cal pyramidal neuron synaptic activity. *J. Neurosci.* 2008; 28(19):4967–4973. [PubMed: 18463250]
29. Hertle DN, Yeckel MF, Hertle DN, Yeckel MF. Distribution of inositol-1,4,5-trisphosphate receptor isotypes and ryanodine receptor isotypes during maturation of the rat hippocampus. *Neuroscience*. 2007; 150(3):625–638. [PubMed: 17981403]
30. Holtzclaw LA, Pandhit S, Bare DJ, et al. Astrocytes in adult rat brain express type 2 inositol 1,4,5-trisphosphate receptors. *Glia*. 2002; 39(1):69–84. [PubMed: 12112377]
31. Sharp AH, Nucifora FC Jr, Blondel O, et al. Differential cellular expression of isoforms of inositol 1,4,5-trisphosphate receptors in neurons and glia in brain. *J. Comp. Neurol.* 1999; 406(2):207–220. [PubMed: 10096607]
32. Reyes RC, Verkhratsky A, Parpura V. Plasmalemmal $\text{Na}^+/\text{Ca}^{2+}$ exchanger modulates Ca^{2+} -dependent exocytotic release of glutamate from rat cortical astrocytes. *ASN Neuro*. 2012; 4(1):e00075. [PubMed: 22268447]
33. Linde CI, Baryshnikov SG, Mazzocco-Spezia A, Golovina VA. Dysregulation of Ca^{2+} signaling in astrocytes from mice lacking amyloid precursor protein. *Am. J. Physiol. Cell Physiol.* 2011; 300(6):C1502–C1512. [PubMed: 21368296]
34. Illes P, Verkhratsky A, Burnstock G, Franke H. P2X receptors and their roles in astroglia in the central and peripheral nervous system. *Neuroscientist*. 2012; 18(5):422–438. [PubMed: 22013151]
35. Shigetomi E, Tong X, Kwan KY, Corey DP, Khakh BS. TRPA1 channels regulate astrocyte resting calcium and inhibitory synapse efficacy through GAT-3. *Nat. Neurosci.* 2012; 15(1):70–80. [PubMed: 22158513]
36. Shirakawa H, Sakimoto S, Nakao K, et al. Transient receptor potential canonical 3 (TRPC3) mediates thrombin-induced astrocyte activation and upregulates its own expression in cortical astrocytes. *J. Neurosci.* 2010; 30(39):13116–13129. [PubMed: 20881130]
37. Parpura V, Basarsky TA, Liu F, Jęftinija K, Jęftinija S, Haydon PG. Glutamate-mediated astrocyte-neuron signalling. *Nature*. 1994; 369(6483):744–747. [PubMed: 7911978]

38. D'Ascenzo M, Fellin T, Terunuma M, et al. mGluR5 stimulates gliotransmission in the nucleus accumbens. *Proc. Natl Acad. Sci. USA*. 2007; 104(6):1995–2000. [PubMed: 17259307]
39. Angulo MC, Kozlov AS, Charpak S, Audinat E. Glutamate released from glial cells synchronizes neuronal activity in the hippocampus. *J. Neurosci*. 2004; 24(31):6920–6927. [PubMed: 15295027]
40. Pasti L, Volterra A, Pozzan T, Carmignoto G. Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes *in situ*. *J. Neurosci*. 1997; 17(20):7817–7830. [PubMed: 9315902]
41. Porter JT, McCarthy KD. Hippocampal astrocytes *in situ* respond to glutamate released from synaptic terminals. *J. Neurosci*. 1996; 16(16):5073–5081. [PubMed: 8756437]
42. Jourdain P, Bergersen LH, Bhaukaurally K, et al. Glutamate exocytosis from astrocytes controls synaptic strength. *Nat. Neurosci*. 2007; 10(3):331–339. [PubMed: 17310248]
43. Parri HR, Gould TM, Crunelli V. Spontaneous astrocytic Ca^{2+} oscillations *in situ* drive NMDAR-mediated neuronal excitation. *Nat. Neurosci*. 2001; 4(8):803–812. [PubMed: 11477426]
44. Cotrina ML, Lin JH, Lopez-Garcia JC, Naus CC, Nedergaard M. ATP-mediated glia signaling. *J. Neurosci*. 2000; 20(8):2835–2844. [PubMed: 10751435]
45. Pascual O, Casper KB, Kubera C, et al. Astrocytic purinergic signaling coordinates synaptic networks. *Science*. 2005; 310(5745):113–116. [PubMed: 16210541]
46. Newman EA. Propagation of intercellular calcium waves in retinal astrocytes and Muller cells. *J. Neurosci*. 2001; 21(7):2215–2223. [PubMed: 11264297]
47. Mothet JP, Pollegioni L, Ouanounou G, Martineau M, Fossier P, Baux G. Glutamate receptor activation triggers a calcium-dependent and SNARE protein-dependent release of the gliotransmitter d-serine. *Proc. Natl Acad. Sci. USA*. 2005; 102(15):5606–5611. [PubMed: 15800046]
48. Henneberger C, Papouin T, Oliet SH, Rusakov DA. Long-term potentiation depends on release of d-serine from astrocytes. *Nature*. 2010; 463(7278):232–236. [PubMed: 20075918]
49. Martineau M, Shi T, Puyal J, et al. Storage and Uptake of d-serine into astrocytic synaptic-like vesicles specify gliotransmission. *J. Neurosci*. 2013; 33(8):3413–3423. [PubMed: 23426669]
50. Stobart JLL, Lu L, Anderson HDI, Mori H, Anderson CM. Astrocyte-induced cortical vasodilation is mediated by d-serine and endothelial nitric oxide synthase. *Proc. Natl Acad. Sci. USA*. 2013
51. ■■■ Takata N, Mishima T, Hisatsune C, et al. Astrocyte calcium signaling transforms cholinergic modulation to cortical plasticity *in vivo*. *J. Neurosci*. 2011; 31(49):18155–18165. [PubMed: 22159127] [*In vivo* study on the role of astrocytic Ca^{2+} in neuronal plasticity.]
52. Chen N, Sugihara H, Sharma J, et al. Nucleus basalis-enabled stimulus-specific plasticity in the visual cortex is mediated by astrocytes. *Proc. Natl Acad. Sci. USA*. 2012; 109(41):E2832–E2841. [PubMed: 23012414]
53. Navarrete M, Perea G, de Sevilla DF, et al. Astrocytes mediate *in vivo* cholinergic-induced synaptic plasticity. *PLoS Biol*. 2012; 10(2):e1001259. [PubMed: 22347811]
54. Denk W, Strickler JH, Webb WW. Two-photon laser scanning fluorescence microscopy. *Science*. 1990; 248(4951):73–76. [PubMed: 2321027]
55. ■■■ Hirase H, Qian L, Bartho P, et al. Calcium dynamics of cortical astrocytic networks *in vivo*. *PLoS Biol*. 2004; 2(4):E96. [PubMed: 15094801] [First paper to study astrocytic Ca^{2+} signaling *in vivo* using two-photon microscopy.]
56. Zipfel WR, Williams RM, Webb WW. Nonlinear magic: multiphoton microscopy in the biosciences. *Nat. Biotech*. 2003; 21(11):1369–1377.
57. ■■■ Nimmerjahn A, Kirchhoff F, Kerr JN, Helmchen F. Sulforhodamine 101 as a specific marker of astroglia in the neocortex *in vivo*. *Nat. Meth*. 2004; 1(1):31–37. [First paper to report selective labeling of astrocytes *in vivo*.]
58. Stosiek C, Garaschuk O, Holthoff K, Konnerth A. *In vivo* two-photon calcium imaging of neuronal networks. *Proc. Natl Acad. Sci. USA*. 2003; 100(12):7319–7324. [PubMed: 12777621]
59. Yang Y, Videny S, Jin L, et al. Molecular comparison of GLT1+ and ALDH1L1+ astrocytes *in vivo* in astroglial reporter mice. *Glia*. 2011; 59(2):200–207. [PubMed: 21046559]
60. Kuzmanovic M, Dudley VJ, Sarthy VP. GFAP promoter drives muller cell specific expression in transgenic mice. *Invest. Ophthalmol. Vis. Sci*. 2003; 44(8):3606–3613. [PubMed: 12882814]

