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Probing the complexities of astrocyte calcium signaling

Eiji Shigetomi[1], **Sandip Patel**[2], and **Baljit S. Khakh**[3,4]

[1]Department of Neuropharmacology, Interdisciplinary Graduate School of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan

[2]Department of Cell and Developmental Biology, University College London, Gower Street, London WC1E 6BT; Department of Neurobiology David Geffen School of Medicine, University of California Los Angeles, Los Angeles, USA CA 90095-1751

[3]Department of Physiology, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, USA CA 90095-1751

[4]Department of Physiology, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, USA CA 90095-1751

Abstract

Astrocytes are abundant glial cells that tile the entire central nervous system and mediate well established functions for neurons, blood vessels and other glia. These ubiquitous cells display intracellular Ca^{2+} signals, which have been intensely studied for 25 years. Recently, the use of improved methods has unearthed the panoply of astrocyte Ca^{2+} signals and a variable landscape of basal Ca^{2+} levels. *In vivo* studies have started to reveal the settings under which astrocytes display behaviorally relevant Ca^{2+} signaling. Studies in mice have emphasized how astrocyte Ca^{2+} signaling is altered in distinct neurodegenerative diseases. Progress in the last few years, fueled by methodological advances, has thus reignited interest in astrocyte Ca^{2+} signaling for brain function and dysfunction.

Keywords

astrocyte; calcium; imaging; GCaMP; AAV

Introduction

Astrocytes tile the central nervous system and may represent ~20-40% of the total number of brain cells [1]. Their highly branched morphology, including proximity to neurons and blood vessels, has been the subject of intrigue, speculation and study ever since astrocytes

Correspondence to BSK: Department of Physiology, David Geffen School of Medicine, University of California, Los Angeles, 10833 Le Conte Avenue, 53-263 CHS, Los Angeles, CA 90095-1751, Fax: 310 206 5661, Tel : 310 825 6258, bkhakh@mednet.ucla.edu.

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were discovered ~150 years ago. It is now well established that astrocytes serve diverse and important roles for the brain to function as an organ. These include roles for astrocytes in ion homeostasis, neurotransmitter clearance, synapse formation/removal, synaptic modulation and contributions to neurovascular coupling, all of which are reviewed elsewhere [2-4]. From these perspectives, attention has focussed on astrocyte intracellular Ca^{2+} signals as a basis to measure, interrogate and ultimately understand their roles within neural circuits [2, 5]. The strong focus on Ca^{2+} is based on the knowledge that Ca^{2+} is a widely used and important second messenger and on the realisation that astrocytes, unlike neurons, are electrically not excitable [6].

The possibility that astrocytes may contribute to neural circuit function was first proposed almost 25 years ago [7, 8], soon after astrocyte Ca^{2+} signals were discovered [5]. Since then astrocyte Ca^{2+} signals have been studied in detail. In this review, we focus on recent insights on the properties of astrocyte Ca^{2+} signals, on emerging studies of astrocyte basal Ca^{2+} levels and on their relation to two exemplar neurodegenerative disease models and behavior. Recent breakthroughs in the astrocyte field over the last few years have been fueled by technical advances that allow Ca^{2+} signals to be studied reliably in physiologically relevant compartments both *ex vivo* in brain slices and *in vivo* in adult mice.

Genetically-encoded calcium indicators (GECIs)

Organic Ca^{2+} indicator dyes, delivered to astrocytes by bulk loading or by intracellular dialysis using patch pipettes, have proven useful to study astrocytes [5]. Fundamental insights have emerged from their use [9-13]. These include the discovery of astrocyte Ca^{2+} signals [14-16] and two decades of work on their relation to neuronal activity [10, 17], including the first studies of astrocyte Ca^{2+} dynamics *in vivo* [18]. However, astrocytes are problematic to load with organic Ca^{2+} indicator dyes in adult brain slices. Moreover, *in vivo* repeated chronic imaging experiments are problematic because the dyes are lost from the cells over time, and organic dyes clearly only reveal the astrocyte's somata [19]. Hence additional approaches were needed. We focus here on recent studies employing GECIs, which comprise a single polypeptide chain of a fluorescent protein (or proteins) and a Ca^{2+} binding motif [20-22]. These largely overcome the aforementioned limitations of organic Ca^{2+} indicator dyes for the study of astrocytes.

Two types of GECIs have been used in astrocyte studies: single wavelength GECIs such as the GCaMP series (Figure 1), and FRET based ratiometric GECIs, such as the Yellow Cameleons [20]. GCaMPs are derived from circularly permuted enhanced GFP, the M13 peptide from myosin light chain kinase and calmodulin (Figure 1) [23]. In the absence of Ca^{2+}, GCaMPs display weak fluorescence, but upon Ca^{2+} binding (to calmodulin) increased green fluorescence is observed. GCaMPs have been optimized in terms of brightness, signal-to-noise, dynamic range, Ca^{2+} affinity and photostability [24-27]. The latest GCaMP6 series developed by the Janelia GENIE Project performs as well as some organic Ca^{2+} indicator dyes [24]. The properties of GCaMPs are further leveraged by targeting them to sub cellular compartments such as the plasma membrane [28-31], mitochondria [32-34] and endoplasmic reticulum [33, 35]. Ratiometric FRET based GECIs are also widely used and have been

improved to tune Ca^{2+} affinity to desirable levels [36]. The design and evolution of GECIs has been reviewed [20, 22].

GECIs have been targeted to astrocytes using several techniques, including *in utero* electroporation, adeno-associated viruses (AAVs), as well as transgenic and knock-in approaches (Table 1). These versatile proteins have features of relevance to astrocytes: i. cell specific targeting increases signal-to-noise, especially in relation to organic dyes [28]; ii. GECIs are stably expressed for weeks without noticeable deleterious effects, permitting chronic repeated imaging experiments *in vivo* [37, 38]; iii. GECIs generally display little bleaching during acute imaging experiments [28]; and iv. GECIs reveal Ca^{2+} signals from entire astrocytes [28]. Thus, GECIs overcome some of the limitations [5, 19] of organic Ca^{2+} indicators to study astrocytes.

