Large-scale recording of astrocyte activity

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Astrocytes are highly ramified glial cells found throughout the central nervous system (CNS). They express a variety of neurotransmitter receptors that can induce widespread chemical excitation, placing these cells in an optimal position to exert global effects on brain physiology. However, the activity patterns of only a small fraction of astrocytes have been examined and techniques to manipulate their behavior are limited. As a result, little is known about how astrocytes modulate CNS function on synaptic, microcircuit, or systems levels. Here, we review current and emerging approaches for visualizing and manipulating astrocyte activity *in vivo*. Deciphering how astrocyte network activity is controlled in different physiological and pathological contexts is crucial for defining their roles in the healthy and diseased CNS.

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Introduction
The adult human brain contains roughly equal numbers of neurons and glial cells [1,2]. Historically, it was believed that there was a clear division of labor among these two cell classes, with glia relegated to performing supportive roles to ensure that neuronal activity can be sustained. In the last few decades, our knowledge about the diverse roles played by different glial cell types has expanded dramatically, and it is now clear that they can exert a profound influence on neuronal synaptic plasticity, excitability, and behavior. Among glial cells, astrocytes are in a unique position to modulate brain activity. They are ubiquitous in all gray and white matter regions [3], they express receptors for neurotransmitters, and they extend highly ramified processes that interact with synapses, nodes of Ranvier, blood vessels, and many other CNS elements. Astrocytes also exhibit structural and functional dynamics on spatial and temporal scales that span several orders of magnitude (from micrometers to millimeters and from milliseconds to weeks). Measuring their dynamics and relating these events to distinct CNS functions remains a significant challenge, requiring development of a wide range of techniques to monitor and manipulate their local and global activity patterns *in vivo* in both physiological and pathological contexts.

In this review, we focus on current and emerging approaches for measuring the activity of astrocytes at the synaptic, microcircuit, and systems levels. Although most of our insight into the physiology and function of astrocytes has come from *in vitro* studies (primary cultures, acute brain slices), emphasis here has been placed on techniques that enable visualization of their dynamics in the intact CNS of live animals, and insights that have been obtained from these studies. We conclude by discussing current technical challenges that need to be overcome to obtain a mechanistic understanding of the many roles of astrocytes in brain function.

Astrocytes in neural circuits
Astrocytes in different regions of the CNS share a number of common features — they have a high resting conductance to potassium and low membrane resistance, they are electrically unexcitable and lack synaptic specializations and long-range projections, they are extensively coupled to each other through gap junctions, they express a high density of glutamate transporters, they form end feet specializations on blood vessels, and they express G-protein coupled receptors that liberate intracellular calcium. Individual astrocytes also establish and maintain distinct territories, defined by their numerous, highly ramified processes, with adjacent cells occupying largely non-overlapping domains in rodents [4–6] (Figure 1d,e). Thin lamellae extend from their processes to wrap neuronal and non-neuronal structures [7], giving rise to their extraordinarily complex morphology (Figure 1f–h). At the tips of their processes they connect to each other through gap junctions; as a result, astrocytes form a vast network of interconnected cells, providing nearly complete coverage of the CNS.

Despite these shared characteristics, astrocytes are not homogenous. For example, fibrous astrocytes in white matter have processes that are more polarized and less complex than protoplasmic astrocytes, their gray matter counterparts, and astrocyte density varies between CNS regions and cell layers [8]. In addition, the complement of receptors and transporters expressed [9] and the extent of

gap junction coupling varies between different regions of the CNS [10], suggesting that they can adapt to the unique requirements of their local environment. Some physiological features, such as gap junction coupling [11], glutamate transporter expression [12], and synapse ensheathment [13] can be modulated on rapid time scales by neuronal activity, while aging and pathological conditions can induce slower but more dramatic phenotypic changes (e.g., reactive astrocytosis) [14]. These structural and functional alterations at the synaptic, microcircuit, and systems level [15••,16,17,18], are believed to help organisms adapt to new environmental demands, and conversely, disturbances in this homeostatic adaptation are likely to contribute to CNS disease [19].

