Genetically encoded calcium indicators and astrocyte calcium microdomains

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The discovery of intracellular Ca\(^{2+}\) signals within astrocytes has changed our view of how these ubiquitous cells contribute to brain function. Classically thought merely to serve supportive functions, astrocytes are increasingly thought to respond to, and regulate, neurons. The use of organic Ca\(^{2+}\) indicator dyes such as Fluo-4 and Fura-2 has proved instrumental in the study of astrocyte physiology. However, progress has recently been accelerated by the use of cytosolic and membrane targeted genetically encoded calcium indicators (GECIs). Herein, we review these recent findings, discuss why studying astrocyte Ca\(^{2+}\) signals is important to understand brain function and summarize work that led to the discovery of TRPA1 channel-mediated near-membrane Ca\(^{2+}\) signals in astrocytes and their indirect neuromodulatory roles at inhibitory synapses in the CA1 stratum radiatum region of the hippocampus. We suggest that the use of membrane targeted and cytosolic GECIs holds great promise to explore the diversity of Ca\(^{2+}\) signals within single astrocytes, and also to study diversity of function for astrocytes in different parts of the brain.

Key words: GECI, GCaMP3, GCaMP5G, astrocyte, process, branchlet, TRPA1, GAT-3, calcium, leaflet, endfoot, branch

Astrocytes are found throughout the brain and their proximity to neurons has been known for over a century (DeFelipe 2009; Kettenmann and others 2008). Astrocyte density in the brain has increased dramatically through evolution (Nedergaard and others 2003). It is now well established that astrocytes serve vital support roles including buffering of K\(^+\) around neurons, regulation of blood flow, clearance of neurotransmitters from synapses, as well as providing nutrients and oxygen (Attwell and others 2010; Iadecola and others 2007; Kofuji and others 2004; Magistretti 2006). In addition to these varied and vital supportive roles, increasing evidence suggests that astrocytes respond to, and regulate, neuronal function (Araque and others
Why we need to study astrocyte intracellular Ca\textsuperscript{2+} signals ([Ca\textsuperscript{2+}]\textsubscript{i})

The possibility that astrocytes may contribute to information processing in neuronal circuits was thoughtfully enunciated twenty years ago (Smith 1992), soon after Ca\textsuperscript{2+} signals were discovered within astrocytes (Charles and others 1991; Cornell-Bell and others 1990; Dani and others 1992). Astrocytes do not display propagated electrical signals analogous to action potentials (APs) in neurons and from an electrophysiological perspective appear silent. Their resting membrane potential rarely deviates by much from the K\textsuperscript{+} equilibrium potential (Mishima and others 2010; Mishima and others 2007). However, astrocytes frequently display spontaneous intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) elevations that can reach the micromolar range at peak height, and are thus easily within the range of [Ca\textsuperscript{2+}]\textsubscript{i}, known to impact cell physiology. Astrocyte [Ca\textsuperscript{2+}]\textsubscript{i} signals can also be evoked by activation of natively expressed G-protein coupled receptors using exogenous agonists or by evoking neurotransmitter release from neuronal synapses (Agulhon and others 2008; Halassa and others 2010). In some circumstances, most notably in vitro, astrocyte [Ca\textsuperscript{2+}]\textsubscript{i} signals may spread to entire astrocytes and even propagate to neighboring astrocytes as [Ca\textsuperscript{2+}]\textsubscript{i} waves (Bowser and others 2007b; Cornell-Bell and others 1990; Dani and others 1992; Koizumi 2010; Scemes 2000). In view of these findings, astrocyte [Ca\textsuperscript{2+}]\textsubscript{i} signals have increasingly been considered to represent a form of excitability that may be
caused by, control, trigger or otherwise be correlated with alterations in neuronal function. Thus, it has been shown that astrocytes respond to neuronal signals in a process called neuron-to-astrocyte signaling, and that astrocytes also regulate neuronal function via a variety of active and supportive mechanisms in a process called astrocyte-to-neuron signaling (Agulhon and others 2008; Halassa and others 2010).

The last few years have witnessed a growth of interest in both astrocyte-to-neuron and neuron-to-astrocyte signaling, heralding the emergence of an exciting research area within neuroscience. In these settings, attention has focused on \([\text{Ca}^{2+}]_i\) dynamics as a form of encoded signal. In particular, in parallel with reports of astrocyte-to-neuron signaling via intercellular connections (Nedergaard 1994), it was shown that \([\text{Ca}^{2+}]_i\) increases caused release of glutamate from astrocytes that in turn affected neurons (Parpura and others 1994; Pasti and others 1997). Subsequent studies provided mounting evidence to suggest that gliotransmitters lead to changes in synapses (Lee and others 2007; Perea and others 2009) and behaviour (Gourine and others 2010; Halassa and others 2009) via astrocyte-to-neuron signaling. However, despite impressive strides, the settings under which brain astrocyte-to-neuron signaling occurs are still not completely clear and this remains an important and ongoing task for the field. In particular, the relative contributions of vesicle and channel mediated release of gliotransmitters is unclear in many brain areas, although recent studies suggest that both mechanisms exist and that both should be considered on a case by case basis (Halassa and others 2009; Lee and others 2010; Pascual and others 2005; Woo and others 2012).

Several recent papers and reviews have considered astrocyte-to-neuron signaling in great depth, offering views and interpretations that both support and refute the existence, importance and prevalence of this mechanism for brain function (Agulhon and others 2008; Halassa and
others 2010; Nedergaard and others 2012). We do not dwell on these issues here, in light of the breadth of these past undertakings.

We offer the parsimonious view that astrocytes may mediate diverse types of responses in different brain nuclei and under different cellular settings, and that there may not be a single binary signal that astrocytes utilize in a way that is comparable to the use of APs by neurons. Indeed, this more nuanced and graded (as opposed to all-or-none) view of astrocyte [Ca\(^{2+}\)]\(_i\) signals seems consistent with the physiology of astrocytes, which display tremendous diversity of form and function in different regions of the brain (Oberheim and others 2012; Sun and others 2011) and, of course, with the exception of endfeet, lack polarized specializations akin to axons and dendrites. Astrocytes also undergo reactive gliosis, which itself is a remarkably heterogeneous response leading to changes in gene expression in a context specific manner (Sofroniew 2009; Zamanian and others 2012). In fact, the latest transcriptome studies show that different inflammatory mediators change astrocyte gene expression in distinct ways and produce strong changes in GPCRs that mobilize Ca\(^{2+}\) from intracellular stores (Hamby and others 2012). This speaks to the possibility that a given population of astrocytes may produce varied functional responses depending on the degree to which they are reactive. Ca\(^{2+}\) mediates myriad responses within cells (Clapham 2007), making it likely that its roles in astrocytes will also be equally diverse and varied. Finally, a pioneering study showed clear morphological differences in astrocytes even within subareas of the dentate gyrus, providing strong evidence for heterogeneity (Kosaka and others 1986). With these considerations in mind, it seems unlikely that a single stereotyped response to [Ca\(^{2+}\)]\(_i\) exists in all astrocytes – simply because all astrocytes are not the same and Ca\(^{2+}\) does multiple things in different settings. It seems reasonable that [Ca\(^{2+}\)]\(_i\) will serve specific functions in distinct settings. Perhaps the key to understanding what these
ubiquitous and endlessly fascinating cells do in the brain is to embrace their diversity of form and function to explore specific questions, within specific brain nuclei and under specific experimental settings.