GECIs are not a panacea, however, and they come with features and considerations that need to be taken into account for each experiment. Thus GECIs, like all Ca^{2+} indicators, have the potential to buffer Ca^{2+} and therefore alter astrocyte physiology. As far as we know, this has not proven to be a problem, but needs to be considered. The concentration of GECIs inside cells is largely unknown and is expected to differ between expression strategies (Table 1). A conservative estimate suggests that GECIs are expressed in the tens of micromolar concentration range [31]. Moreover, the affinity of available GECIs should be considered for each specific experiment; this parameter can be tuned by mutagenesis [39]. The on- and off-kinetics of many GECIs are slower than those of organic indicator dyes, but the GCaMP6 series is the fastest [24]. Of note, high expression of GFP in astrocytes is known to cause astrogliosis [40] and expression of GECIs over several months decreases the health of neurons [41]. Thus, long-term GECI expression in astrocytes may be problematic, especially with AAV-based expression methods [41]. Hence, GECI expression strategies need to be validated on a case-by-case basis as in published studies for cortex, hippocampus and striatum [28, 38, 42-44]. In addition, although AAV-mediated GECI delivery has proven powerful, one has to consider that surgical delivery of AAVs unavoidably damages brain tissue, and thus the cells that are imaged are usually located distally to the microinjection site. This strategy works well for large brain structures such as cortex, hippocampus and striatum, but may be problematic for small brain nuclei. In these cases, independent assessment of damage is needed. *In utero* electroporation has clear advantages for studying astrocytes during development, in neonatal mice and for rats for which gene targeting strategies are less well developed [31]. With the use of Cre-dependent knock-in mouse lines, one needs to consider the specificity of the Cre line and the proportion of astrocytes targeted in a given brain area. For example, GLAST-Cre/ERT2 targets GECIs to ∼35% of astrocytes in the cortex, ∼40% in the CA1 region of the hippocampus and close to 100% of Bergmann glia in the cerebellum [37, 38]. Most glial fibriliary acidic protein (GFAP)-Cre lines result in expression within populations of neurons and astrocytes and are not suited for expressing GECIs selectively within astrocytes [45, 46].

Ca^{2+} signals were recently compared for hippocampal astrocytes when GCaMP3 or GCaMP6f were expressed using AAV or using GCaMP3 and GCaMP6f knock-in mice [38, 43]. The data were indiscernible. Furthermore, GECIs have been deployed in several studies

and no untoward effects have been reported [26, 28-32, 37, 38, 42-44, 47-55]. In future astrocyte studies, appropriate controls will still be needed.

Astrocyte Ca^{2+} signals

Ca^{2+} signaling is a mature field in cell biology. Nonetheless, despite progress, major questions concerning astrocyte Ca^{2+} signaling remain to be satisfactorily addressed [5]. This is probably because multiple potential sources of Ca^{2+} have not been explored and hence not convincingly ruled in or out (Figure 2, Key Figure). These include receptors, channels, exchangers and pumps on the plasma membrane, as well as within intracellular organelles such as mitochondria, endoplasmic reticulum, Golgi and acidic organelles (Figure 2). These sources and sinks exploit Ca^{2+} concentration gradients between cellular compartments [58-60]. In going forward, it will be important to consider the possibility that astrocytes, like other cells, contain multiple sources of Ca^{2+} and that the relative contributions of each may change depending on the brain area [1, 61, 62]. Notably, recent cell specific proteomics shows that ~85% of proteins are shared between major brain cell types [63], implying that several plausible Ca^{2+} sources known to exist in other cell types merit detailed study in astrocytes (Figure 2).

Ca^{2+} release from intracellular stores—GECIs have been particularly valuable to study Ca^{2+} signals in astrocyte branches and branchlets that have previously been difficult to explore [64]. A major mechanism for generating Ca^{2+} signals is through activation of inositol triphosphate (IP_3) receptors which mediate Ca^{2+} release from the ER (Figure 2). Three isoforms of IP_3 receptors exist in vertebrates (IP3R1-3). IP3R2s are enriched in astrocytes [65] and because astrocytes respond to many G-protein coupled receptor (GPCR) agonists [66, 67] this IP3R2 mediated intracellular Ca^{2+} release pathway was expected to dominate. In accord, astrocytes that lacked IP3R2s appeared to lack spontaneous and GPCR-mediated Ca^{2+} signals [68] that were measured using organic Ca^{2+} indicators and Ca^{2+} signaling was reported to be “obliterated” in 100% of hippocampal astrocytes [68, 69]. The finding that the *Ip3r2*$^{-/-}$ mice also displayed no obvious neuronal deficits led the authors to the conclude that astrocyte Ca^{2+} signals were not involved in neuronal and behavioral responses [69, 70]. However, recent evaluations in brain slices and *in vivo* using the fast GECI GCaMP6f to measure Ca^{2+} signals in wild type and homozygous *Ip3r2*$^{-/-}$ adult mice, have provided new insights [38]. First, the overall pattern of Ca^{2+} signals within astrocytes was similar between hippocampal astrocytes in slices and cortical astrocytes *in vivo* for both wild type and *Ip3r2*$^{-/-}$ mice [38]. This shows that Ca^{2+} signals were not the consequence of the method employed, i.e. they were not detectably caused or altered by brain slice procedures. This is an important point, given past suggestions that astrocyte Ca^{2+} signaling was altered in brain slices under conditions used by some investigators [71]. Second, standardized semi-automated analyses of wild type and *Ip3r2*$^{-/-}$ mice showed IP3R2-independent Ca^{2+} signaling within astrocyte branches [38, 43]. Similar residual Ca^{2+} signals in astrocyte branches have been reported by others [48] and available data suggest that most physiological Ca^{2+} signaling actually occurs mainly in astrocyte branches and branchlets [28, 43, 72]. Consistent with these data, roles for IP3R1 and 3 in mediating residual Ca^{2+} signals in astrocytes from *Ip3r2*$^{-/-}$ mice has been suggested [73]. Strong stimuli were required to elicit somatic Ca^{2+} signals [43, 48, 72]. Consistent with these data, somatic Ca^{2+}