Different scales of astrocyte functional dynamics

Astrocyte networks are particularly well positioned to integrate both neuronal and non-neuronal signals to regulate diverse CNS functions, such as neural network excitability and metabolism, on various spatial and temporal scales [20,21••]. In particular, astrocytes express a rich repertoire of G-protein coupled receptors for neurotransmitters, and in some regions, ligand-gated ion channels (NMDA and AMPA receptors), providing a means to modulate their physiology in response to local neural activity and global shifts in brain states. However, little is known about the types of information that astrocytes extract from these events or how astrocytes use this information to modify their behavior.

Although astrocytes express ligand-gated and voltage-gated ion channels, receptors, and electrogenic transporters, they do not exhibit large deviations in membrane potential in response to neuronal stimulation [22], due to their low resistance and high conductance to potassium, which effectively clamps their membrane potential at the potassium equilibrium potential. As a result, astrocyte activity has been largely invisible to electrophysiological methods, such as extracellular unit recording, that are used to monitor neuronal activity *in vivo*. The development of fluorescent indicators for calcium that can be loaded into cells led to the discovery that astrocytes exhibit dynamic changes in intracellular calcium [23,24] that are markedly enhanced by neurotransmitters. Distinct forms of calcium signaling have been described that involve activation of calcium-permeable plasma membrane channels and/or release of calcium from internal stores [25,26]. These astrocyte calcium transients are widely considered to be a form of glial excitability, but they do not exhibit a stereotyped waveform like action potentials, and thus the functional outcomes of this activity are likely to be more diverse and nuanced. Although it is certain that elevations in calcium are not the only form of activity exhibited by astrocytes, the ability to detect these changes has provided key insight into astrocyte dynamics and neuron–astrocyte interactions *in vivo*.

Microdomain activity

Astrocytic processes exhibit spatially confined (less than a few micrometers in diameter) calcium transients with an average duration of a few seconds [27,28] (Figure 2a,b); *in vivo*, this activity may be confined due to structurally isolated regions within the astrocyte arbor, termed microdomains. Microdomain activity has been observed in both anesthetized and awake animals and persists when neural activity and most, if not all, neurotransmitter receptors have been blocked, and is therefore thought to be intrinsically generated [29–31]. Moreover, activity in different microdomains is largely uncorrelated in the absence of stimulation [27,30], but can be synchronized when surface receptors are activated. However, because only a small region of the astrocyte, and thus only a tiny fraction of microdomains in a given cell, can be measured within the focal plane of a two-photon microscope, which is the predominant tool for monitoring astrocyte calcium signaling *in vivo*, a complete picture of their diversity and spatiotemporal evolution (i.e., in the z-direction) is lacking. Additionally, given the limited spatial resolution of two-photon microscopy much of the fine-scale dynamics of microdomain activity have not been resolved.

Somatic activity

In addition to spatially localized microdomain activity, astrocytes exhibit pronounced somatic calcium transients, particularly when animals experience strong sensory input or when astrocytes are directly stimulated with neuromodulators (Figure 2a,b,d). These somatic events result from IP3-mediated release of calcium from internal stores [32•,33•], are less frequent and exhibit somewhat slower kinetics than microdomain events [34,35•]. Both neuronal activity-dependent and activity-independent transients have been described, with the frequency and degree of temporal correlation depending on brain region [30,36]. Although it is tempting to speculate that activity in microdomains, like synaptic events in neurons, summate to produce somatic events if a threshold is exceeded, the precise functional relationship between microdomain activity and somatic events has not yet been established. Sensory-evoked activity in astrocytic somata tends to be sparse, variable, and stimulus-dependent [37,38••,39]. Due to tissue scattering and absorption, a lack of efficient long-wavelength calcium indicators, and technical challenges associated with imaging large tissue volumes, optical recording of large-scale astrocyte (somatic) activity is currently limited to a few hundred micrometers beneath the pial surface and relatively low (a few Hz) sampling rates. Thus, the relationship of these local events to neuronal and glial cell activity in other brain regions remains largely unexplored.