61. Nolte C, Matyash M, Pivneva T, et al. GFAP promoter-controlled EGFP-expressing transgenic mice. A tool to visualize astrocytes and astrogliosis in living brain tissue. *Glia*. 2001; 33(1):72–86. [PubMed: 11169793]
62. Helmchen F, Denk W. Deep tissue two-photon microscopy. *Nat. Meth.* 2005; 2(12):932–940.
63. Ding, S. *In vivo* imaging of Ca^{2+} signaling in astrocytes using two-photon laser scanning fluorescent microscopy.. In: Milner, R., editor. *Astrocytes*. Humana Press; NY, USA: 2012. p. 545-554.
64. Tian GF, Takano T, Lin JH, et al. Imaging of cortical astrocytes using 2-photon laser scanning microscopy in the intact mouse brain. *Adv. Drug Deliv. Rev.* 2006; 58(7):773–787. [PubMed: 17045697]
65. Tian L, Hires SA, Mao T, et al. Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nat. Meth.* 2009; 6(12):875–881.
66. Zariwala HA, Borghuis BG, Hoogland TM, et al. A Cre-dependent GCaMP3 reporter mouse for neuronal imaging in vivo. *J. Neurosci.* 2012; 32(9):3131–3141. [PubMed: 22378886]
67. Akerboom J, Chen TW, Wardill TJ, et al. Optimization of a GCaMP calcium indicator for neural activity imaging. *J. Neurosci.* 2012; 32(40):13819–13840. [PubMed: 23035093]
68. Shigetomi E, Bushong EA, Haustein MD, et al. Imaging calcium microdomains within entire astrocyte territories and endfeet with GCaMPs expressed using adeno-associated viruses. *J. Gen. Physiol.* 2013; 141(5):633–647. [PubMed: 23589582]
69. Xie Y, Wang T, Sun GY, Ding S. Specific disruption of astrocytic Ca^{2+} signaling pathway in vivo by adeno-associated viral transduction. *Neuroscience*. 2010; 170(4):992–1003. [PubMed: 20736051] [Study using adeno-associated virus to selectively express transgenes and inhibit astrocytic Ca^{2+} signaling pathway.]
70. Tong X, Shigetomi E, Looger LL, Khakh BS. Genetically encoded calcium indicators and astrocyte calcium microdomains. *Neuroscientist*. 2013; 19(3):274–291. [PubMed: 23264008]
71. Takata N, Hirase H. Cortical layer 1 and layer 2/3 astrocytes exhibit distinct calcium dynamics in vivo. *PLoS ONE*. 2008; 3(6):e2525. [PubMed: 18575586]
72. Kuga N, Sasaki T, Takahara Y, Matsuki N, Ikegaya Y. Large-scale calcium waves traveling through astrocytic networks *in vivo*. *J. Neurosci.* 2011; 31(7):2607–2614. [PubMed: 21325528]
73. Sasaki T, Kuga N, Namiki S, Matsuki N, Ikegaya Y. Locally synchronized astrocytes. *Cerebral Cortex*. 2011; 21(8):1889–1900. [PubMed: 21212170]
74. Haas B, Schipke CG, Peters O, Söhl G, Willecke K, Kettenmann H. Activity-dependent ATP-waves in the mouse neocortex are independent from astrocytic calcium waves. *Cerebral Cortex*. 2006; 16(2):237–246. [PubMed: 15930372]
75. Nimmerjahn A, Mukamel EA, Schnitzer MJ. Motor behavior activates Bergmann glial networks. *Neuron*. 2009; 62(3):400–412. [PubMed: 19447095] [Study on Bergmann glial Ca^{2+} responses to motor behavior in awake and behaving animals.]
76. Dombeck DA, Khabbaz AN, Collman F, Adelman TL, Tank DW. Imaging large-scale neural activity with cellular resolution in awake, mobile mice. *Neuron*. 2007; 56(1):43–57. [PubMed: 17920014]
77. Takano T, Tian GF, Peng W, et al. Astrocyte-mediated control of cerebral blood flow. *Nat. Neurosci.* 2006; 9(2):260–267. [PubMed: 16388306] [First in vivo study on astrocytic Ca^{2+} signaling on neurovascular coupling.]
78. Tian GF, Azmi H, Takano T, et al. An astrocytic basis of epilepsy. *Nat. Med.* 2005; 11:973–981. [PubMed: 16116433]
79. Winship IR, Plaa N, Murphy TH. Rapid astrocyte calcium signals correlate with neuronal activity and onset of the hemodynamic response in vivo. *J. Neurosci.* 2007; 27(23):6268–6272. [PubMed: 17554000]
80. Ghosh A, Wyss MT, Weber B. Somatotopic astrocytic activity in the somatosensory cortex. *Glia*. 2013; 61(4):601–610. [PubMed: 23339077]
81. Schummers J, Yu H, Sur M. Tuned responses of astrocytes and their influence on hemodynamic signals in the visual cortex. *Science*. 2008; 320(5883):1638–1643. [PubMed: 18566287] [Study on astrocytic response to visual stimulation in vivo.]