signals were largely reduced in *Ip3r2*$^{-/-}$ mice, but those in branches were only partially reduced (Figure 3) [38]. However, astrocyte Ca^{2+} signals evoked by neuronal glutamate release were abolished in *Ip3r2*$^{-/-}$ mice [43]. Taken together, these findings suggest that the use of organic Ca^{2+} indicator dyes, which mainly reveal somatic compartments, led to the simplistic conclusion that spontaneous and GPCR-mediated Ca^{2+} signaling in astrocytes was abolished in *Ip3r2*$^{-/-}$ mice. The discovery of residual Ca^{2+} signals in *Ip3r2*$^{-/-}$ mice now necessitates the need for additional ways to impair Ca^{2+} signaling, for reevaluation of potential neuronal and cerebrovascular consequences [2] and for exploration of the full gamut of potential Ca^{2+} signals (Figure 2).

Ca^{2+} influx signals in astrocytes—Astrocytes interact with neurons via small finger-like structures [74, 75], which we term leaflets and branchlets. These display diameters on the tens of nanometer scale and are expected to have high surface area to volume ratios [76] and hence cannot be imaged directly with light microscopy. In addition, evanescent wave microscopy has shown that astrocytes display numerous near membrane Ca^{2+} signals [29, 30]. In light of these facts, the Lck tag has been used to recruit GECIs to the plasma membrane of astrocytes *in vitro* and *in vivo* following delivery with AAVs [28, 47] or *in utero* electroporation [31]. The Lck-tag is a 26 amino acid polypeptide including tandem myristoylation and palmitoylation domains - it results in essentially complete labeling of the astrocyte plasma membrane [77]. Lck-GCaMP3 revealed microdomain Ca^{2+} signals which occurred spontaneously and in a seemingly random fashion. These were termed “spotty Ca^{2+} signals” [29]. Pharmacological and genetic evaluations showed that spotty Ca^{2+} signals were mediated by Ca^{2+} influx through TRPA1 channels [30]. Subsequent slice experiments showed that they contributed to basal Ca^{2+} levels, which were necessary for the constitutive release of D-serine into the extracellular space [47] and for γ-aminobutyric acid (GABA) transporter expression on the plasma membrane [30]. The wide use of GECIs has revealed other examples of broadly similar spotty Ca^{2+} signals in astrocytes in brain slices and *in vivo*; frequently they are called microdomains. However, their molecular basis remains to be determined (Figure 2). For example, in the CA3 region of the hippocampus, TRPA1 channels did not mediate Ca^{2+} microdomains and those observed in stratum radiatum astrocytes were only partially blocked by a TRPA1 antagonist [43, 47]; TRPV1 channels could be usefully explored in this context based on cell culture studies [78]. Region specific differences may explain why astrocytes in different circuits display Ca^{2+} signals from different sources, of differing duration and separable reliance on neuronal action potential firing [13, 43, 79]. Evaluations with GCaMP6f in the hippocampus show that ∼50% of spontaneous Ca^{2+} signals are dependent on transmembrane fluxes within astrocyte branches, but few if any are dependent on this pathway in the somata [38]. The spotty transmembrane fluxes probably locally elevate Ca^{2+} concentration by ∼300 nM, whereas G-protein coupled receptors globally elevate Ca^{2+} to at least 1 μM [29]. Further detailed molecular and biophysical analyses would be insightful.

Basal Ca^{2+} levels of astrocytes—In addition to Ca^{2+} signals, the basal Ca^{2+} level of cells is expected to be physiologically important. The basal level of Ca^{2+} within cultured hippocampal astrocytes was estimated to be ∼120 nM using ratiometric Fura-2 imaging [29]. However, these methods are prone to several well-known artifacts when used in

complex tissues such as brain slices and *in vivo* (e.g. light absorption/scattering, movement, concentration inhomogeneities). As a result, the astrocyte basal Ca^{2+} concentration was estimated by 2-photon laser scanning microscopy (2PLSM) and fluorescence lifetime imaging microscopy (FLIM), an advanced method that measures the average time a fluorophore spends in the excited state before returning to the ground state [80]. The method overcomes many of the problems mentioned above, but requires specialized equipment. FLIM was used to measure the Ca^{2+} concentration of cortical astrocytes using Oregon Green BAPTA-1 (OGB-1) delivered by bulk loading *in vivo* [81]. The resting concentration of Ca^{2+} was found to be ∼84 nM, but was significantly elevated to ∼155 nM in mouse models of Alzheimer's disease. The potential molecular pathways that determine basal Ca^{2+} levels in cortical astrocytes are currently unknown (Figure 2).

FLIM has recently been used with aplomb in a recent study to map astrocyte basal Ca^{2+} concentrations in hippocampal brain slices and for the cortex *in vivo* [82]. The study exploited the observation that the fluorescence lifetime of OGB-1 increases with increasing concentration of Ca^{2+} in the nanomolar range [83, 84]. A number of fundamental observations were made. First, the basal Ca^{2+} concentration of astrocytes was markedly higher than that of neurons in hippocampal brain slices. Second, the Ca^{2+} concentration in astrocytes that were loaded with OGB-1 via gap junction coupling was ∼100 nM near the soma, but increased to ∼125 nM towards the edges of single astrocytes, i.e. in areas that correspond to branches (Figure 4) where there is greater preponderance of Ca^{2+} fluxes [28, 38, 43, 72]. Third, the astrocyte basal Ca^{2+} concentration decreased dramatically between the ages of 14 and 21 days throughout the cell, but nonetheless maintained an increasing gradient outward from the soma (Figure 4). This recalls previous observations on the differences between young and adult mice for Ca^{2+} signaling [85]. Fourth, when basal Ca^{2+} was measured in many dozens of astrocytes, the cells segregated into two populations: one with a mean basal Ca^{2+} concentration of ∼70-75 nM and another at ∼120-130 nM. This observation was unexpected and made possible only because of FLIM. Fifth, there was remarkable concordance across the board for data gathered *in vivo* and in acute brain slices, again suggesting that the judicious use of brain slices is a useful way to study astrocytes within semi intact preparations.