**The need for improved methods to study diverse astrocyte Ca\(^{2+}\) signals**

To fully understand the physiology of astrocyte [Ca\(^{2+}\)]\(_i\) signals one needs methods to selectively monitor and evoke [Ca\(^{2+}\)]\(_i\) signals within genetically specified astrocytes. Herein, we focus on recent work from our group on the use of genetically encode Ca\(^{2+}\) indicators (GECIs) to image [Ca\(^{2+}\)]\(_i\) within astrocytes and how the use of membrane tethered GECIs allowed us to discover a novel form of near membrane [Ca\(^{2+}\)]\(_i\) signal in hippocampal astrocytes. The physiology of this [Ca\(^{2+}\)]\(_i\) signal is just beginning to be explored. We do not discuss ways to evoke astrocyte [Ca\(^{2+}\)]\(_i\) signals, but refer the readers to general approaches developed to elevate [Ca\(^{2+}\)]\(_i\) using modified G-protein coupled receptors (GPCRs) (Agulhon and others 2010; Conklin and others 2008) and optogenetics (Zhang and others 2011), as well as a specific method that has been shown to work beautifully for astrocytes (Li and others 2012). We restrict our focus to monitoring [Ca\(^{2+}\)]\(_i\) signals within astrocytes, which we believe is the first step needed before we can devise the right strategies to trigger physiologically relevant [Ca\(^{2+}\)]\(_i\) signals and explore their causative roles in circuits.

The development and use of [Ca\(^{2+}\)]\(_i\) imaging techniques has been central to explore the physiology of astrocytes and has led to an explosion in our understanding of these cells. Astrocyte [Ca\(^{2+}\)]\(_i\) signaling has been shown to occur *in vitro* within astrocyte cultures (Bowser and others 2007a; Charles and others 1991; Cornell-Bell and others 1990; Dani and others 1992; Nedergaard 1994; Parpura and others 1994), in acute slice preparations (Agulhon and others
2010; Bacci and others 1999; Bowser and others 2004; Fellin and others 2004; Fiacco and others 2007; Fiacco and others 2004; Nett and others 2002; Parri and others 2001; Pasti and others 1997; Perea and others 2007; Porter and others 1995a; Porter and others 1995b; Porter and others 1996; Sasaki and others 2011a; Sasaki and others 2011b; Shigetomi and others 2008; Shigetomi and others 2012), in vivo for anesthetized animals (Hirase and others 2004; Hoogland and others 2009; Navarrete and others 2012; Petzold and others 2008; Sasaki and others 2011a; Schummers and others 2008; Takata and others 2011; Thrane and others 2011; Wang and others 2006), in freely behaving animals (Nimmerjahn and others 2009) and within astrocytes from humans soon after biopsy removal of brain tissue (Oberheim and others 2009). Since Ca$^{2+}$ is a ubiquitous second messenger, these findings draw attention to [Ca$^{2+}$]$_i$ signaling as a prevalent feature of astrocytes and raise important questions about the contribution of Ca$^{2+}$ signaling to astrocyte interactions with other cells. These include other types of astrocytes, neurons, microglia, NG2 cells and blood vessels.

The need for improved methods to study astrocyte [Ca$^{2+}$]$_i$ signals is not driven by a technical quest, but rather by knowledge of existing biology showing distinct types of [Ca$^{2+}$]$_i$ signals that differ in their spatial extent and in their duration. One recent study is of particular note. Nimmerjahn et al. reported three different types of Ca$^{2+}$ signals in Bergmann glia, which are specialized astrocytes in the cerebellum (Nimmerjahn and others 2009). By imaging [Ca$^{2+}$]$_i$ signals from awake and freely moving animals they found evidence for ‘Ca$^{2+}$ flares’ that extend to more than 100 cells and occur during locomotion, ‘Ca$^{2+}$ bursts’ that expand radially as waves encompassing ~40 cells and ‘Ca$^{2+}$ sparklets’, which are local subcellular [Ca$^{2+}$]$_i$ signals partly independent of neuronal activity (Nimmerjahn and others 2009). These studies provide elegant proof that astrocyte [Ca$^{2+}$]$_i$ signals are a diverse group of events and that the physiological roles
of each type will need to be explored in depth before we can surmise their physiological roles for the function of neural circuits.

In order to study astrocyte Ca\textsuperscript{2+} dynamics, bulk-loading of membrane-permeable organic Ca\textsuperscript{2+} indicator dyes has been extensively used because of the ease and relative specificity of the procedure for loading astrocytes in brain slices (Porter and others 1995a; Porter and others 1995b). This method remains powerful for studying [Ca\textsuperscript{2+}]\textsubscript{i} signals in the somata and main thick branches of astrocytes (see Box 1 for a note on the nomenclature we use in this review). However, these techniques suffer from severe limitations. The mechanism of astrocyte-specific labeling is unclear, and likely represents a ‘plumbing’ effect rather than a cell-surface protein-dependent uptake mechanism. As such, cell labeling can be quite uneven. Most importantly, it fails to report on finer branchlets perhaps because of the high-background noise within the neuropil and/or inadequate loading of Ca\textsuperscript{2+} indicator dyes into these small compartments. Indeed, quantitative Sholl analysis of astrocyte morphology suggests that as much as 90% of an astrocyte’s area was not sampled by bulk-loading methods (Fig 1) (Reeves and others 2011). This is an important realization because astrocytes interact with neurons and blood vessels via their fine extensions and endfeet, respectively (Bergersen and others 2012; Bushong and others 2002; Mishchenko and others 2010; Ventura and others 1999). Electron microscopy reconstruction of astrocyte processes and endfeet revealed an extraordinarily complex network of small filaments (Mathiisen and others 2010). Therefore, it is unlikely that the full extent of astrocyte [Ca\textsuperscript{2+}]\textsubscript{i} signals has been studied in domains that are considered to be the sites of interaction with other cells (Reeves and others 2011). It seems likely that this disconnect between where astrocyte [Ca\textsuperscript{2+}]\textsubscript{i} signals have been measured (i.e. the soma) and where they underlie physiological responses (at the fine branchlets and leaflets; Box 1) has contributed to an
incomplete picture and perhaps even led to the current controversy regarding the role of astrocytes in neuronal circuits (Agulhon and others 2008). In light of this, measuring \([\text{Ca}^{2+}]_i\) signals with improved resolution, at all relevant cellular substructures and within genetically defined populations of astrocytes is an important and worthy goal.