82. Petzold GC, Albeanu DF, Sato TF, et al. Coupling of neural activity to blood flow in olfactory glomeruli is mediated by astrocytic pathways. *Neuron*. 2008; 58(6):897–910. [PubMed: 18579080] [Study on astrocytic responses to odor stimulation in vivo.]
83. Fiacco TA, Agulhon C, Taves SR, et al. Selective stimulation of astrocyte calcium in situ does not affect neuronal excitatory synaptic activity. *Neuron*. 2007; 54(4):611–626. [PubMed: 17521573]
84. Agulhon C, Fiacco TA, McCarthy KD. Hippocampal short- and long-term plasticity are not modulated by astrocyte Ca^{2+} signaling. *Science*. 2010; 327(5970):1250–1254. [PubMed: 20203048] [Important study showing astrocytic Ca^{2+} has no effect on synaptic plasticity.]
85. Zonta M, Angulo MC, Gobbo S, et al. Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. *Nat. Neurosci*. 2003; 6(1):43–50. [PubMed: 12469126]
86. Gordon GR, Choi HB, Rungta RL, Ellis-Davies GC, MacVicar BA. Brain metabolism dictates the polarity of astrocyte control over arterioles. *Nature*. 2008; 456(7223):745–749. [PubMed: 18971930]
87. Mishra A, Hamid A, Newman EA. Oxygen modulation of neurovascular coupling in the retina. *Proc. Natl Acad. Sci. USA*. 2011; 108(43):17827–17831. [PubMed: 22006332]
88. Attwell D, Buchan AM, Charkpak S, Lauritzen M, MacVicar BA, Newman EA. Glial and neuronal control of brain blood flow. *Nature*. 2010; 468(7321):232–243. [PubMed: 21068832]
89. Calcinaghi N, Jolivet R, Wyss MT, et al. Metabotropic glutamate receptor mGluR5 is not involved in the early hemodynamic response. *J. Cereb. Blood Flow Metab*. 2011; 31(9):e1–e10. [PubMed: 21731033]
90. Chuquet J, Hollender L, Nimchinsky EA. High-resolution *in vivo* imaging of the neurovascular unit during spreading depression. *J. Neurosci*. 2007; 27(15):4036–4044. [PubMed: 17428981]
91. Choi D. Glutamate neurotoxicity and diseases of the nervous system. *Neuron*. 1988; 1:623–634. [PubMed: 2908446]
92. Medvedeva YV, Lin B, Shuttleworth CW, Weiss JH. Intracellular Zn^{2+} accumulation contributes to synaptic failure, mitochondrial depolarization, and cell death in an acute slice oxygen-glucose deprivation model of ischemia. *J. Neurosci*. 2009; 29(4):1105–1114. [PubMed: 19176819]
93. Tanaka E, Yamamoto S, Kudo Y, Mihara S, Higashi H. Mechanisms underlying the rapid depolarization produced by deprivation of oxygen and glucose in rat hippocampal CA1 neurons in vitro. *J. Neurophysiol*. 1997; 78(2):891–902. [PubMed: 9307122]
94. Lo EH, Dalkara T, Moskowitz MA, Lo EH, Dalkara T, Moskowitz MA. Mechanisms, challenges and opportunities in stroke. *Nat. Rev. Neurosci*. 2003; 4(5):399–415. [PubMed: 12728267]
95. Kasischke KA, Vishwasrao HD, Fisher PJ, Zipfel WR, Webb WW. Neural activity triggers neuronal oxidative metabolism followed by astrocytic glycolysis. *Science*. 2004; 305(5680):99–103. [PubMed: 15232110]
96. Brown AM. Brain glycogen re-awakened. *J. Neurochem*. 2004; 89(3):537–552. [PubMed: 15086511]
97. Rossi DJ, Brady JD, Mohr C. Astrocyte metabolism and signaling during brain ischemia. *Nat. Neurosci*. 2007; 10(11):1377–1386. [PubMed: 17965658]
98. Gurer G, Gursoy-Ozdemir Y, Erdemli E, Can A, Dalkara T. Astrocytes are more resistant to focal cerebral ischemia than neurons and die by a delayed necrosis. *Brain Pathol*. 2009; 19:630–641. [PubMed: 18947334]
99. Nedergaard M, Dirnagl U. Role of glial cells in cerebral ischemia. *Glia*. 2005; 50(4):281–286. [PubMed: 15846807]
100. Barber PA, Demchuk AMH. Biochemistry ischemic stroke. *Adv. Neurol*. 2003; 92:151–164. [PubMed: 12760178]
101. Swanson RA, Ying W, Kauppinen TM. Astrocyte influences on ischemic neuronal death. *Curr. Mol. Med*. 2004; 4(2):193–205. [PubMed: 15032713]
102. Kimelberg HK. Astrocytic swelling in cerebral ischemia as a possible cause of injury and target for therapy. *Glia*. 2005; 50(4):389–397. [PubMed: 15846797]
103. Panickar KS, Norenberg MD. Astrocytes in cerebral ischemic injury: morphological and general considerations. *Glia*. 2005; 50(4):287–298. [PubMed: 15846806]