The realization that astrocyte basal Ca^{2+} levels are an important physiological attribute of astrocytes raises the all-important issue of their functional roles. In relation to this, work with brain slices prepares us for the possibility that changes in the basal Ca^{2+} concentration of astrocytes may have neuronal consequences by virtue of neurotransmitter uptake and D-serine release [30, 47]. Basal Ca^{2+} levels in astrocytes also play a key role in maintaining tonic blood flow by regulation of arteriole diameter [86]. More work is needed, but it is likely that additional functions of basal Ca^{2+} levels will emerge, for example setting of the sensitivity of Ca^{2+} sensitive Ca^{2+} release channels to activation upon GPCR engagement. The cytosol as an “excitable medium” in astrocytes might substitute for excitable behavior at the plasma membrane.

Astrocyte Ca^{2+} signaling in health and disease

Astrocyte Ca^{2+} signals in relation to behavior—Smith invoked the idea that astrocyte Ca^{2+} signals may affect the function of neural circuits [7, 8] and ultimately behavior. The use of GECIs and 2PLSM is now permitting exploration of this concept *in vivo* for whole astrocytes and entire fields of view containing many astrocytes, i.e. at sub cellular and macroscopic levels of investigation. The most relevant experiments are performed in awake behaving mice, because of the discovery that anesthesia largely abrogates astrocyte Ca^{2+} signals [87-89]. Astrocytes display robust Ca^{2+} elevations during locomotion. These were correlated in the cortex and Bergmann glia and reflect brain wide changes [37, 88]. The available data suggest that astrocytes respond to the endogenous release of noradrenaline during arousal or heightened vigilance states [37, 90]. Noradrenaline acts on α1 adrenoceptors, which in the simplest interpretation are located on astrocytes [37, 38, 90-93], although other neuromodulators such as acetylcholine may also contribute [94, 95]. α1 adrenoceptors could conceivably be located on another cell type and impact upon astrocytes indirectly. The responses were thus driven by a slow neuromodulator and were global in the sense that all astrocytes in the imaged field of view displayed elevations. Although abundant spontaneous Ca^{2+} signals in branches were observed [38], there was little evidence for local Ca^{2+} signals triggered selectively during startle responses, which implies that noradrenaline acts broadly as a volume transmitter. This effect of endogenously released noradrenaline was mainly, but not completely mediated by Ca^{2+} release from intracellular IP3R2-dependent Ca^{2+} stores [38]. Thus, a startle-evoked slow response persisted in astrocyte branches in the *Ip3r2*$^{-/-}$ mice [38]. Interestingly, responses of layer 2/3 visual cortex astrocytes to light were enhanced markedly by enforced locomotion [37], which suggests that local (visual stimulation) and global (locomotion/arousal) signals may act synergistically through as yet unknown molecular mechanisms. However, further studies are needed to determine if astrocyte responses during arousal have any relation to the change in gain of primary visual cortex layer 2/3 neurons during locomotion [96, 97], or if astrocyte responses mediated by noradrenaline can affect cortical synaptic function. The kinetics of the astrocyte responses appear too slow to affect the gain change of cortical neurons [37, 38], but refined tools to abolish astrocyte Ca^{2+} signals selectively and locally in the cortex are needed to explore this issue. Moreover, given that astrocytes are considered to be heterogeneous [62], future studies will need to evaluate astrocyte Ca^{2+} responses *in vivo* in genetically defined populations of astrocytes and also in deeper brain structures during ethologically relevant behaviors. Such studies will benefit immensely from the use of wearable endoscopes in combination with red shifted GECIs to permit imaging deeper into tissue [98]. Calcium integrators such as CaMPARI are also likely to be valuable [99].

Astrocyte Ca^{2+} signaling and neurodegenerative disease—It has been proposed that glia contribute to brain disease [100], and more specifically that astrocyte Ca^{2+} signals may participate in neurological and psychiatric disorders [101]. We emphasize recent evaluations in mouse models of two distinct neurodegenerative diseases in which astrocyte Ca^{2+} signaling was altered in separable ways.

Pioneering studies using FLIM in a mouse model of familial Alzheimer's disease (AD) showed that astrocytes displayed higher basal astrocyte Ca^{2+} levels and increased transient

Ca^{2+} signals, which may represent a heightened Ca^{2+} signaling phenotype [81]. However, although it has been proposed that astrocyte Ca^{2+} signals can be triggered by action potential firing in some brain areas [5], within the cortex *in vivo* neither normal nor heightened Ca^{2+} signaling in AD model mice was the result of action potential firing in neurons [102]. The best available data show that heightened Ca^{2+} signaling arose because reactive astrocytes were located near β-amyloid plaques and because they displayed enhanced extracellular ATP-dependent P2Y1-receptor mediated Ca^{2+} signaling [102]. In support, heightened Ca^{2+} signaling could be blocked by pharmacological interventions that blocked ATP release from connexin hemi channels and by P2Y1 receptors antagonists [102]. Taken together, these data indicate that heightened astrocyte Ca^{2+} signals that accompany pathology in mouse models of AD may be caused by a combination of ATP release and/or increased P2Y1 receptor expression by a population of reactive astrocytes located near plaques. If so, P2Y1 receptors may represent a novel therapeutic target for AD. In addition, it is of great interest, and worthy of further evaluations *in vivo*, that direct application of low amounts of amyloid-β peptides to astrocytes can enhance astrocyte Ca^{2+} signals via a mechanism involving α7 nicotinic receptors [103, 104].