Loading of membrane-impermeable organic \(\text{Ca}^{2+}\) indicator dyes via glass patch pipettes has also been used to measure astrocyte \(\text{Ca}^{2+}\) signals (Di Castro and others 2011; Fiacco and others 2007; Fiacco and others 2004; Grosche and others 1999; Nett and others 2002; Panatier and others 2011). Direct pipette filling leads to fuller, and more target cell-specific, labeling of processes than bulk loading, although long, thin processes may remain unlabeled. A pioneering study was conducted by Grosche et al for Bergmann glia in the cerebellum (Grosche and others 1999). They found intracellular store-mediated microdomain \([\text{Ca}^{2+}]_i\) signals as a result of repetitive stimulation of parallel fibers. More recently, Panatier et al (Panatier and others 2011) used a similar approach to dialyze organic \(\text{Ca}^{2+}\) indicator dyes into hippocampal astrocytes in brain slices and found that glutamate released from neurons evoked by single action potentials could evoke compartmentalized \(\text{Ca}^{2+}\) signals within astrocyte processes. Similarly, Di Castro et al (Di Castro and others 2011) found two types of localized \(\text{Ca}^{2+}\) signals in hippocampal astrocytes in the molecular layer of the dentate gyrus, which they termed ‘focal’ and ‘expanded’ \(\text{Ca}^{2+}\) signals. Focal signals were action potential-independent, confined signals with a size of \(~4.2\ \mu\text{m}\) and duration of \(~700\ \text{ms}\). In contrast, expanded signals were larger \(~12.9\ \mu\text{m}\), lasted longer \(~3.2\ \text{s}\) and were driven by action potentials. Overall, Panatier et al and Di Castro et al developed upon the work of Grosche et al and found evidence for local \([\text{Ca}^{2+}]_i\) signals within astrocyte branches that they concluded were important for regulating basal synaptic transmission in a gliotransmitter-dependent manner.
Overall, patch-mediated dialysis of astrocytes with Ca\textsuperscript{2+} indicator dyes represents an improvement over the use of bulk loading, however it is still far from a panacea and introduces several problems of its own. \textit{First}, patch mediated dialysis of Ca\textsuperscript{2+} indicator dyes does not provide information on astrocyte branchlets and leaflets: Panatier \textit{et al} and Di Castro \textit{et al} studied processes that were only 5-20 µm from the soma and between 1.2 and 1.4 µm wide, i.e. these main branches were far larger than the fine less than hundreds of nanometer scale branchlets and leaflets that are widely considered to be the sites of interaction with synapses based on electron microscopy studies (Bergersen and others 2012; Bushong and others 2002; Harris and others 2012; Kosaka and others 1986; Mishchenko and others 2010; Ventura and others 1999; Witcher and others 2010). \textit{Second}, Di Castro \textit{et al} used quite high amounts of Ca\textsuperscript{2+} indicator dye (0.2 mM), leaving open the possibility that this procedure altered [Ca\textsuperscript{2+}], buffering. \textit{Third}, by definition, the act of dialyzing cells not only introduces the exogenous dye into them, but also washes out intracellular components from within. This is a concern for all such experiments, but is particularly relevant to the study of astrocytes, which are known to couple extensively via gap junctions into a syncytium. Indeed, astrocytes in the molecular layer of the dentate gyrus form sponge-like conglomerates via fine leaflets (Kosaka and others 1986). One wonders therefore if the act of patching and dialyzing single astrocytes actually alters their functions in a way that is more perturbing than for other more weakly coupled cells? It is noteworthy that patch pipette mediated dialysis is often used as a way to perturb astrocytes (e.g. to wash in toxins and Ca\textsuperscript{2+} buffers), meaning that coincidental wash out must happen when using the very same method to deliver Ca\textsuperscript{2+} indicator dyes, which ideally should be innocuous. McCarthy and colleagues have advocated brief dialysis times with high dye concentrations (Agulhon and others 2010; Nett and others 2002), which of course will help, but even this does
not remedy the problem. Indeed this suggestion is a persuasive argument that the problem of dialysis is real and should not be ignored (Nett and others 2002). Irrespectively, the work by Panatier et al and Di Castro et al does suggest that astrocytes may signal locally to neurons, and therefore represents a call for improved methods to study local \([Ca^{2+}]_i\), signals non-invasively in astrocytes using methods that remedy some or all of the aforementioned shortcomings of organic Ca\(^{2+}\) indicator dyes.

**Genetically encoded calcium indicators and fluorescence imaging**

Genetically encoded calcium indicators (GECIs) circumvent many of the problems presented by small molecule dyes. GECIs are typically composed of a Ca\(^{2+}\)-binding domain and 1-2 fluorescent proteins (FPs), the properties of which are modulated in a Ca\(^{2+}\)-dependent fashion, leading to a change in fluorescence excitation/emission wavelengths, intensities, or Förster resonance energy transfer (FRET). Fluorescent proteins, such as the widely used GFP, are autocatalytic, requiring no exogenous cofactors. As such, they, and sensors derived from them, may be encoded in single polypeptide chains, and targeted via cell type-specific promoters to particular populations of cells, and/or via designed fusion proteins to specific sub-cellular compartments. Expressed protein probes can be repeatedly imaged over weeks and months, allowing visualization of long-term processes such as development and learning. Delivery of transgene from viral infection or *in utero* electroporation can target large volumes of cells, and the labeling that results from endogenous expression is far more uniform than from small molecule injection. Labeling density may be carefully titrated depending on the application, from all cells in a region to sparse, individually resolvable cells. *In vivo* electroporation (Boutin and
others 2008) is a recent technique facilitating the uniform loading of single cells with genetically encoded reporters.

The most relevant GECI properties for neuroscience applications include: signal-to-noise ratio (SNR), Ca²⁺ affinity, rise and decay kinetics, dynamic range, photostability, compatibility with long-term imaging, and targeting to specified locations and fusion proteins. The most optimized GECI scaffold is the GCaMP scaffold, initially engineered in 2001 (Nakai and others 2001), with numerous subsequent improvements (e.g. GCaMP1.6, GCaMP2, GCaMP3, GCaMP5 and GCaMP-HS) that have been recently reviewed (Tian and others 2012). The determination of the high-resolution structures of the Ca²⁺-bound and Ca²⁺-unbound forms of the sensor (Akerboom and others 2009; Wang and others 2008) has significantly improved efforts to improve its properties, as rational design and focused-library screening have largely validated the relevance of the crystal structures to in situ performance. Current GCaMP variants can reliably detect single APs in acute mouse brain slice, and bursts of ~3 APs in vivo (GCaMP3 (Tian and others 2009); GCaMP5 (Akerboom and others 2012)). Imaging specific populations of ~100-200 neurons over weeks to months with GCaMP3 has revealed circuit-level changes in behavioral task-dependent neuronal activity (Huber and others 2012).

Astrocyte imaging places somewhat different restrictions on GECI performance criteria than for those for optimal imaging in neurons. For instance, neuronal membranes are highly electrically excitable, with fast rise and decay rates of the voltage signal controlled by the action potential machinery. Voltage-gated Ca²⁺ channels opened by APs result in large, fairly rapid [Ca²⁺] fluxes propagated through dendrites and the soma. Astrocytes are not electrically excitable, except at their specialized endfeet (see Box 1), and as such Ca²⁺ must enter through ligand-gated channels (and gap junctions to neighboring astrocytes), or be released from
intracellular stores. These lower levels of \([\text{Ca}^{2+}]\) flux demand either higher-affinity GECIs or the targeting of the GECIs to the source of the \(\text{Ca}^{2+}\) entry. Given the absence of the AP excitation mechanism in astrocytes, \(\text{Ca}^{2+}\) fluxes are likely to be slower in both rise and decay than in neurons, lowering the bar for GECI kinetics. Indeed even the signals termed “fast” typically last >0.5 s within astrocytes.

Obviously, there are different demands for \textit{in vitro} and \textit{in vivo} imaging, as well. \textit{In vivo} imaging dramatically degrades SNR, particularly as imaging depth increases. Two-photon imaging can bleach probes at much higher rates than one-photon illumination, particularly for targeted, slowly-diffusing fluorophores. Scattering, autofluorescence, hemodynamics and animal-motion artifacts all significantly pollute imaging channels. As \textit{in vivo} samples behave as light sinks, phototoxicity concerns require lower laser powers, which directly competes with probe fluorescence output. Furthermore, mammals typically have body temperatures of 34-37 °C, increasing the speed at which \(\text{Ca}^{2+}\) signals propagate relative to room temperature that is often used for \textit{in vitro} studies.