104. Pekny M, Nilsson M. Astrocyte activation and reactive gliosis. *Glia*. 2005; 50(4):427–434. [PubMed: 15846805]
105. Szydlowska K, Tymianski M. Calcium, ischemia and excitotoxicity. *Cell Calcium*. 2010; 47(2): 122–129. [PubMed: 20167368]
106. Wang T, Cui W, Xie YZ. Controlling the volume of the focal cerebral ischemic lesion through photothrombosis. *Am. J. Biomed. Sci*. 2010; 2:33–42.
107. Zhang W, Xie Y, Wang T, et al. Neuronal protective role of PBEF in a mouse model of cerebral ischemia. *J. Cereb. Blood Flow Metab*. 2010; 30(12):1962–1971.
108. Li H, Zhang N, Sun G, Ding S. Inhibition of the group I mGluRs reduces acute brain damage and improves long-term histological outcomes after photothrombosis-induced ischemia. *ASN Neuro*. 2013 doi:10.1042/ AN20130002 (Epub ahead of print).
109. Risher WC, Croom D, Kirov SA. Persistent astroglial swelling accompanies rapid reversible dendritic injury during stroke-induced spreading depolarizations. *Glia*. 2012; 60(11):1709–1720. [PubMed: 22821441]
110. Risher WC, Ard D, Yuan J, Kirov SA. Recurrent spontaneous spreading depolarizations facilitate acute dendritic injury in the ischemic penumbra. *J. Neurosci*. 2010; 30(29):9859–9868. [PubMed: 20660268]
111. Winship IR, Murphy TH. *In vivo* calcium imaging reveals functional rewiring of single somatosensory neurons after stroke. *J. Neurosci*. 2008; 28(26):6592–6606. [PubMed: 18579732]
112. Zhang S, Boyd J, Delaney K, Murphy TH. Rapid reversible changes in dendritic spine structure in vivo gated by the degree of ischemia. *J. Neurosci*. 2005; 25(22):5333–5338. [PubMed: 15930381]
113. Dunwiddie TV, Masino SA. The role and regulation of adenosine in the central nervous system. *Annu. Rev. Neurosci*. 2001; 24:31–55. [PubMed: 11283304]
114. Takatsuru Y, Eto K, Kaneko R, et al. Critical role of the astrocyte for functional remodeling in contralateral hemisphere of somatosensory cortex after stroke. *J. Neurosci*. 2013; 33(11):4683–4692. [PubMed: 23486942] [Studied the changes of in vivo astrocytic Ca²⁺ signaling in ipsilateral and contralateral hemispheres in the chronic period of photothrombosis.]
115. Howard RS, Holmes PA, Koutroumanidis MA. Hypoxic-ischaemic brain injury. *Pract. Neurol*. 2011; 11(1):4–18. [PubMed: 21239649]
116. Hoogland TM, Kuhn B, Göbel W, et al. Radially expanding transglial calcium waves in the intact cerebellum. *Proc. Natl Acad. Sci. USA*. 2009; 106(9):3496–3501. [PubMed: 19211787]
117. Mathiesen C, Brazhe A, Thomsen K, Lauritzen M. Spontaneous calcium waves in Bergman glia increase with age and hypoxia and may reduce tissue oxygen. *J. Cereb. Blood Flow Metab*. 2013; 33(2):161–169. [PubMed: 23211964] [Ca²⁺ signaling of Bergmann glial cells in hypoxia.]
118. Li P, Murphy TH. Two-photon imaging during prolonged middle cerebral artery occlusion in mice reveals recovery of dendritic structure after reperfusion. *J. Neurosci*. 2008; 28(46):11970–11979. [PubMed: 19005062]
119. Buckingham SC, Robel S. Glutamate and tumor-associated epilepsy: glial cell dysfunction in the peritumoral environment. *Neurochem. Int*. 2013 doi:10.1016/j. neuint.2013.01.027 (Epub ahead of print).
120. Sutula T, Cascino G, Cavazos J, Parada I, Ramirez L. Mossy fiber synaptic reorganization in the epileptic human temporal lobe. *Ann. Neurol*. 1989; 26(3):321–330. [PubMed: 2508534]
121. Houser CR. Neuronal loss and synaptic reorganization in temporal lobe epilepsy. *Adv. Neurol*. 1999; 79:743–761. [PubMed: 10514861]
122. Houser CR, Esclapez M. Vulnerability and plasticity of the GABA system in the pilocarpine model of spontaneous recurrent seizures. *Epilepsy Res*. 1996; 26(1):207–218. [PubMed: 8985701]
123. Houser CR, Swartz BE, Walsh GO, Delgado-Escueta AV. Granule cell disorganization in the dentate gyrus: possible alterations of neuronal migration in human temporal lobe epilepsy. *Epilepsy Res. Suppl*. 1992; 9:41–48. [PubMed: 1285913]
124. Houser CR. Morphological changes in the dentate gyrus in human temporal lobe epilepsy. *Epilepsy Res. Suppl*. 1992; 7:223–234. [PubMed: 1466768]

125. de Lanerolle NC, Kim JH, Robbins RJ, Spencer DD. Hippocampal interneuron loss and plasticity in human temporal lobe epilepsy. *Brain Res.* 1989; 495(2):387–395. [PubMed: 2569920]
126. Babb TL, Kupfer WR, Pretorius JK, Crandall PH, Levesque MF. Synaptic reorganization by mossy fibers in human epileptic fascia dentata. *Neuroscience.* 1991; 42(2):351–363. [PubMed: 1716744]
127. Babb TL, Brown WJ, Pretorius J, Davenport C, Lieb JP, Crandall PH. Temporal lobe volumetric cell densities in temporal lobe epilepsy. *Epilepsia.* 1984; 25(6):729–740. [PubMed: 6510381]
128. Fonseca CG, Green CR, Nicholson LF. Upregulation in astrocytic connexin 43 gap junction levels may exacerbate generalized seizures in mesial temporal lobe epilepsy. *Brain Res.* 2002; 929(1):105–116. [PubMed: 11852037]
129. Garzillo CL, Mello LE. Characterization of reactive astrocytes in the chronic phase of the pilocarpine model of epilepsy. *Epilepsia.* 2002; 43(Suppl. 5):107–109. [PubMed: 12121303]
130. Lemos T, Cavalheiro EA. Suppression of pilocarpine-induced status epilepticus and the late development of epilepsy in rats. *Exp. Brain Res.* 1995; 102(3):423–428. [PubMed: 7737389]
131. Cohen-Gadol AA, Pan JW, Kim JH, Spencer DD, Hetherington HH. Mesial temporal lobe epilepsy: a proton magnetic resonance spectroscopy study and a histopathological analysis. *J. Neurosurg.* 2004; 101(4):613–620. [PubMed: 15481715]
132. Borges K, Gearing M, McDermott DL, et al. Neuronal and glial pathological changes during epileptogenesis in the mouse pilocarpine model. *Exp. Neurol.* 2003; 182(1):21–34. [PubMed: 12821374]
133. Seifert G, Steinhäuser C. Neuron–astrocyte signaling and epilepsy. *Exp. Neurol.* 2013; 244:4–10. [PubMed: 21925173]
134. Hinterkeuser S, Schroder W, Hager G, et al. Astrocytes in the hippocampus of patients with temporal lobe epilepsy display changes in potassium conductances. *Eur. J. Neurosci.* 2000; 12(6):2087–2096. [PubMed: 10886348]
135. Martinez-Hernandez A, Bell KP, Norenberg MD. Glutamine synthetase: glial localization in brain. *Science.* 1977; 195(4284):1356–1358. [PubMed: 14400]
136. Lanerolle N, Lee TS, Spencer D. Astrocytes and epilepsy. *Neurotherapeutics.* 2010; 7(4):424–438. [PubMed: 20880506]
137. Wetherington J, Serrano G, Dingledine R. Astrocytes in the epileptic brain. *Neuron.* 2008; 58(2):168–178. [PubMed: 18439402]
138. Fellin T, Gomez-Gonzalo M, Gobbo S, Carmignoto G, Haydon PG. Astrocytic glutamate is not necessary for the generation of epileptiform neuronal activity in hippocampal slices. *J. Neurosci.* 2006; 26(36):9312–9322. [PubMed: 16957087]
139. Aronica E, van Vliet EA, Mayboroda OA, Troost D, da Silva FHL, Gorter JA. Upregulation of metabotropic glutamate receptor subtype mGluR3 and mGluR5 in reactive astrocytes in a rat model of mesial temporal lobe epilepsy. *Eur. J. Neurosci.* 2000; 12(7):2333–2344. [PubMed: 10947812]
140. Carmignoto G, Haydon PG. Astrocyte calcium signaling and epilepsy. *Glia.* 2012; 60(8):1227–1233. [PubMed: 22389222]
141. Gómez-Gonzalo M, Losi G, Chiavegato A, et al. An excitatory loop with astrocytes contributes to drive neurons to seizure threshold. *PLoS Biol.* 2010; 8(4):e1000352. [PubMed: 20405049]
142. Sword J, Masuda T, Croom D, Kirov SA. Evolution of neuronal and astroglial disruption in the peri-contusional cortex of mice revealed by *in vivo* two-photon imaging. *Brain.* 2013; 136(5):1446–1461. [PubMed: 23466395]
143. Watts L, Sprague S, Zheng W, et al. Purinergic 2Y1 receptor stimulation decreases cerebral edema and reactive gliosis in a traumatic brain injury model. *J. Neurotrauma.* 2013; 30(1):55–66. [PubMed: 23046422]
144. Ren Z, Iliff JJ, Yang L, et al. ‘Hit & run’ model of closed-skull traumatic brain injury (TBI) reveals complex patterns of post-traumatic AQP4 dysregulation. *J. Cereb. Blood Flow Metab.* 2013; 33:834–845. [PubMed: 23443171]
145. Choo AM, Miller WJ, Chen YC, et al. Antagonism of purinergic signalling improves recovery from traumatic brain injury. *Brain.* 2013; 136(1):65–80. [PubMed: 23293266] [Study that discovered that astrocytes exhibited ATP-induced Ca²⁺ waves after traumatic brain injury.]