Several previous studies have implicated astrocytes in Huntington's disease (HD) [105-109]. Astrocyte Ca^{2+} signals have thus recently been explored in a mouse model of HD[110]. Notably, at the ages tested, HD model mice did not display astrogliosis [109] and thus the underlying changes that occur are separable from those observed in AD model mice, which are strongly associated with astrogliosis [102]. The experiments were conducted on striatal astrocytes in adult mice at early stages of symptom onset before overt tissue loss [110]. Wild type striatal astrocytes displayed extensive spontaneous Ca^{2+} signals, but did not respond reliably with Ca^{2+} elevations during synaptic glutamate release from cortical inputs [110]. In contrast, in HD model mice, spontaneous Ca^{2+} signals were significantly reduced in amplitude, frequency and duration, but astrocytes responded robustly to cortical stimulation with evoked action potential dependent Ca^{2+} signals [110]. The available data suggest that these evoked Ca^{2+} signals were accompanied by prolonged extracellular glutamate levels that activated mGluR2/3 receptors on astrocytes to cause Ca^{2+} release from intracellular stores. In this view, astrocyte engagement was tightly gated by Glt1 glutamate transporters, which presumably normally function to clear extracellular glutamate before it can reach astrocyte mGluR2/3 receptors. Interestingly, Ca^{2+} and glutamate signaling in astrocytes from HD model mice was rescued by astrocyte specific restoration of Kir4.1, which past studies show was reduced in HD and contributed to disease phenotypes [109]. These data imply that astrocyte contributions to HD model mice involve intimate functional interactions between two homeostatic pathways: K^+ buffering and glutamate clearance. The reduction of these in HD results in dramatically altered Ca^{2+} signaling, but it remains to be determined if altered astrocyte Ca^{2+} signaling directly contributes to neuronal deficits in HD or if these are mainly modulated by altered K^+ and glutamate levels. Nonetheless, early astrocyte dysfunctions that precede astrogliosis may represent novel therapeutic targets in HD. More broadly, it is of interest mechanistically that two neurodegenerative diseases are accompanied by distinctly altered astrocyte Ca^{2+} signaling [81, 102, 110]. This raises the possibility that astrocyte Ca^{2+} signaling contributes in very specific ways to specific brain disorders.

Concluding remarks

An important goal of modern neuroscience is to understand how neurons and neural circuits give rise to complex behaviors. This is one of the oldest questions in neurobiology, but the tools of modern biology now make it experimentally tractable. It is tempting to also ask how astrocytes contribute to neural circuits that underlie behavior. Such experiments are certainly needed and are underway [17, 111], but one ought to also consider the possibility that astrocytes may largely provide an appropriate substrate and milieu for neurons to perform their multifarious operations. Such astrocyte functions could be indispensable [100], but perhaps not directly related to the computations neurons perform to give rise to behavior *per se*. If this proves to be the case, and experiments are needed to prove or refute this idea, it may instead be productive to ask how astrocyte dysfunction drives neural circuit malfunction and how this contributes to phenotypic manifestations of disease. In other words, perhaps astrocyte dysfunctions drive disease mechanisms, but in otherwise healthy tissue astrocytes normally provide sustenance and a fabric on which neural circuits form and work. In these settings, astrocytes may be amenable to selective pharmacological, genetic and remote manipulation, or even replacement [112], with the goal of producing desirable effects within neural circuits to modify disease symptoms and outcomes. Early examples of such strategies are emerging and could be explored in other disorders [102, 109, 110, 112]. We believe this is a useful and new conceptual framework within which to consider astrocyte Ca^{2+} signals, however, it should also be considered that astrocytes regulate synaptic function as well as participate in and contribute to global brain states. Both of these existing ideas have been extensively reviewed already [17, 113]. Future research will be immeasurably advanced with the detailed understanding of astrocyte molecular mechanisms and the availability of methods with which to study additional aspects of astrocyte signaling. Many important questions remain unanswered in this field (see Outstanding Questions).

The last few years have witnessed vigorous debate on the physiological role(s) of astrocyte Ca^{2+} signals and if, how, why and when they contribute to neural circuit function. A summary of the relevant issues is provided by Bazargani and Attwell [2]. Recent advances with monitoring Ca^{2+} signals selectively within physiologically relevant astrocyte compartments in brain slices and *in vivo* for adult mice have now provided a more nuanced view of astrocyte Ca^{2+} signals and their relations to synaptic and neural circuit function. Many of these insights derive from the use of much improved methods. Taken together, these technique driven insights are fueling a new wave of astrocyte research [2] and stimulating new ideas. We are reminded of Sydney Brenner's insightful adage: "*Progress in science depends on new techniques, new discoveries and new ideas, probably in that order*".

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Highlights

- Astrocytes display intracellular Ca^{2+} signals
- Improved methods have unearthed new types of astrocyte Ca^{2+} signals
- Astrocyte basal Ca^{2+} levels are variable
- *In vivo* studies suggest astrocytes display behaviorally relevant Ca^{2+} signaling
- Astrocyte Ca^{2+} signaling is altered in neurodegenerative diseases