Localized multi-cellular waves of activity

Cortical cerebellar astrocytes (termed Bergmann glia) exhibit radially-expanding, ATP-dependent calcium waves that engage groups of astrocytic microdomains

within an ellipsoid volume tens of micrometers in diameter around their focal site of origin [31,40] (Figure 2c). This activity is reminiscent of the intercellular 'calcium waves' that can be induced in cultured astrocytes upon focal application of glutamate or mechanical stimulation [23,24,41]. These calcium waves depend on calcium release from internal stores, have an average duration of only a few seconds, appear to be independent of sensory input, and occur in both anesthetized and awake animals. Their frequency is low (tens to hundreds of mHz/mm^2) but may increase with age and reductions in tissue oxygen tension [42]. Differences in tissue preparation may contribute to the variable incidence of these events, as less invasive procedures show seemingly fewer and spatially less clustered events. Hence, the mechanisms that induce the release of ATP from astrocytes and the functional role of these multi-cellular calcium waves remains to be determined. Notably, this activity is rarely observed in the cerebral cortex [30,38••,43•] (but see [44]). *In vitro*, calcium wave propagation requires repetitive release of ATP and sequential activation of P2Y purinergic receptors as the wave spreads outward [45,46]; although gap junctions can modulate the speed and extent of propagation, they are not required. The smaller spread of these waves *in vivo* may reflect a higher abundance of extracellular nucleotidases, enzymes which hydrolyze ATP to adenosine.

Large-scale concerted activity

Two-photon imaging in primary somatosensory and anterior cerebellar cortex of head-restrained, mobile mice revealed that astrocytes show behavioral state-dependent and neuronal activity-dependent concerted calcium increases across areas several hundred micrometers in diameter with an average duration of many seconds [31,44] (Figure 2d,e). These concerted transients, which involve most, but perhaps not all astrocytes or astrocytic processes, also appear to depend on calcium release from internal stores. Their amplitude and probability of occurrence depends on inter-stimulus interval [31,38••], perhaps resulting from receptor desensitization or calcium store depletion. This large-scale calcium activity in astrocytes can be initiated by aversive stimuli [38••,43•], locomotion [31,38••], or direct stimulation of the nucleus basalis [32•] where cholinergic neurons are located, or locus coeruleus [47], where noradrenergic neurons are located. Using fiber photometry, which allows bulk but not cellular-level measurements, [38••], it was shown that concerted astrocytic calcium increases were synchronized across anterior cerebellar and primary (V1) visual cortices (Figure 2e). Thus, it appears that different neuromodulatory inputs can trigger widespread activation of astrocytes, in keeping with the diffuse, highly ramified nature of these projections. This study also reported that while visual stimulation alone was ineffective in evoking concerted astrocytic calcium responses in V1 of awake, resting mice, calcium transients in astrocytes were enhanced beyond that

produced by locomotion alone when visual stimuli were applied during locomotion, suggesting that norepinephrine can alter the responsiveness of astrocytes to local circuit activity. Visual stimuli-independent but locomotion-enhanced activity has also been described for cortical neurons (e.g., vasoactive intestinal peptide-positive V1 neurons, though in their case nicotinic inputs from basal forebrain were identified as the anatomical source of neuromodulation [48]). Although these studies suggest that widespread activation of astrocyte networks occurs when neuromodulators are released, the full three-dimensional extent of large-scale concerted astrocytic activity and its relationship to neuromodulatory projections and local neural network activity remains unknown. Approaches providing increased fields of view or simultaneous multi-site imaging, greater depth penetration, and fast volume sampling, would help define the patterns of astrocyte activity induced by neuromodulators in different behavioral contexts.