146. Nagele RG, Wegiel J, Venkataraman V, Imaki H, Wang KC, Wegiel J. Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease. *Neurobiol. Aging*. 2004; 25(5): 663–674. [PubMed: 15172746]
147. Kuchibhotla KV, Goldman ST, Lattarulo CR, et al. Abeta plaques lead to aberrant regulation of calcium homeostasis *in vivo* resulting in structural and functional disruption of neuronal networks. *Neuron*. 2008; 59(2):214–225. [PubMed: 18667150]
148. Busche MA, Eichhoff G, Adelsberger H, et al. Clusters of hyperactive neurons near amyloid plaques in a mouse model of Alzheimer's disease. *Science*. 2008; 321(5896):1686–1689. [PubMed: 18802001]
149. Busche MA, Chen X, Henning HA, et al. Critical role of soluble amyloid- β for early hippocampal hyperactivity in a mouse model of Alzheimer's disease. *Proc. Natl Acad. Sci. USA*. 2012; 109(22):8740–8745. [PubMed: 22592800]
150. Abramov AY, Canevari L, Duchen MR. Changes in intracellular calcium and glutathione in astrocytes as the primary mechanism of amyloid neurotoxicity. *J. Neurosci*. 2003; 23(12):5088–5095. [PubMed: 12832532]
151. Kuchibhotla KV, Lattarulo CR, Hyman BT, Bacskaï BJ. Synchronous hyperactivity and intercellular calcium waves in astrocytes in Alzheimer mice. *Science*. 2009; 323(5918):1211–1215. [PubMed: 19251629] [Study that discovered that astrocytes exhibit intercellular Ca²⁺ waves *in vivo* in a mouse Alzheimer's disease model.]
152. Sathe K, Maetzler W, Lang JD, et al. S100B is increased in Parkinson's disease and ablation protects against MPTP-induced toxicity through the RAGE and TNF- α pathway. *Brain*. 2012; 135(11):3336–3347. [PubMed: 23169921]
153. Gan L, Vargas MR, Johnson DA, Johnson JA. Astrocyte-specific overexpression of nrf2 delays motor pathology and synuclein aggregation throughout the CNS in the alpha-synuclein mutant (A53T) mouse model. *J. Neurosci*. 2012; 32(49):17775–17787. [PubMed: 23223297]
154. Barat E, Boisseau S, Bouyssieres C, Appaix F, Savasta M, Albrieux M. Subthalamic nucleus electrical stimulation modulates calcium activity of nigral astrocytes. *PLoS ONE*. 2012; 7(7):e41793. [PubMed: 22848608]
155. Hsiao HY, Chern Y. Targeting glial cells to elucidate the pathogenesis of Huntington's disease. *Mol. Neurobiol*. 2010; 41(2–3):248–255. [PubMed: 20107928]
156. Arregui L, Benitez J, Razgado L, Vergara P, Segovia J. Adenoviral astrocyte-specific expression of BDNF in the striata of mice transgenic for Huntington's disease delays the onset of the motor phenotype. *Cell Mol. Neurobiol*. 2011; 31(8):1229–1243. [PubMed: 21681558]
157. Valenza M, Leoni V, Karasinska JM, et al. Cholesterol defect is marked across multiple rodent models of Huntington's disease and is manifest in astrocytes. *J. Neurosci*. 2010; 30(32):10844–10850. [PubMed: 20702713]
158. Faideau M, Kim J, Cormier K, et al. In vivo expression of polyglutamine-expanded huntingtin by mouse striatal astrocytes impairs glutamate transport: a correlation with Huntington's disease subjects. *Hum. Mol. Genet*. 2010; 19(15):3053–3067. [PubMed: 20494921]
159. Bradford J, Shin JY, Roberts M, et al. Mutant huntingtin in glial cells exacerbates neurological symptoms of Huntington disease mice. *J. Biol. Chem*. 2010; 285(14):10653–10661. [PubMed: 20145253]
160. Yang Q, Feng B, Zhang K, et al. Excessive astrocyte-derived neurotrophin-3 contributes to the abnormal neuronal dendritic development in a mouse model of fragile X syndrome. *PLoS Genet*. 2012; 8(12):e1003172. [PubMed: 23300470]
161. Jacobs, S.; Cheng, C.; Doering, L. Probing astrocyte function in fragile X syndrome.. In: Denman, RB., editor. *Modeling Fragile X Syndrome*. Springer; Berlin, Germany: 2012. p. 15-31.
162. Higashimori H, Morel L, Huth J, et al. Astroglial FMRP-dependent translational down-regulation of mGluR5 underlies glutamate transporter GLT1 dysregulation in the fragile X mouse. *Hum. Mol. Genet*. 2013; 22(10):2041–2054. [PubMed: 23396537]
163. Maragakis NJ, Rothstein JD. Mechanisms of disease: astrocytes in neurodegenerative disease. *Nat. Clin. Pract. Neuro*. 2006; 2(12):679–689.