**GCaMPs and the Lck tag: discovery of spotty \([\text{Ca}^{2+}]_i\) signals**

We recently sought to develop a non-invasive method to measure \([\text{Ca}^{2+}]_i\) signals in astrocytes using GCaMP variants (Hires and others 2008; Tian and others 2009). In the past, we had enjoyed some success imaging \(\text{Ca}^{2+}\) near the inner mouth of an ion channel (Chaumont and others 2008; Richler and others 2008) and in light of this experience we focused on using membrane targeted GECIs. There were also three reasons from an astrocyte perspective. \textit{First,} work with astrocyte-neuron co-cultures and total internal reflection fluorescence microscopy (TIRFM) has shown that astrocytes display near membrane \([\text{Ca}^{2+}]_i\) signals that were not observed
with conventional wide field microscopy (Shigetomi and others 2009; Shigetomi and others 2010b). However, TIRFM can only be used in cell culture and so another method was needed to explore near membrane [Ca^{2+}]_i signals in astrocytes in brain slices and *in vivo*. Second, rapid switching between TIRFM and wide field microscopy revealed that many cytosolic [Ca^{2+}]_i signals failed to elevate Ca^{2+} near the plasma membrane, implying that cytosolic signals could not be used as surrogate measure of events near the plasma membrane. Third, we were cognizant of the fact that the sites of interaction between neurons and astrocytes were likely to be small tubular structures that by definition have high surface areas and low internal volumes (Hama and others 2004; Ventura and others 1999), implying that a membrane targeted GECI may be better than a cytosolic one to image [Ca^{2+}]_i signals within them. Based on these considerations, we generated Lck-GCaMP2 (Shigetomi and others 2010b), which we improved further to generate Lck-GCaMP3 (Shigetomi and others 2010a) (Fig 2). In this context, “Lck” refers to a 26 amino acid peptide from the Lck tyrosine kinase that has been shown to be a strong membrane tether (Benediktsson and others 2005; Zlatkine and others 1997). Consistent with our expectations, we found that Lck-GCaMP2 was ~10 fold better than cytosolic GCaMP2 for measuring near membrane [Ca^{2+}]_i signals, that it was evenly and strongly distributed throughout the plasma membrane and that it diffused as expected within the plasma membrane, implying there were no gross barriers to hinder its diffusion into fine branchlets (Shigetomi and others 2010b). On the other hand, there do appear to be significant barriers to the diffusion of cytosolic dyes into branchlets via the cytosolic bulk (Reeves and others 2011). Lck-GCaMP3 shares all these features of Lck-GCaMP2, with the additional boon that it displays a ~2-3 fold better signal-to-noise ratio because of three designer point mutations (Shigetomi and others 2010a). Most recently, we made Lck-GCaMP5G and have shown that it improves the peak response size
(dF/F) for astrocyte Ca\textsuperscript{2+} signals by a further ~2-fold over Lck-GCaMP3 because it carries three new point mutations (Akerboom and others 2012). However, Lck-GCaMP5G tends to be dimmer than Lck-GCaMP3 (i.e. resting F is lower) and does not reveal new signals that were undetected by Lck-GCaMP3 (Akerboom and others 2012). Thus overall, Lck-GCaMP3 and Lck-GCaMP5G can be used to study near membrane Ca\textsuperscript{2+} signals in astrocytes (Fig 2). Our data suggest that membrane tethered GECIs are far better than cytosolic dyes because they place the GECI in the membrane of fine processes where Ca\textsuperscript{2+} fluxes are likely to be greatest. Our use of a membrane tether was reminiscent of the use of other designer tags to place GECIs near sites of interest. For example GECIs have been targeted to neurotransmitter release sites in neurons with great success (Akerboom and others 2012; Dreosti and others 2011; Dreosti and others 2009). Table 1 summarizes the GECIs that have been used for astrocyte studies and provides details on how to obtain the plasmids.

Introduction of Lck-GCaMP2, 3 and 5G into cultured hippocampal astrocytes revealed a novel type of microdomain Ca\textsuperscript{2+} signal, which we call ‘spotty Ca\textsuperscript{2+} signals’ (Shigetomi and others 2010a; Shigetomi and others 2010b; Shigetomi and others 2012). Spotty Ca\textsuperscript{2+} signals (measured at room temperature) occurred randomly and frequently at numerous spots with an average size of ~5 µm and a duration of ~3.7 s (Fig 3). Spotty Ca\textsuperscript{2+} signals are due to Ca\textsuperscript{2+} entry from the extracellular space and are resistant to strategies that block or deplete Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores (Shigetomi and others 2012). Interestingly, spotty Ca\textsuperscript{2+} signals occur without any overt stimulation and they are not affected by activation of a variety of GPCRs with exogenous agonists (Shigetomi and others 2012). Taken together, these findings suggest that the mechanisms underlying spotty Ca\textsuperscript{2+} signals are different from those for the more classical GPCR-mediated Ca\textsuperscript{2+} signals frequently studied within astrocytes (Fiacco and others 2009).
Moreover, the spotty Ca^{2+} signals are not caused by the expression of Lck-GCaMPs because similar signals could be observed using organic Ca^{2+} indicator dyes and TIRFM (Shigetomi and others 2009; Shigetomi and others 2010b). However, the spotty Ca^{2+} signals are much easier to observe with Lck-GCaMPs and could also be studied with conventional wide field and multi-photon microscopy. When using wide field imaging, spotty Ca^{2+} signals are easily and reliably detected by membrane-targeted GECIs, but infrequently using cytosolic GECIs, implying that membrane targeted GECIs are closer to the transmembrane source of local Ca^{2+} and that diffusion of the cytosolic GECIs may be suboptimal to detect near membrane events (Shigetomi and others 2009; Shigetomi and others 2010a; Shigetomi and others 2010b; Shigetomi and others 2012).

**Diversity of Ca^{2+} signals in astrocytes**

Astrocytes constitute a diverse ‘cell type’, the breadth of which is only now beginning to be understood. Long thought of as a homogeneous population of ‘glue’ or ‘support’ cells, astrocytes are now recognized to come in different flavors. The glial fibrillary acidic protein (GFAP), previously believed to be a universal astrocyte marker, is now acknowledged to label only a subset of astrocytes; the aldehyde dehydrogenase enzyme Aldh1L1 is the current best pan-astrocyte marker (Cahoy and others 2008).

Even within a given ‘type’ of astrocyte, calcium signaling physiology may also be quite diverse. Although there are only a handful of available studies, the resting \([Ca^{2+}]\); level of astrocytes is thought to be \(\sim 100\) nM (Kuchibhotla and others 2009). Since astrocytes express a large variety of GPCRs that mobilize Ca^{2+} from intracellular stores, fluctuations in the resting \([Ca^{2+}]\); level of astrocytes have been extensively studied in the somata during pharmacological
and physiological activation of GPCRs (Fiacco and others 2009). Activation of GPCRs causes inositol 1,4,5 triphosphate (IP₃) production through phospholipase C (PLC) leading to opening of IP₃ receptor Ca²⁺ channels on the endoplasmic reticulum (ER) to release Ca²⁺ from intracellular stores. Interestingly, although there are three types of IP₃ receptor, only type 2 receptors (i.e. IP₃R2) are expressed predominantly within astrocytes from the cortex and hippocampus. Accordingly, IP₃R2 knockout mice do not show spontaneous [Ca²⁺]ᵢ elevations and lack [Ca²⁺]ᵢ signals evoked by GPCR activation in the somata of hippocampal and cortical astrocytes loaded with organic calcium indicator dyes (Navarrete and others 2012; Petravicz and others 2008; Takata and others 2011). Thus the molecular basis of a well studied form of astrocyte [Ca²⁺]ᵢ signal is release from intracellular stores via type 2 IP3 receptors; the physiological roles of these [Ca²⁺]ᵢ signals for neuron-astrocyte and astrocyte-neuron communication have been extensively reviewed (Fiacco and others 2009).