There is increasing appreciation that astrocytes in different regions of the CNS exhibit different functional characteristics [49]; for example, astrocytes in the cortex have been shown to express NMDA receptors [50], while those in the hippocampus do not appear to [22]. However, the pattern of activity exhibited by astrocytes *in vivo* in regions other than the superficial cortical layers remains largely unexplored. Long-wavelength multi-photon microscopy [51–53], which experiences less light attenuation, in combination with red-shifted calcium indicators, sparse tissue labeling, and adaptive optics approaches, which correct for tissue-induced wavefront distortions, will allow minimally invasive optical recordings from deeper brain regions. However, imaging depth is ultimately limited by fluorophore brightness, out-of-focus background fluorescence generation, the objective/detector's limited light collection angle, and other factors [54]. Optical recording from brain regions beyond the imaging depth limit has been achieved by aspirating overlying tissue, implanting a biocompatible tissue-stabilizing transparent gel or glass window-bearing guide tube, and imaging with a long working distance objective or gradient index (GRIN) lens [55–57]. Alternatively, optical components such as micro-prisms can be implanted directly into the brain to deliver and capture light [58]. Although these approaches have yielded new insight into the relationship between neural activity and behavior [55,56,59], they may be ill-suited for the study of normal astrocyte activity. Astrocytes are highly sensitive to tissue damage, and exhibit widespread and prolonged structural and functional changes following CNS injury [14,60]. Approaches that induce less inflammatory responses or can image far beyond the glial scar are needed.

Another largely unexplored issue is whether astrocyte activity patterns change over the course of hours, days, or weeks in response to changes in life experience. Long-term imaging studies in cortical and deep brain regions of