164. Nagai M, Re DB, Nagata T, et al. Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. *Nat. Neurosci.* 2007; 10(5):615–622. [PubMed: 17435755]
165. di Giorgio FP, Carrasco MA, Siao MC, Maniatis T, Eggan K. Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. *Nat. Neurosci.* 2007; 10(5):608–614. [PubMed: 17435754]
166. Charles AC, Merrill JE, Dirksen ER, Sandersont MJ. Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. *Neuron.* 1991; 6(6): 983–992. [PubMed: 1675864]
167. Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, Kater SB. ATP released from astrocytes mediates glial calcium waves. *J. Neurosci.* 1999; 19(2):520–528. [PubMed: 9880572]
168. Davidson BL, Breakefield XO. Viral vectors for gene delivery to the nervous system. *Nat. Rev. Neurosci.* 2003; 4(5):353–364. [PubMed: 12728263]
169. Cetin A, Komai S, Eliava M, Seeburg PH, Osten P. Stereotaxic gene delivery in the rodent brain. *Nat. Protoc.* 2007; 1(6):3166–3173. [PubMed: 17406580]
170. Cearley CN, Wolfe JH. Transduction Characteristics of adeno-associated virus vectors expressing cap serotypes 7, 8, 9, and Rh10 in the mouse brain. *Mol. Ther.* 2006; 13(3):528–537. [PubMed: 16413228]
171. Davidson BL, Stein CS, Heth JA, et al. Recombinant adeno-associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system. *Proc. Natl Acad. Sci. USA.* 2000; 97(7):3428–3432. [PubMed: 10688913]
172. Cearley CN, Wolfe JH. A Single injection of an adeno-associated virus vector into nuclei with divergent connections results in widespread vector distribution in the brain and global correction of a neurogenetic disease. *J. Neurosci.* 2007; 27(37):9928–9940. [PubMed: 17855607]
173. Peel AL, Klein RL. Adeno-associated virus vectors: activity and applications in the CNS. *J. Neurosci. Methods.* 2000; 98(2):95–104. [PubMed: 10880823]
174. Tenenbaum L, Chtarto A, Lehtonen E, et al. Recombinant AAV-mediated gene delivery to the central nervous system. *J. Gene Med.* 2004; 6(Suppl. 1):S212–S222. [PubMed: 14978764]
175. Lawlor PA, Bland RJ, Mouravlev A, Young D, During MJ. Efficient gene delivery and selective transduction of glial cells in the mammalian brain by AAV serotypes isolated from nonhuman primates. *Mol. Ther.* 2009; 17(10):1692–1702. [PubMed: 19638961]
176. Lin X, Varnai P, Csordas G, et al. Control of calcium signal propagation to the mitochondria by inositol 1,4,5-trisphosphate-binding proteins. *J. Biol. Chem.* 2005; 280(13):12820–12832. [PubMed: 15644334]
177. Kanemaru K, Okubo Y, Hirose K, Iino M. Regulation of neurite growth by spontaneous Ca^{2+} oscillations in astrocytes. *J. Neurosci.* 2007; 27(33):8957–8966. [PubMed: 17699677]
178. Park F. Lentiviral vectors: are they the future of animal transgenesis? *Physiol. Genomics.* 2007; 31(2):159–173. [PubMed: 17684037]
179. Cannon JR, Sew T, Montero L, Burton EA, Greenamyre JT. Pseudotype-dependent lentiviral transduction of astrocytes or neurons in the rat substantia nigra. *Exp. Neurol.* 2011; 228(1):41–52. [PubMed: 21056560]
180. Dittgen T, Nimmerjahn A, Komai S, et al. Lentivirus-based genetic manipulations of cortical neurons and their optical and electrophysiological monitoring *in vivo*. *Proc. Natl Acad. Sci. USA.* 2004; 101(52):18206–18211. [PubMed: 15608064]
181. Colin, AI; Faideau, M.; Dufour, N., et al. Engineered lentiviral vector targeting astrocytes *in vivo*. *Glia.* 2009; 57(6):667–679. [PubMed: 18942755]
182. Kuhn B, Hoogland TM, Wang SS. Injection of recombinant adenovirus for delivery of genetically encoded calcium indicators into astrocytes of the cerebellar cortex. *Cold Spring Harb. Protoc.* 2011; 2011(10):1217–1223. [PubMed: 21969619]
183. Figueiredo M, Lane S, Tang F, et al. Optogenetic experimentation on astrocytes. *Exp. Physiol.* 2011; 96(1):40–50. [PubMed: 21041318]
184. Wang CY, Wang S. Astrocytic expression of transgene in the rat brain mediated by baculovirus vectors containing an astrocytespecific promoter. *Gene Ther.* 2006; 13(20):1447–1456. [PubMed: 16724097]