Outstanding Questions

Molecular/Cellular

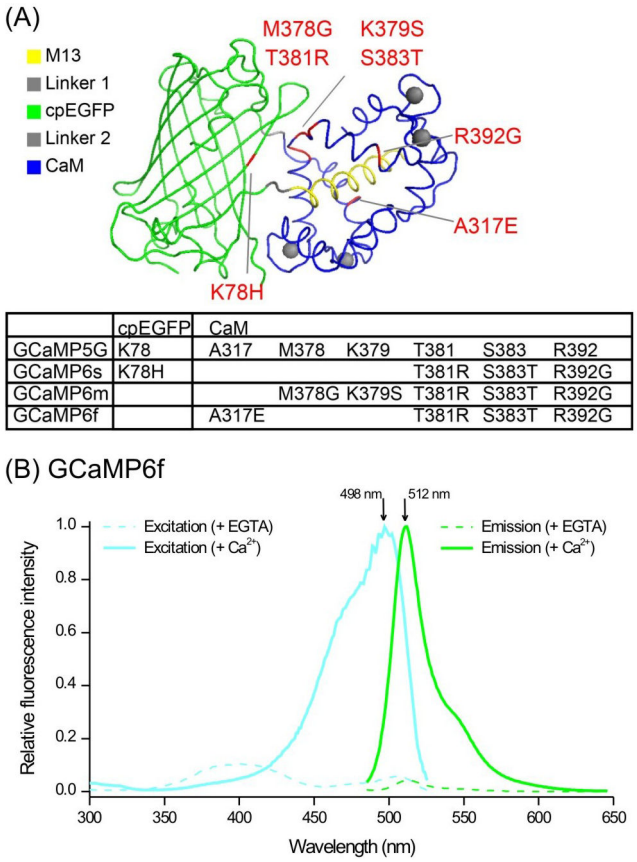
- What are the molecular pathways that mediate various Ca^{2+} signals in astrocytes?
- What are the role(s) of organelles, other than ER, for astrocyte Ca^{2+} signals?
- What is the full set of molecular and physiological processes within astrocytes that Ca^{2+} controls?
- Can new genetic methods be developed to broadly suppress astrocyte Ca^{2+} signals in defined brain areas?

Circuits

- Are the functions of basal Ca^{2+} levels separable from those of Ca^{2+} elevations?
- What are the correlative relations between astrocyte Ca^{2+} signals and neural circuit function and mouse behavior?
- What are the causative functions of astrocyte Ca^{2+} signals for circuits and behavior?
- Do astrocyte Ca^{2+} signals serve distinct functions in distinct circuits?

Disease

- Can astrocyte biology be exploited to produce desirable effects in neural circuits associated with specific brain disorders?
- Do altered astrocyte Ca^{2+} signals contribute to specific neurodegenerative diseases?



	cpEGFP	CaM					
GCaMP5G	K78	A317	M378	K379	T381	S383	R392
GCaMP6s	K78H				T381R	S383T	R392G
GCaMP6m			M378G	K379S	T381R	S383T	R392G
GCaMP6f		A317E			T381R	S383T	R392G

Figure 1. GCaMPs

(A) Structure of GCaMP showing the modular nature of the molecule and the locations of mutations in different GCaMP6 variants relative to GCaMP5G. The panel is reproduced from the paper reporting the development of GCaMP6f [24]. **(B)** Excitation and emission spectra of GCaMP6f in solution in the absence and presence of Ca^{2+}. The spectra were provided by the Janelia GENIE Project; further biophysical properties have been published [24].