Figure 1
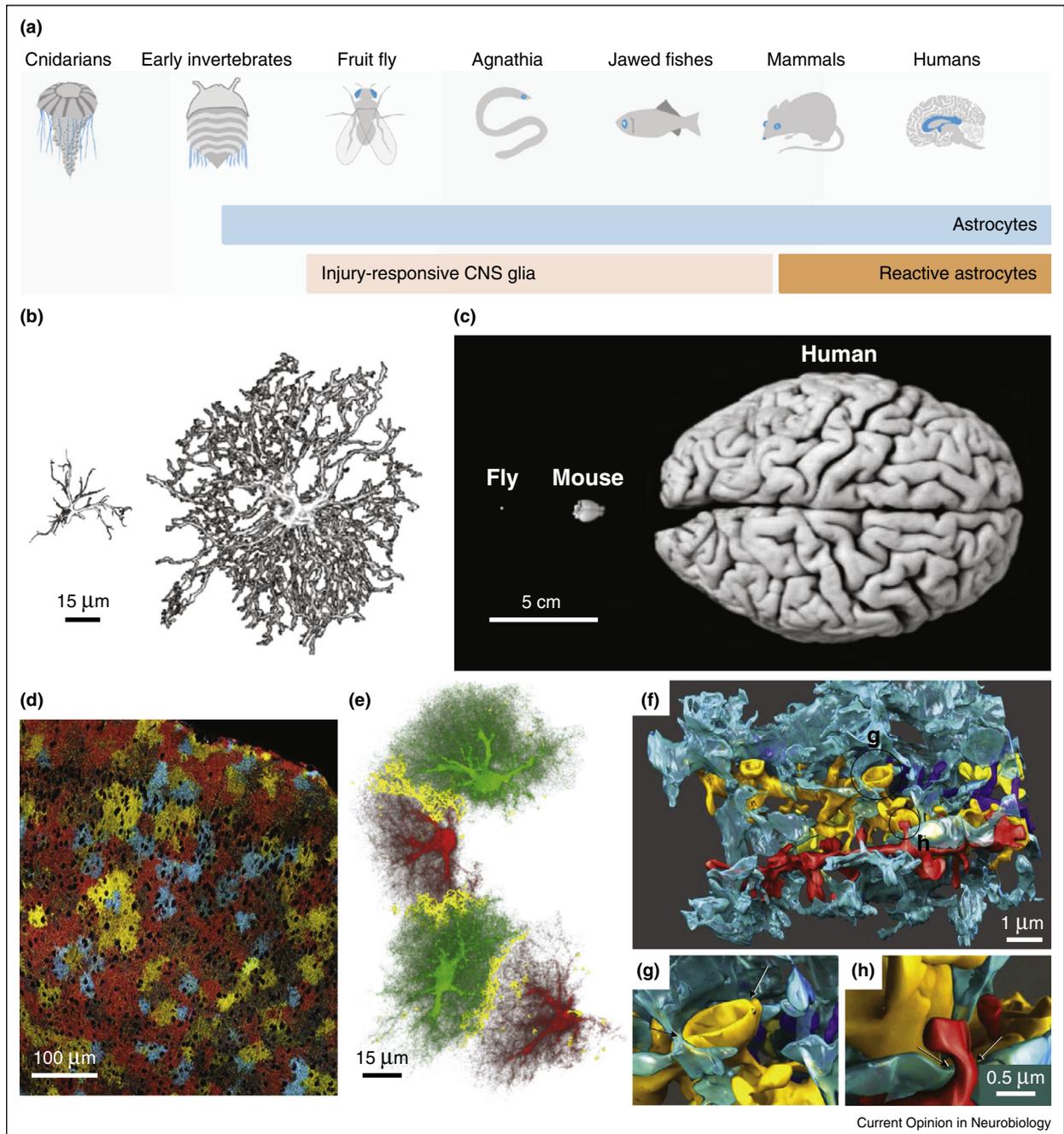
Astrocyte complexity across spatial scales and species. **(a)** Astrocytes are found in both vertebrate and invertebrate species, and their size and complexity increases with phylogeny (b). **(b)** Computer drawings show three-dimensional (3D) reconstructions of mouse (left) and human (right) cortical astrocytes based on glial fibrillary acidic protein (GFAP) immunostaining. Note that GFAP-positive filaments are restricted to the cell body and main processes of astrocytes, representing only a small fraction (∼15%) of the cell's actual volume. Cortical astrocytes with different morphologies are present in the human brain (not shown), suggesting that greater diversification has occurred with evolution. **(c)** Brain size changes with phylogeny. Because scattering and absorption restrict fluorescence imaging depth, a smaller proportion of astrocytes can be visualized in the larger brains of higher organisms *in vivo*. **(d)** Fluorescence image shows cross-section through the cortex of a 'Brainbow' mouse, in which three different fluorophores were expressed stochastically in astrocytes. Dark round areas represent primarily neuronal cell bodies, illustrating the extraordinary coverage of the CNS by astrocytes. In a given brain region, astrocytes are extensively coupled through gap junctions. **(e)** Image shows 3D reconstruction of four dye-filled astrocytes in mouse dentate gyrus. Within gap junction-coupled networks, individual astrocytes (green or red) occupy distinct territories that exhibit minimal overlap (yellow) with those of neighboring astrocytes. **(f)** Image shows 3D reconstruction from electron microscopy data of four dendrites (red, yellow, gold, purple) and protrusions of a nearby astrocytic process (blue) in rat hippocampus. Processes of individual astrocytes exhibit highly intricate lamellar protrusions.

behaving mice have revealed complex learning-related changes in neuronal activity patterns, such as enhanced temporal correlation of activity among neurons that respond to similar aspects of a learned task [61], increases in task-related population activity despite variable single-neuron responses [62], and neuron type-dependent and layer-dependent changes in ensemble activity across trials [63,64]. Given the close interrelationship between astrocytic and neuronal activity, changes in the spatiotemporal excitation patterns of astrocytes may also occur following training, as a consequence of the change in activity patterns of nearby neurons or as a result of intrinsic adaptive changes. Indeed, recent studies suggest that secretion from astrocytes can alter ensemble neuronal network activity such as gamma oscillations [65••] and influence cyclical behaviors such as sleep [66,67] (but see [68•,69]). The development of new transgenic mouse lines in which genetically encoded calcium indicators (GECIs) can be expressed stably in astrocytes will help to define astrocyte network activity on more prolonged time scales [35•,38••,70•,71], and enable simultaneous optical monitoring of astrocyte and neuronal activity through cell-specific expression of different-colored GECIs [72,73].