185. Mank M, Santos AF, Drenth S, et al. A genetically encoded calcium indicator for chronic in vivo two-photon imaging. *Nat. Meth.* 2008; 5(9):805–811.
186. Ortinski PI, Dong J, Mungenast A, et al. Selective induction of astrocytic gliosis generates deficits in neuronal inhibition. *Nat. Neurosci.* 2010; 13(5):584–591. [PubMed: 20418874]
187. Zhang H, Yang B, Mu X, et al. Several rAAV vectors efficiently cross the blood–brain barrier and transduce neurons and astrocytes in the neonatal mouse central nervous system. *Mol. Ther.* 2011; 19(8):1440–1448. [PubMed: 21610699]
188. Duque S, Joussemet B, Riviere C, et al. Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neurons. *Mol. Ther.* 2009; 17(7):1187–1196. [PubMed: 19367261]
189. Foust KD, Nurre E, Montgomery CL, Hernandez A, Chan CM, Kaspar BK. Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nat. Biotech.* 2009; 27(1):59–65.
190. Platel JC, Dave KA, Gordon V, Lacar B, Rubio ME, Bordey A. NMDA receptors activated by subventricular zone astrocytic glutamate are critical for neuroblast survival prior to entering a synaptic network. *Neuron.* 2010; 65(6):859–872. [PubMed: 20346761]
191. Lacar B, Young SZ, Platel JC, Bordey A. Imaging and recording subventricular zone progenitor cells in live tissue of postnatal mice. *Front. Neurosci.* 2010; 4:43. [PubMed: 20700392]
192. Li X, Zima AV, Sheikh F, Blatter LA, Chen J. Endothelin-1-induced arrhythmogenic Ca^{2+} signaling is abolished in atrial myocytes of inositol-1,4,5-trisphosphate(IP_3)-receptor type 2-deficient mice. *Circ. Res.* 2005; 96(12):1274–1281. [PubMed: 15933266]
193. Zheng W, Talley Watts L, Holstein DM, Wewer J, Lechleiter JD. $P2Y_{1R}$ -initiated, IP_3R -dependent stimulation of astrocyte mitochondrial metabolism reduces and partially reverses ischemic neuronal damage in mouse. *J. Cereb. Blood Flow Metab.* 2013; 33(4):600–611.
194. Dong QP, He JQ, Chai Z. Astrocytic Ca^{2+} waves mediate activation of extrasynaptic NMDA receptors in hippocampal neurons to aggravate brain damage during ischemia. *Neurobiol. Dis.* 2013; 58(0):68–75. [PubMed: 23702310]
195. Halassa MM, Florian C, Fellin T, et al. Astrocytic modulation of sleep homeostasis and cognitive consequences of sleep loss. *Neuron.* 2009; 61(2):213–219. [PubMed: 19186164]
196. Cao X, Li LP, Wang Q, et al. Astrocyte-derived ATP modulates depressive-like behaviors. *Nat. Med.* 2013; 19(6):773–777. [PubMed: 23644515]
197. Hines DJ, Haydon PG. Inhibition of a SNARE-sensitive pathway in astrocytes attenuates damage following stroke. *J. Neurosci.* 2013; 33(10):4234–4240. [PubMed: 23467341]
198. Picciotto MR, Wickman K. Using knockout and transgenic mice to study neurophysiology and behavior. *Physiol. Rev.* 1998; 78(4):1131–1163. [PubMed: 9790572]
199. Mori T, Tanaka K, Buffo A, Wurst W, Kuhn R, Gotz M. Inducible gene deletion in astroglia and radial glia – a valuable tool for functional and lineage analysis. *Glia.* 2006; 54(1):21–34. [PubMed: 16652340]
200. Saito T, Nakatsuji N. Efficient gene transfer into the embryonic mouse brain using in vivo electroporation. *Dev. Biol.* 2001; 240(1):237–246. [PubMed: 11784059]
201. Fukuchi-Shimogori T, Grove EA. Neocortex patterning by the secreted signaling molecule FGF8. *Science.* 2001; 294(5544):1071–1074. [PubMed: 11567107]
202. Tabata H, Nakajima K. Efficient *in utero* gene transfer system to the developing mouse brain using electroporation: visualization of neuronal migration in the developing cortex. *Neuroscience.* 2001; 103(4):865–872. [PubMed: 11301197]
203. LoTurco J, Manent JB, Sidiqi F. New and improved tools for *in utero* electroporation studies of developing cerebral cortex. *Cerebral Cortex.* 2009; 19(Suppl. 1):i120–i125. [PubMed: 19395528]
204. Garcia-Marques J, Lopez-Mascaraque L. Clonal identity determines astrocyte cortical heterogeneity. *Cerebral Cortex.* 2013; 23(6):1463–1472. [PubMed: 22617854]
205. Willig KI, Kellner RR, Medda R, Hein B, Jakobs S, Hell SW. Nanoscale resolution in GFP-based microscopy. *Nat. Meth.* 2006; 3(9):721–723.
206. Rust MJ, Bates M, Zhuang X. Subdiffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Meth.* 2006; 3(10):793–796.

Executive summary

- The properties of spontaneous astrocytic Ca^{2+} signaling *in vivo* under normal condition are affected by age, animal species, wakefulness of animal and the location of astrocytes in the brains.
- Intercellular Ca^{2+} waves in astrocytes *in vivo* can be evoked by various approaches including sensory stimulation and direct pharmacological activation of G-protein coupled receptors.
- Enhanced spontaneous Ca^{2+} signaling featured as intercellular waves among astrocytes can be observed *in vivo* in the experimental models of ischemia, status epilepticus, traumatic brain injury and Alzheimer's disease. The mechanisms underlying astrocytic Ca^{2+} hyperexcitability are different in different brain disorders, and need to be further investigated.
- It remains to be established as to whether astrocytes also exhibit Ca^{2+} hyperexcitability *in vivo* in many other neurodegenerative diseases.
- Molecular genetic approaches including viral transduction, genetically modified mice, and *in utero* electroporation to selectively express or delete transgenes in astrocytes are powerful tools to study the mechanisms of neuron–glia interactions and the role of astrocytes in synaptic plasticity and brain disorders.

Figure 1. Glial fibrillary acidic protein expression in protoplasmic and fibrous astrocytes and the distribution of protoplasmic astrocytes in the cortex and hippocampus in the mouse brain
(A) GFAP expression (arrows) in protoplasmic astrocytes in the cortex (upper), fibrous astrocytes in corpus callosum (the region between the dashed lines; middle) and protoplasmic astrocytes in the CA1 region of hippocampus (lower). Notice the thick process and high expression levels of glial fibrillary acidic protein in the corpus callosum. S100b staining in the **(B)** cortex and **(C)** hippocampus of a mouse brain slice. Notice there is no difference in cell densities between the two regions. All the images were taken from confocal microscopy and are the maximum projection images.
 GFAP: Glial fibrillary acidic protein.

Figure 2. Tripartite synapse and phospholipase C/inositol 1,4,5-triphosphate-mediated Ca^{2+} release signaling pathway in astrocytes

(A) PLC/ IP_3 -mediated Ca^{2+} release signaling pathway in astrocytes. Following upstimulation of GPCRs, IP_3 is liberated following the activation of PLC astrocytes. Astrocytes then release Ca^{2+} from internal stores through the opening of IP_3R ion channels.