In contrast to the store-mediated signals, our findings with Lck-GCaMPs suggest that the spotty Ca²⁺ signals represent an additional [Ca²⁺]ᵢ signal that is spatially restricted to near membrane regions, including within astrocytic processes (Shigetomi and others 2010b; Shigetomi and others 2012). Furthermore, pharmacological analyses show that spotty Ca²⁺ signals are distinct from global [Ca²⁺]ᵢ signals (Shigetomi and others 2010b). This is of interest because empirical data shows that distinct sources or types of [Ca²⁺]ᵢ signal may serve distinct physiological roles within astrocytes. Thus, photolysis of caged Ca²⁺ within astrocytes is known to cause both slow inward currents (mediated by extrasynaptic NMDA receptors) and facilitation of excitatory postsynaptic currents (EPSCs) onto neurons (Fellin and others 2004; Perea and others 2007; Sasaki and others 2011a). In contrast, photolysis of caged-IP₃, which elevates [Ca²⁺]ᵢ levels, only facilitates EPSCs (Fiacco and others 2004). Moreover, two almost identical
[Ca\textsuperscript{2+}]\textsubscript{i} signals evoke different forms of astrocyte single vesicle exocytosis in cell culture (Bowser and others 2007a) and two almost identical [Ca\textsuperscript{2+}]\textsubscript{i} signals lead to distinct effects on pyramidal neurons in acute brain slices (Shigetomi and others 2008). Additionally, Gordon et al. (Gordon and others 2009) reported that local [Ca\textsuperscript{2+}]\textsubscript{i} signals within astrocyte processes are related to functional interactions with hypothalamic neurons, whereas somatic astrocyte [Ca\textsuperscript{2+}]\textsubscript{i} signals are not always correlated with neuronal effects. Taken together, these data imply that the details, such as the source, location and dynamics of [Ca\textsuperscript{2+}]\textsubscript{i} are important and may dictate, or at least regulate, how astrocytes respond to [Ca\textsuperscript{2+}]\textsubscript{i} elevations in terms of their effects on neurons. More generally, as discussed earlier, astrocyte [Ca\textsuperscript{2+}]\textsubscript{i} signals do not appear to be all-or-none binary signals, like action potentials in neurons. With these considerations in mind, we set out to identify the molecular basis of the spotty Ca\textsuperscript{2+} signals measured with Lck-GCaMPs in astrocyte-neuron co-cultures because we thought they may be physiologically relevant for astrocytes.

By combining a pharmacological screening approach with the use of siRNAs, we discovered that spotty Ca\textsuperscript{2+} signals in astrocyte-neuron co-cultures were mediated by TRPA1 channels (Shigetomi and others 2012). As far as we know, the TRPA1 mediated [Ca\textsuperscript{2+}]\textsubscript{i} signaling pathway has not been previously reported in past astrocyte studies, which have focused largely on [Ca\textsuperscript{2+}]\textsubscript{i} elevations due to store mediated Ca\textsuperscript{2+} release (Agulhon and others 2010; Agulhon and others 2008). The discovery of functional TRPA1 channels in astrocytes and their contribution to near membrane and basal [Ca\textsuperscript{2+}]\textsubscript{i} signals adds to the richness and diversity of astrocyte [Ca\textsuperscript{2+}]\textsubscript{i} signals in astrocytes (Gordon and others 2009; Shigetomi and others 2008), which also includes Ca\textsuperscript{2+} influx via ionotropic receptors such as Ca\textsuperscript{2+} permeable AMPA receptors, NMDA receptors and P2X receptors (Nedergaard and others 2012) as well as reverse activation of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers (Nedergaard and others 2012).
TRPA1 channels contribute to basal calcium levels in astrocytes

Transient receptor potential (TRP) channels are a large family of non-selective cation channels (Wu and others 2010) that are grouped into six subfamilies based on sequence homology: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPML (mucolipin) and TRPP (polycystin). As a family, TRP channels are opened by a diverse range of physiological and pathophysiological stimuli, functioning as thermosensors, mechanosensors and chemosensors. Most TRP channels are permeable to Ca$^{2+}$ and contribute transmembrane Ca$^{2+}$ fluxes. Because of their multimodality and their Ca$^{2+}$ permeability the functional properties, biophysical mechanisms and physiological roles of TRP channels represent a fascinating area of biology (Wu and others 2010).

A wide variety of TRP channels could potentially be expressed in cultured astrocytes based on mRNA and protein expression studies. The pioneering transcriptome analysis by Cahoy et al. (Cahoy and others 2008) showed TRPC1-2 and TRPM2-7 mRNA expression within acutely dissociated and immunopanned astrocytes that most likely reflect astrocytes in vivo (Cahoy and others 2008). However, several TRP channels were not seen to be astrocyte enriched and were under (or within) the limits of detection, including TRPA1 (Cahoy and others 2008). Of the TRP channels, mRNAs of TRPM3 and TRPM7 were highly expressed in astrocytes, although the functional significance of these in astrocytes is currently unknown. In light of these careful transcriptome studies, we were surprised to find functional evidence for TRPA1 expression within astrocytes from astrocyte-neuron co-cultures and within acute brain slices (Shigetomi and others 2012). There is no immediately satisfying explanation for this difference between our functional studies and past mRNA expression analysis. However, several lines of
evidence suggest that astrocytes express functional TRPA1 channels in culture and in acute brain slices.

First, spotty Ca\(^{2+}\) signals in astrocytes were reduced by siRNAs against TRPA1 and were almost completely abolished by the specific TRPA1 blocker, HC 030031. Second, activation of TRPA1 by low concentrations of AITC (the pungent ingredient in wasabi) increased the number of spotty Ca\(^{2+}\) signals. Third, several known TRPA1 activators/agonists caused global Ca\(^{2+}\) increases in astrocytes. Fourth, AITC-evoked Ca\(^{2+}\) elevations were reduced by both siRNA against TRPA1 and HC 030031. Fifth, AITC-evoked currents in astrocytes were reduced by siRNA against TRPA1. Sixth, TRPA1 protein expression was detected by Western blotting. Seventh, blockade of TRPA1 reduced basal [Ca\(^{2+}\)]\(_i\) levels in astrocytes within cultures and acute slices. Eighth, the effect of TRPA1 blockade on astrocytes in acute hippocampal slices was significantly reduced in TRPA1 deletion (Trpa1\(^{-/-}\)) mice.

We suggest low-level expression of TRPA1 mRNAs may be sufficient to account for the spotty Ca\(^{2+}\) signals. In accord, TRPA1 is a relatively high-conductance cation channel with high Ca\(^{2+}\) permeability (fractional Ca\(^{2+}\) current \(\sim 17\%\); (Nilius and others 2011)) and TRPA1 undergoes pore-dilation, which further increases Ca\(^{2+}\) permeability (Chen and others 2009). Additionally, TRPA1 channels are also regulated by Ca\(^{2+}\), both through channel desensitization and through the function of an intracellular EF-hand Ca\(^{2+}\)-binding domain (Wu and others 2010). This potentially establishes complex feedback loops between TRPA1, Ca\(^{2+}\), and other channels. A further issue also merits mention. It is now clear that the act of culturing astrocytes changes their complement of mRNAs (Cahoy and others 2008; Foo and others 2011; Zamanian and others 2012) and so it is possible that the TRPA1 responses were affected by such changes in our astrocyte-neuron co-cultures. However, this would not explain our data from acute brain slices.
The Barres lab has made tremendous progress in developing a culture system that maintains purified astrocytes in a native \textit{in vivo} like state (Foo and others 2011). This represents the state-of-the-art for work on pure astrocyte cultures, but a similar preparation for astrocyte-neuron co-cultures does not currently exist, and our goals were to study astrocytes with neurons, as in the brain. On balance, our findings, and perhaps all data from astrocyte-neuron co-cultures, should be interpreted with these considerations in mind (Foo and others 2011). One looks forward to further advances with cell culture methodology that preserve astrocytes with \textit{in vivo} like properties when co-cultured with neurons.