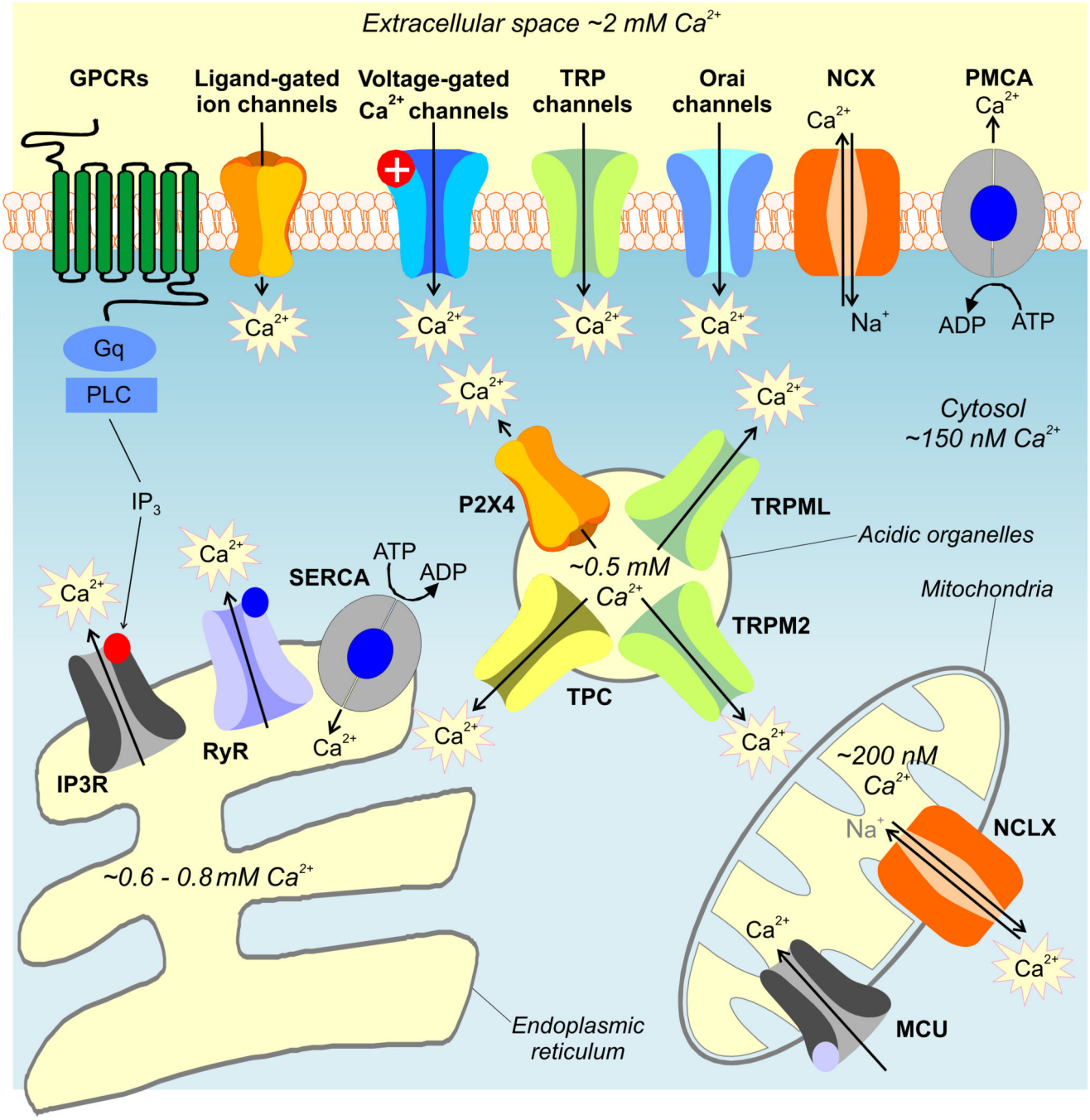


Figure 2. Key Figure: Ca^{2+} transport in cells

The cartoon shows various sources and sinks for Ca^{2+} in cells. In going forward, these need to be considered carefully in studies of astrocytes. Changes in cytosolic Ca^{2+} are dynamically regulated through interplay between Ca^{2+} channels, pumps and transporters. A variety of Ca^{2+} channels exist which are expressed on both the plasma membrane and the membrane of organelles. Voltage-gated Ca^{2+} channels are highly selective plasma membrane proteins that mediate Ca^{2+} signals in excitable cells in response to membrane depolarization. A number of neurotransmitters mediate Ca^{2+} signals through less-selective ligand-gated ion

channels. These include cell surface P2X receptors activated by ATP and the NMDA class of ionotropic receptors for glutamate (NMDAR). Nicotinic acetylcholine (AChR) and 5-HT3 (5HT3R) receptors are Ca^{2+}-permeable members of the cys-loop family of ligand-gated ion channels activated by acetylcholine and 5-HT, respectively. TRP channels constitute a large family of widely expressed ion channels. They are divided into 6 families in mammals. Their Ca^{2+} selectivity varies widely and they are activated by a number of intracellular and extracellular cues including heat (e.g. TRPV1), cold (e.g. TRPM8), pungent chemicals (TRPA) and Ca^{2+} itself (TRPP2). TRPC channels are activated upon receptor-mediated signaling downstream of phospholipase C signaling via diacylglycerol in some cases. Orai channels are highly Ca^{2+}-selective channels activated by the ER Ca^{2+} sensor STIM upon depletion of ER Ca^{2+} stores. Plasma membrane Ca^{2+} ATPase (PMCA) pumps and sodium-calcium exchangers (NCX) transport Ca^{2+} out of the cytosol. IP_3 receptors are intracellular Ca^{2+}-permeable channels localized predominately to the ER where they mediate Ca^{2+} signals in response to IP_3 produced upon phospholipase C activation, which occurs downstream of GPCR activation. Receptor stimulation also elevates levels of, cyclic ADP-ribose (cADPR) which activates the related ryanodine receptors (RyR). The ER Ca^{2+} stores are filled by sarco-endoplasmic reticulum Ca^{2+} ATPases (SERCA The Golgi complex (not shown) also acts as a dynamic Ca^{2+} store housing IP_3 and ryanodine receptors for Ca^{2+} release and secretory pathway ATPases (together with SERCA) for filling. In addition to the ER, a number of acidic organelles which include lysosomes also serve as Ca^{2+} stores. These acidic Ca^{2+} stores express two-pore channels (TPCs), activated by the intracellular messenger NAADP, and TRPM2 activated by ADP-ribose (which additionally activates plasma membrane TRPM2). TRP mucolipins (TRPML) and P2X4 receptors are also expressed on lysosomes. Ca^{2+} is also dynamically regulated by mitochondria. Ca^{2+} is rapidly taken up by the mitochondrial uniporter (MCU) which is a Ca^{2+}-permeable channel localized to the inner mitochondrial membrane. Ca^{2+} is slowly released back into the cytosol by the actions of a mitochondrially targeted sodium-calcium changer (NCLX). The Ca^{2+} concentrations for the intracellular compartments were taken from published work [58-60].