(B) The astrocytic process together with pre- and post-synaptic regions of neurons constitutes a synapse – that is, a tripartite synapse. The local microdomains of astrocytic processes can be excited by neuronal activity through glutamate or other neurotransmitters released from the synaptic cleft by activating GPCRs. Ca^{2+} release from internal stores can

mediate glutamate or other chemical transmitter release, a process called 'gliotransmission', which signals back to the neuron by activating neuronal receptors through the tripartite synapse.

DAG: Diacyl glycerol; ER: Endoplasmic reticulum; GPCR: G-protein coupled receptor; IP₃: Inositol 1,4,5-trisphosphate; IP₃R: Inositol 1,4,5-trisphosphate receptor; PIP₂: Phosphatidylinositol 4,5-bisphosphate; PLC: Phospholipase C.

Figure 3. *In vivo* imaging of astrocytic Ca^{2+} signal using two-photon microscopy
 (Ai) The skull of the mouse was attached to a metal plate beneath the objective of a two-photon microscope. (ii) A craniotomy (1.8 mm in diameter) was made on the mouse cortex, where fluorescent dyes were incubated or injected. (B) Surface incubation of (i) fluo-4 acetoxymethyl leads to the specific labeling of astrocytes with the indicator, which is confirmed by (ii) SR101 labeling. (C) A bolus injection of (i) fluo-4 acetoxymethyl leads to simultaneously labeling of astrocytes and neurons. Astrocytes can be identified by (ii) SR101 labeling. An alternative way to label astrocytes with Ca^{2+} indicators is to express genetically encoded Ca^{2+} indicators using viral transduction and transgenic mice. Reproduced with permission from [63].

Figure 4. Microdomain-limited Ca^{2+} signaling versus intercellular Ca^{2+} waves in astrocytes *in vivo*

(A–C) Microdomain-limited Ca^{2+} signaling in astrocytes. (A) Image of a fluo-4-labeled astrocyte with processes numbered and (B) the time courses of $\Delta F/F_0$ showing asynchronous Ca^{2+} signals within processes (1, 2 and 3) and S. The data were recorded from the cortex of a 2-month-old adult mouse anesthetized with urethane. (C) Illustration of microdomain-limited Ca^{2+} signal in astrocytes. Under anesthesia and nonpathological condition, astrocytes occasionally exhibit spontaneous Ca^{2+} signaling in the microdomains of processes. Ca^{2+} signals (black arrows) in the microdomains are not synchronized and randomly occur. Some individual astrocytes also exhibit low frequency somatic Ca^{2+} increase (red arrow), which is not necessarily synchronized with microdomain Ca^{2+} signals in the same cell. (D–F) Intercellular Ca^{2+} waves in astrocytes. (D) Astrocytes labeled as Cell 1–4 exhibit (E) synchronized somatic Ca^{2+} increase known as intercellular Ca^{2+} wave. The data were recorded from the cortex of a 2-month-old adult mouse anesthetized with urethane in the presence of 0.5 mM ATP. (F) Illustration of intercellular Ca^{2+} wave in astrocytic network. With pharmacological and sensory stimulations, and under certain pathological conditions, astrocytes exhibit cell-wide Ca^{2+} increase within an individual astrocyte, for instance synchronized Ca^{2+} increases in the processes (black arrows) and the soma of an astrocyte (red arrow), as well as intercellular Ca^{2+} waves in astrocytic network (red arrows). This phenomenon was also observed in astrocytes in awake, healthy mice.

$\Delta F/F_0$: Background subtracted fluorescence change; S: Soma.

(E) Adapted with permission from [11].

Figure 5. Astrocytes exhibit enhanced Ca^{2+} signaling after photothrombosis

Images of astrocytes loaded with fluo-4 (A) before and (B) after photothrombosis. Time courses of somatic Ca^{2+} oscillation of individual astrocytes expressed as $\Delta F/F_0$ (C) before and (D) after photothrombosis. The boxed regions correspond to the images in (A) and (B) as indicated. (E) Time course of somatic Ca^{2+} signal in astrocytes developed after photothrombosis.

$\Delta F/F_0$: Background subtracted fluorescence change.

Adapted with permission from [11].

Figure 6. Status epilepticus stimulates enhanced astrocytic Ca^{2+} excitability and astrocytic Ca^{2+} -dependent depolarization shift

Time courses of astrocytic Ca^{2+} signal (three examples from three mice in each condition) recorded (A) 3 days following a subthreshold injection of pilocarpine (control) or (B) 3 days after SE. (C) Photolysis of caged Ca^{2+} nitrophenyl ethylene glycol tetraacetic acid in an astrocyte elicits a local depolarization shift in the presence of 1 μM tetrodotoxin. Sequence of pseudocolor images of an astrocyte loaded with nitrophenyl-EGTA/acetoxymethyl and fluo-4/acetoxymethyl (upper panel). Ultraviolet pulses targeting the astrocyte elevates cytosolic Ca^{2+} and triggers a spontaneous depolarization shift with a latency of 1.3 s. Scale bar, 10 μm . Time courses of astrocytic Ca^{2+} changes and field potential (lower panel). Black arrow indicates the delivery of ultraviolet pulses. Numbers on the Ca^{2+} trace represent images in the upper panel.

F/F: Fluorescence change from baseline level; F/F_0 : Background subtracted fluorescence change; SE: Status epilepticus.

(A & B) Adapted with permission from [8]. (C) Adapted with permission from [78].

Figure 7. Intercellular astrocytic Ca^{2+} waves in mice with cortical plaques

(A) Maximum projection of two-photon images of a blood vessel labeled by (i) dextran-rhodamine, amyloid b plaque labeled by (ii) thioflavin and (iii) their overlay. (B) Time-lapse Fluorescence change from baseline level images and (C) cell-resolved time course of Ca^{2+} activity in an amyloid b precursor protein/ presenilin transgenic mouse. The white arrow in the second panel points to the initiator astrocyte, and the sequential panels show the propagation of the wave.

(B & C) Adapted with permission from [151].

Figure 8. Specific transgene expression in astrocytes using adeno-associated virus transduction
(A) Linear feature map of expressing plasmid of recombinant adeno-associated virus2/5 vector. The plasmid contained an astrocyte-specific glial fibrillary acidic protein promoter *gfaABC₁D* and transgene *p130PH* with fusion protein mRFP. **(B–D)** Maximal projection images of mRFP-p130PH expressing astrocytes in hippocampus CA1 regions stained with antibodies against **(B)** glial fibrillary acidic protein, **(C)** NeuN and **(D)** Iba1. Poly A: SV40 late polyadenylation signal sequence. ITR: Inverted terminal repeat. Adapted with permission from [69].