An important aspect of TRPA1-mediated spotty Ca$^{2+}$ signals within astrocytes is that they contribute to basal cytosolic Ca$^{2+}$ levels. Thus, inhibition of TRPA1 channels significantly reduces basal [Ca$^{2+}$]$_i$ levels from $\sim$120 to $\sim$60 nM (Shigetomi and others 2012). It remains feasible that the spotty Ca$^{2+}$ signals may also regulate astrocyte [Ca$^{2+}$]$_i$ levels locally within microdomains, but the available data instead suggest that they largely contribute to setting bulk intracellular basal [Ca$^{2+}$]$_i$ levels. An additional feature of the TRPA1 mediated spotty Ca$^{2+}$ signals that set them apart from other astrocyte [Ca$^{2+}$]$_i$ signals is that we found no evidence to suggest that they were regulated by, or required, neuronal activity or GPCR activation (Shigetomi and others 2012). This lack of regulation by mechanisms that are known to regulate intracellular store mediated [Ca$^{2+}$]$_i$ signals further suggests that the function of TRPA1 may be to regulate basal [Ca$^{2+}$]$_i$ levels, rather than be regulated by neuronal function, i.e. at this juncture it seems unlikely that TRPA1 channels contribute to neuron-to-astrocyte communication in a way directly analogous to GPCR mediated signals (Agulhon and others 2008). Further experiments to prove or refute these hypotheses are certainly needed. Moreover, given that TRPA1 channels are multimodal receptors activated by diverse stimuli (Wu and others 2010), it will also be important
to evaluate if astrocyte TRPA1 channels are regulated by other processes, such as blood flow (Attwell and others 2010), brain injury and disease (Kremeyer and others 2010).

We would be remiss if we did not mention other TRP channels in astrocytes, although our focus by necessity is TRPA1. A variety of approaches for astrocytes from different parts of the brain have provided evidence for the expression of TRPC1, TRPC3, TRPC6, TRPV1 and TRPV4 channels within astrocytes with important functional roles (Akita and others 2011; Benfenati and others 2011; Beskina and others 2007; Butenko and others 2012; Golovina 2005; Grimaldi and others 2003; Huang and others 2010; Lanciotti and others 2012; Liu and others 2009; Malarkey and others 2008; Shirakawa and others 2010; Wu and others 2010). Broadly, this emerging data set speaks to the potential importance of the TRP channel family for astrocytes and calls for more detailed explorations of their patho-physiological roles in specific brain nuclei. TRPM3 and TRPM7 seem to be particularly interesting based on transcriptome analysis (Cahoy and others 2008; Lovatt and others 2007).

**TRPA1 channels in astrocytes indirectly modulate inhibitory synapses**

We explored the role of astrocyte TRPA1 channels on synaptic transmission in the stratum radiatum region of the CA1 area of the mouse hippocampus. Pharmacological blockade of TRPA1 channels decreased the amplitude of GABAergic miniature inhibitory postsynaptic currents (mIPSCs) onto interneurons by ~20%, but did not affect mIPSCs onto pyramidal neurons (Shigetomi and others 2012). The effect of TRPA1 blockade on interneuron mIPSCs was mimicked by clamping astrocyte [Ca²⁺]ᵢ to ~35 nM, but was unaffected when [Ca²⁺]ᵢ was clamped close to its resting level at around ~120 nM, implying that basal [Ca²⁺]ᵢ was important and that the act of patching astrocytes *per se* was not (Shigetomi and others 2012). Importantly,
the effect of TRPA1 blockade on mIPSC amplitudes onto interneurons was occluded by prior astrocyte dialysis with BAPTA to strongly buffer \([\text{Ca}^{2+}]_i\) levels in the astrocyte syncytium, suggesting that TRPA1 blockade with HC 030031 acted via astrocytes and contributed to their basal \([\text{Ca}^{2+}]_i\). Moreover, applications of HC 030031 did not affect mIPSCs in mice lacking TRPA1 channels, indicating that the drug acted on TRPA1 to produce its effect rather than some other mechanism (Shigetomi and others 2012). Taken together, these data provide strong evidence for the functional consequences of TRPA1 expression within astrocytes.

During the course of the aforementioned studies it became clear that astrocyte dialysis with BAPTA or TRPA1 blockade also resulted in enhanced GABA\(_A\) receptor mediated tonic currents in interneurons that were sensitive to the competitive antagonist bicuculline. This finding suggested elevated GABA levels in the extracellular space during BAPTA dialysis and TRPA1 blockade, which may cause the tonic currents and lead to decreases in the amplitude of mIPSCs through desensitization of synaptic GABA\(_A\) channels. Indeed, blocking GAT-3 GABA transporters, which are enriched within astrocytes, mimicked the effects of astrocyte BAPTA dialysis and TRPA1 blockade, and the effects were mutually occlusive, suggesting that BAPTA dialysis and TRPA1 blockade both converge on GAT-3 (Shigetomi and others 2012). We found no evidence that other GABA transporters were involved (i.e. GAT-1 and GAT-2). Our findings were consistent with the widely accepted view that GABA transporters regulate the spillover of GABA into extrasynaptic regions (Beenhakker and others 2010) and accordingly we found robust expression of GAT-3 in both the somata and processes of astrocytes recalling past work showing that GAT-3 is the major type of astrocyte GABA transporter (Minelli and others 1995; Minelli and others 1996; Ribak and others 1996).
Taken together our functional analyses in brain slices suggests that the major function of astrocyte TRPA1 channels is to regulate basal \([Ca^{2+}]_i\) levels, which are permissive for the proper functioning and/or surface expression of GAT-3 (Shigetomi and others 2012). Once TRPA1 is blocked or astrocyte \(Ca^{2+}\) is buffered, GAT-3 function and/or surface expression is decreased leading to elevated extracellular GABA levels and reduced mIPSCs onto interneurons due to desensitization of GABA\(_A\) receptors (Fig 4). In this context, astrocyte \([Ca^{2+}]_i\) levels are important for astrocyte-neuron communication, but the mechanism appears to involve a classical homeostatic function of astrocytes, namely clearance of neurotransmitter rather than calcium-dependent gliotransmission. Also, the effect of astrocyte \([Ca^{2+}]_i\) levels is indirect and neuromodulatory, rather than directly stimulatory or inhibitory.

We noted earlier that \(Ca^{2+}\) entry through TRPA1 channels contributes to basal \([Ca^{2+}]_i\) levels in astrocytes, and we further found that \(Ca^{2+}\) entry also acted locally to regulate GAT-3 (Shigetomi and others 2012). Thus, we compared GAT-3 mediated responses after dialyzing astrocytes with BAPTA and EGTA, which differ in their association kinetics for \(Ca^{2+}\) (Soeller and others 2004). Estimation of length constants with these buffers suggests that GAT-3 is located within about 250 nm of the source of \(Ca^{2+}\) that regulates it (i.e. TRPA1). This unexpected finding strongly implies the existence of localized \(Ca^{2+}\) signaling in astrocytes, which may be particularly important for the physiology of fine astrocyte branches, branchlets and leaflets. The finding that \(Ca^{2+}\) acts locally in astrocytes to regulate GAT-3 recalls work by Henneberger et al, which demonstrated the importance of nanodomain astrocyte \(Ca^{2+}\) signals for the regulation of long-term potentiation through calcium-dependent D-serine release (Henneberger and others 2010). However, in this latter case the source of \(Ca^{2+}\) remains to be identified.
Areas for future exploration

Our findings on TRPA1 channels and their impact on inhibitory synapses were made possible because of the ability of Lck-GCaMP3 to reveal novel spotty Ca\(^{2+}\) signals. These findings now lead to three classes of question.