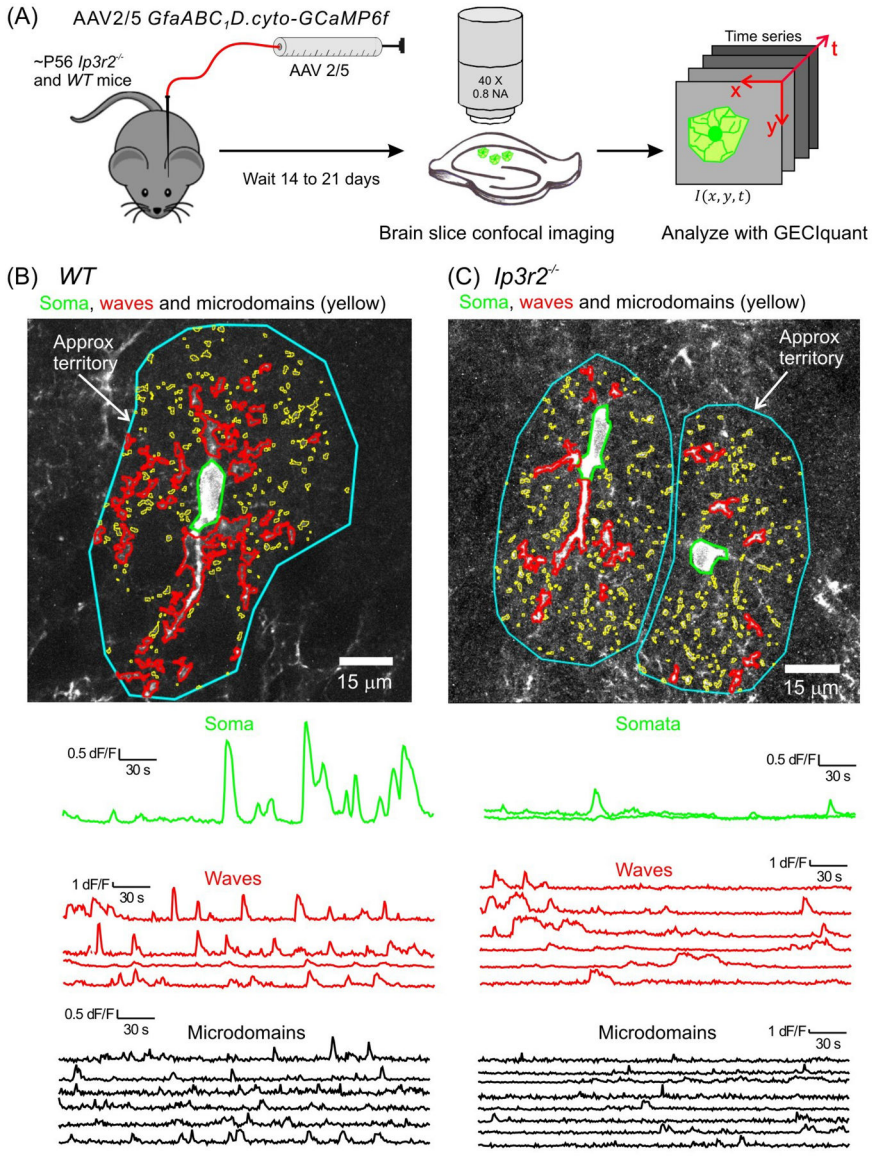


Figure 3. Ca^{2+} signals in hippocampal astrocytes from wild type and *Ip3r2*$^{-/-}$ mice

(A) Schematic illustrating the experimental approach. AAVs were microinjected *in vivo*, brain slices were imaged ~ 2 weeks later and Ca^{2+} signals analyzed in a semi automated manner using custom software called GECIquant. **(B)** Representative images and traces for Ca^{2+} signals measured in an astrocyte from a *WT* mouse. Three predominant types of Ca^{2+} signal are demarcated: somatic signals (green), waves (red) and microdomains (yellow). Approximate territory boundaries are outlined in blue. **(C)** As in B, but for two astrocytes from an *Ip3r2*$^{-/-}$ mouse; somatic signals are largely absent, but many signals persist in branches. Figure from a recent paper [38].

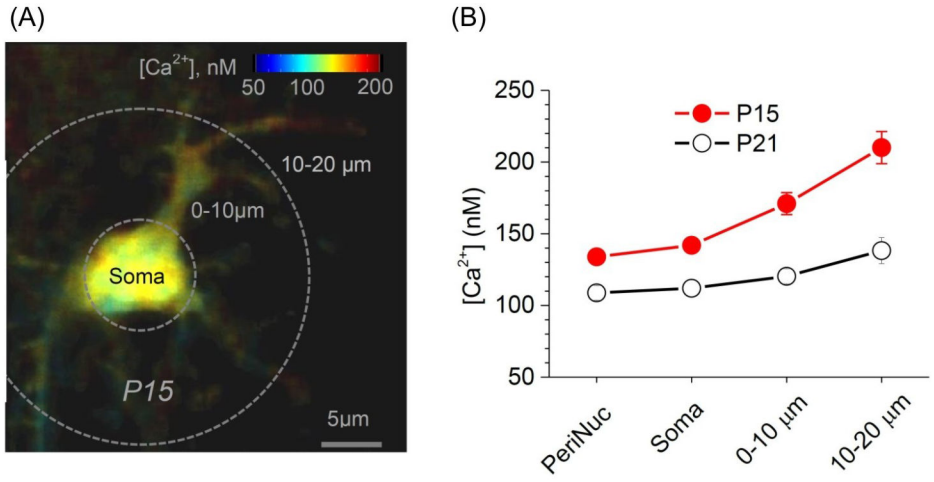


Figure 4. Astrocyte basal Ca^{2+} levels are developmentally regulated

(A) Characteristic Ca^{2+} concentration map in a gap junction-coupled astrocyte from a mouse aged 15 days. Note that the Ca^{2+} concentration increases towards the branches. **(B)** Distribution of resting Ca^{2+} in cellular compartments of gap junction-coupled astrocytes from images such as those in A. Two developmental ages are shown. The image is reproduced (with permission) from Figure 5 of a recent paper [82].

Table 1
Strategies to express GECIs in astrocytes

GECI	Expression approach	Ref
Cytosolic Yellow cameleon 3.60 mice	Transgenic mice driven by the S100β promoter	[49]
Cytosolic GCaMP3 and GCaMP6f	AAV 2/5 using the GfaABC$_1$D promoter, *in utero* electroporation	[28, 31, 38, 42-44, 47]
Membrane tethered GCaMP3 and GCaMP6f (Lck tagged)	AAV 2/5 and GfaABC$_1$D promoter, *in utero* electroporation	[28, 31, 43, 44, 47]
Cytosolic GCaMP3 mice	Knock-in at the *ROSA26* locus	[37, 41]
Cytosolic YC-nano50 mice	Knock-in using tetracycline transactivator (tTa)-tet operator (tetO) strategy at *Actb* locus	[48]
Cytosolic GCaMP5G mice	Knock-in at the *Polr2a* locus	[53]
Cytosolic GCaMP6f and GCaMP6s mice	Knock-in mice at the *ROSA26* and *TIGRE* loci	[38, 56]

Note: GCaMP3, GCaMP5G and GCaMP6f display Ca^{2+} affinities of 345, 447 and 375 nM, and k_{off} values of 2.57, 2.52 and 3.93 s^{-1}, respectively [24]. The Ca^{2+} affinity for YC-nano50 is 50 nM [48] and that for Yellow cameleon 3.6 is 250 nM [57].