The first class of question centers on the use of GECIs to study astrocyte [Ca\(^{2+}\)]\(_i\) signals within intact preparations and \textit{in vivo}. Thus, it will be important to extend our approaches with cytosolic and membrane targeted GECIs with \textit{in vivo} viral expression methods as well as by using transgenic and knock-in expression (Zariwala and others 2012). Our published data strongly suggest these approaches will reveal a new vista of [Ca\(^{2+}\)]\(_i\) signaling in astrocytes that has been missed with conventional bulk loading methods. Specifically, we predict the existence of large numbers of [Ca\(^{2+}\)]\(_i\) signals in branches and branchlets, but it is not clear if any of the available methods will reveal [Ca\(^{2+}\)]\(_i\) signals within the extremely fine astrocyte leaflets that contact synapses (Box 1). Nonetheless, GECIs may provide the metric needed to explore astrocyte functions within intact brain circuits with precision and without the unavoidable problems of organic Ca\(^{2+}\) indicator dyes. Additionally, the Lck tag appears to be a wonderful way to recruit fluorescent proteins and sensors to astrocyte plasma membranes and reveal their complex morphology (Benediktsson and others 2005; Shigetomi and others 2010b). Perhaps this approach could be exploited to specifically study astrocyte structural plasticity in the context of physiology, disease and injury. Structural remodeling of astrocytes is an exciting area that could be fruitfully explored with the Lck tag (Sun and others 2011).

Although GECIs appear ideal to explore astrocytes, there are limitations. For example, one needs to develop methods to express them within specified astrocytes without changing the physiology of the cells. One needs to ensure that expression of the GECI does not alter Ca\(^{2+}\).
handling or interfere with other processes. Finally, one needs to ensure expression of the GECI is reliable and stable over the time course of the experiment at hand. It is likely these requirements can be met by the use of adeno associated viruses (Xie and others 2010) and knock-in mice using Cre LoxP technology. Floxed STOP mice expressing cytosolic GCaMP3 from the Rosa26 locus are already available (Zariwala and others 2012) and it is likely other mice will soon be made, allowing our approaches with GECIs in vitro to be extended in vivo. Given the strict demands of in vivo imaging discussed above, it is quite possible that further improvements in GECI performance will be required to detect the full complement of in vivo astrocyte calcium responses. Just as Lck-GCaMP3 and Lck-GCaMP5G revealed complexities of spotty calcium signals not seen with Lck-GCaMP2, next-generation GECIs may uncover additional intricacies or even new classes of Ca\(^{2+}\) signals. It is possible that a next-generation GECI, expressed in the cytoplasm, could reveal similar, or improved, signals relative to the current Lck-targeted versions, which would improve compatibility with existing transgenic mice and reagents. Lastly, further classes of genetically encoded sensors, such as for extracellular glutamate or GABA, combined with GECIs in other color channels (\textit{i.e.} red), will open new doors to investigate the underlying physiology of astrocytes and neuron-astrocyte two-way communication.

The second class of question centers on the discovery of TRPA1 channels in astrocytes. Specifically, what other functions do astrocyte TRPA1 channels serve? Do they contribute to synaptic plasticity, neurovascular coupling and/or metabolic support for neurons? Is there an endogenous ligand for TRPA1 channels in astrocytes? Our findings to date suggest that TRPA1 channels have a high open probability at rest and can give rise to spotty Ca\(^{2+}\) signals without the need to invoke the existence of an endogenous ligand secreted by, or contained within, astrocytes (Shigetomi and others 2012). However, since TRPA1 channels are activated by a whole host of
molecular species, these data do not rule out the possibility that an appropriate ligand may be released under some circumstances to open astrocyte TRPA1 channels, during ischemia, inflammation and disease, for example. Reliable antibodies against TRPA1 channels are also needed to start systematic anatomical and electron microscopy studies. Additionally, genetic approaches such as cell-specific and inducible deletion of TRPA1 channels may allow us to investigate the roles of these channels in the function of microcircuits and behavior without having to rely on pharmacological approaches. These endeavors would benefit from technical refinements that are desperately needed, such as improved and specific Cre reporter mice that target distinct astrocyte subpopulations.

The third class of question centers on astrocyte GAT-3. Specifically, what are the molecular scale mechanisms that determine why Ca\(^{2+}\) entry through TRPA1 regulates functional expression of GAT-3 on the astrocyte surface? What is the cellular substructure that places TRPA1 and GAT-3 within a working distance of ~250 nm? Why does blocking GAT-3 function largely affect mIPSCs onto interneurons and not those onto pyramidal neurons? Do the slice results on mIPSC modulation extend to true, *in vivo* effects on inhibitory modulation? We hypothesize the existence of closer functional and/or structural interactions of astrocytes with interneurons than with pyramidal neurons in the hippocampus CA1 region: it will be important to test this hypothesis with GAT-3 reporter mice. Since GABA is intimately involved in regulating synaptic inhibition within microcircuits, it will also be important to evaluate if astrocyte TRPA1 or GAT-3 contribute to neuronal hyperexcitability such as during epileptiform activity.

Another general area that could be usefully pursued concerns definition: we need to agree on the magnitude and kinetics of signals that constitute *bona fide* \([\text{Ca}^{2+}]_i\) signals, so that we can agree on what each of us in the field is studying. In this respect, the recently reported focal
signals appear to be quite small (Di Castro and others 2011) and one wonders if they have previously been missed as noise. A better and agreed definition of what a [Ca\textsuperscript{2+}]\textsubscript{i} signal is would mitigate such ambiguities.

Perhaps the most exciting class of question that could now be addressed concerns astrocyte diversity. Specifically diversity of Ca\textsuperscript{2+} signals within single astrocytes and diversity of astrocyte functions within different parts of the brain. It seems quite implausible to us that some of the most populous cells in the brain are a single homogenous morass (i.e. glue). The use of cytosolic and membrane targeted GECIs offers unique opportunities to study astrocyte diversity in genetically specified cell populations and thus begin to explore the fine details and richness of astrocyte biophysics and biology in the brain.
Box 1: On astrocyte morphology nomenclature: branches, branchlets and leaflets.

It has been known since the time of Cajal that astrocytes are highly branched and ramified cells (DeFelipe 2009; Kettenmann and others 2008), a feature that has been repeatedly demonstrated in subsequent anatomical studies, most notably for human astrocytes (Oberheim and others 2009). However, as discussed in the main text, most [Ca²⁺] imaging studies have focused on astrocyte somata, which are easy to identify and evaluate. As optical methods have improved, focus has shifted to the study of astrocyte processes located at variable distances away from the soma (Agulhon and others 2010; Di Castro and others 2011; Grosche and others 1999; Reeves and others 2011) and in one case to the study of “enlargements” on astrocyte processes (Panatier and others 2011). In such studies, the term “astrocyte process” has been used to discriminate the signals from those recorded in the somata, but it remains unclear exactly what this means in particular studies because astrocytes possess many, perhaps hundreds, of processes.

Defining what is meant by “astrocyte process” is important in order to understand and thoughtfully compare [Ca²⁺] signals between studies and also to make inferences about the relationship of [Ca²⁺] signals to synapses, which are contacted by the myriad terminal endings of astrocytes and seemingly not by thick processes (Bergersen and others 2012; Bushong and others 2002; Harris and others 2012; Mishchenko and others 2010; Ventura and others 1999; Witcher and others 2010).

We suggest that the term “astrocyte process” should evolve into several related terms that convey more meaningful information about the astrocyte sub compartment being studied within intact preparations such as brain slices and in vivo. We provide some working definitions below.

**Branches** are the major processes emanating from the astrocyte soma and the focus of recent attention (Di Castro and others 2011; Grosche and others 1999; Panatier and others 2011; Reeves and others 2011). These probably do not number more than 8 per astrocyte and display diameters on the 1-2 micrometer scale (Reeves and others 2011) and have also been called “stem processes” in the literature.

**Branchlets** are the finer secondary, tertiary and higher order structures that emanate from branches. The precise number of these is presently unknown, but they display diameters on the sub micrometer scale (Reeves and others 2011). Conventional diffraction limited light microscopy may not be ideal to study the finest branchlets satisfactorily, although they can be seen clearly as distinct from branches.

**Leaflets** are the very terminal extensions of astrocyte branchlets that contact synapses (Bergersen and others 2012; Bushong and others 2002; Harris and others 2012; Mishchenko and others 2010; Ventura and others 1999; Witcher and others 2010). The precise numbers of these per astrocyte is currently unknown, but based on electron microscopy studies they display dimensions on the tens of nanometer scale (Hama and others 2004). By our definition, leaflets would be the same structures as those variously termed “astrocyte lamellae”, “astrocyte sheets”, “veillike lamellae”, “peripheral astrocyte processes” and “astrocyte fingers” (Derouiche and others 2001). Direct comparison of light microscopy with high voltage electron microscopy shows that leaflets cannot be imaged with diffraction-limited light microscopy (Hama and others 2004). Moreover, in the dentate gyrus molecular layer leaflets have dimensions of tens of nanometers at sites of interaction with dendrites (Kosaka and others 1986), i.e. they are far smaller than the micrometer scale thick branches that have been imaged by 2-photon microscopy (Di Castro and others 2011).

**Endfeet** are specialized distal extensions of astrocytes that contact the vasculature. One expects that each astrocyte bears only one or two branches with enlarged endfeet. Endfeet appear to represent a truly polarized compartment of astrocytes with a well accepted function.

The more general term of “astrocyte processes” is still useful and could be used loosely, for example to describe astrocytes in cell culture where they have lost their native morphology or when the location of a particular signal does not matter or encompasses all processes (e.g. “process retraction”).
We finish by adding that our suggested nomenclature is, of course, not presented as the final word on the matter. However, we hope it may serve as a basis for further discussion and hopefully the field will converge on definitions of astrocyte compartments that can be accepted and used as a basis to explore and study astrocyte physiology with a common and understood language. Our logic in suggesting better terminology is simple: if we want to determine how astrocytes contribute to neuronal circuits, then we must know how the signals being measured by different laboratories relate to each other from an anatomical perspective and also to the sites of interaction with synapses. This is currently unclear from the existing literature and deserves to be addressed.
Table 1: Summary of the available GECI plasmids and mice to study astrocyte calcium signals.

<table>
<thead>
<tr>
<th>GECI</th>
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<th>Reference</th>
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<td>Khakh lab</td>
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<td>In vivo transgenic mouse tools</td>
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<tr>
<td>S100(\beta) - YC 3.60</td>
<td>–</td>
<td>Russell lab</td>
<td>(Atkin and others 2009)</td>
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<tr>
<td>ROSA26-CAG-floxedSTOP-GCaMP3</td>
<td>JAX Mice: Ai38, #014538</td>
<td>Chen lab</td>
<td>(Zariwala and others 2012)</td>
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Notes on table: – indicates that it is currently unknown if these reagents are available openly. The original GCaMP2 plasmid was developed by the Kotlikoff lab (Tallini and others 2006) and subsequently modified by mutagenesis to generate GCaMP3 and GCaMP5G (sometimes abbreviated simply as GCaMP5) (Akerboom and others 2012; Tian and others 2009). All three GCaMPs have been tagged with the strong membrane tether called Lck (Akerboom and others 2012; Shigetomi and others 2010a; Shigetomi and others 2010b; Shigetomi and others 2012). All the plasmids listed in this table are freely available from Addgene and can be ordered online using the listed Addgene IDs (www.addgene.org). We refer the readers to the main text for the advantages of each type of GECI for imaging calcium signals in astrocytes and their branches and branchlets. In brief, GCaMP5G displays the largest dF/F, but has lower F than GCaMP3. Both GCaMP5G and GCaMP3 detect an equivalent number of spotty Ca\(^{2+}\) signals in astrocytes and we conclude that both are about equally useful in this regard for detecting slow astrocyte signals, although GCaMP5G is significantly better than GCaMP3 for detecting fast neuronal signals (Akerboom and others 2012).
Figure 1: Bulk loading of organic Ca\textsuperscript{2+} indicator dyes does not reveal the full morphology of astrocytes as judged by confocal microscopy, morphological reconstructions and quantitative Sholl analysis.

A. Representative maximum projection images from confocal z-series for astrocytes loaded with Fluo-4AM, labeled with SR101 or dialyzed with Alexa-488. B. 2D representations of astrocytes loaded with calcium indicator dyes (OGB-AM, Fluo-4AM) and those labeled with SR101 and with antibodies against GFAP, as well as an astrocyte dialyzed with Alexa-488. In the Alexa-488 image, the approximate position of the patch-pipette is shown with a red asterisk. C. Average Sholl plots from reconstructions such as those shown in A. D. Average Alexa-488 Sholl plot (orange line and axis) superimposed on the number of calcium transients measured at increasing distances from the soma (black bars) for Fluo-4 loaded astrocytes. The scale bars in A are 15 \textmu m. The figure is reproduced here from our recent paper (Reeves and others 2011).
Figure 2: Cartoon of Lck-GCaMP3 and GCaMP3 GECIs.
A. Schematic representation of Lck-GCaMP3 and a cartoon of how it is recruited to the plasma membrane of astrocytes. B. As in A, but in this case for cytosolic GCaMP3. Note in this case, the GECI is not recruited to the plasma membrane. In both cases the GECI gets brighter when Ca$^{2+}$ is bound. The cartoons are based on our published work Lck-GCaMP2, Lck-GCaMP3 and Lck-GCaMP5G, as discussed in the text (Akerboom and others 2012; Shigetomi and others 2010a; Shigetomi and others 2010b; Shigetomi and others 2012).
Figure 3: Spotty Ca^{2+} signals imaged with Lck-GCaMP3 in astrocytes.

A. Images of an astrocyte expressing Lck-GCaMP3. The left image shows basal fluorescence of Lck-GCaMP3 from a single astrocyte in cell culture. The right image shows a maximum projection image of a 300-frame movie acquired at 1Hz: note many spotty Ca^{2+} signals are seen. Eight regions of interest are shown (as 1–8). The intensity profiles of these eight ROIs are shown in B. C. Shows still frames between 141 and 240 s and between 171 and 270 s of the graph in panel B for ROI 4 and ROI 6, respectively. The time between images is 1 s. D. This panel shows images of microdomain Ca^{2+} signals for ROI 1 and ROI 3. Graph on the right shows the full width of half maxima (FWHM) of the events (~5.0 µm). Based on data such as these, the spotty Ca^{2+} signals were considered to be microdomains. The figure is reproduced from our recent study (Shigetomi and others 2012).
Figure 4: Summary cartoon showing how astrocyte TRPA1 channels indirectly modulate GABAergic inhibitory synapses by regulating the ability of GAT-3 GABA transporters to clear GABA from the extracellular space (see text for details). Extracellular GABA levels increase when TRPA1 channels are blocked, because GAT-3 GABA transporters are likely endocytosed.
References


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