Although we do not yet know if or how learning alters astrocyte activity patterns, it is clear that calcium signaling in astrocytes is not static. Evolving changes in the spatiotemporal activity patterns of astrocytes have been observed in many diseases and following traumatic injury to the CNS [74]. For example, astrocytes in mouse models of Alzheimer's disease exhibit higher resting calcium levels, a higher frequency of calcium transients, and intercellular waves that propagate outward from amyloid deposits [75]. In ischemic stroke, astrocytes at and near the lesion site show larger amplitude, higher frequency, and more synchronized, calcium wave-like activity during the acute phase [76]; changes in astrocytic calcium responses were also seen in the contralateral hemisphere [77], suggesting that their activity can be altered without direct injury. In addition, astrocytes show region-dependent structural changes during early and late post-ischemic phases [78,79]. Similarly complex structural and functional alterations are seen in other cell types [80–82]. However, most of these cellular-level changes in astrocytes have been recorded in cortical areas and represent between-animal comparisons, rather than longitudinal studies of the transformation of individual astrocytes. Although long-wavelength and multi-site multi-photon imaging approaches are likely to increase the depth and area that can be monitored in the future, large parts of the affected tissue will remain inaccessible with these high-resolution techniques. One way to bridge this gap is to combine high-resolution imaging with lower-resolution, whole-tissue imaging approaches [82,83,84••], allowing cellular level observations to be related to systems level changes and behavioral phenotypes.

## Defining the role of astrocytes in neural networks

Although fluorescent indicator imaging has revealed the remarkable diversity of astrocyte activity patterns in intact circuits, much remains to be learned about the pathways involved in generating various forms of astrocytic excitation, and their downstream functional consequences for astrocytes (e.g., acute metabolic regulation or long-term gene expression changes), and for surrounding neuronal and non-neuronal cells (e.g., neuronal excitability or blood flow changes).

To bridge this gap in our understanding, a variety of *in vivo* approaches are being employed to interrogate and manipulate astrocyte activity in healthy animals. For example, *in vivo* pharmacology has revealed that some forms of excitation, particularly spatially localized events within astrocytes, do not depend on neuronal activity, and for the ones that do, the signaling pathways that contribute to their generation [31,38••,43•]. In addition, transgenic approaches have shown that large-scale, correlated activity in astrocyte networks rely on IP3-mediated release of calcium from internal stores as a result of G-protein coupled receptor activation [32•,33•,85]. They have also provided evidence that calcium excitation can lead to local neuromodulatory effects and changes in animal behavior [65••,67,70•]. Transgenic manipulation is not always possible or practical; thus, development of approaches to acutely alter gene expression in astrocytes, such as viral infection [17] or *in utero* electroporation [86] provide additional opportunities for mechanistic studies of astrocyte functions *in vivo*.

Nevertheless, interrogating the behavior of astrocytes and defining the consequences of their activity in live animals remains challenging. For example, with *in vivo* pharmacological experiments it is difficult to control the concentration and sites of influence, limiting conclusions that can be made regarding the receptors and intracellular pathways involved in a response. Additionally, given our limited knowledge about *in vivo* drug actions [87] and the expression of receptors in other cell types [88,89], pharmacological interventions may lead to unexpected side effects (e.g., affect ion channel surface expression [90]). Exogenous fluorescent indicators can themselves

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(Figure 1 Legend Continued) (g) and **(h)** Astrocytic protrusions contact some but not all neuronal boutons or spines. Images are blow-ups of regions indicated in (f). (d–h) Note that images are snapshots taken at a particular point in the animal's life. Astrocytes remain structurally and functionally plastic in the adult brain. See text for more details. (a–b), (f–h) adapted from [107,130,131] with permission from Elsevier, and John Wiley and Sons. (c) Courtesy of Dr. Frank Hirth, King's College London. (d) Courtesy of Jean Livet, Joshua Sanes and Jeff Lichtman, Harvard University, and adapted from [5] with permission from Nature Publishing Group. (e) Reprinted from [132], copyright (2006) National Academy of Sciences, U.S.A.

Figure 2
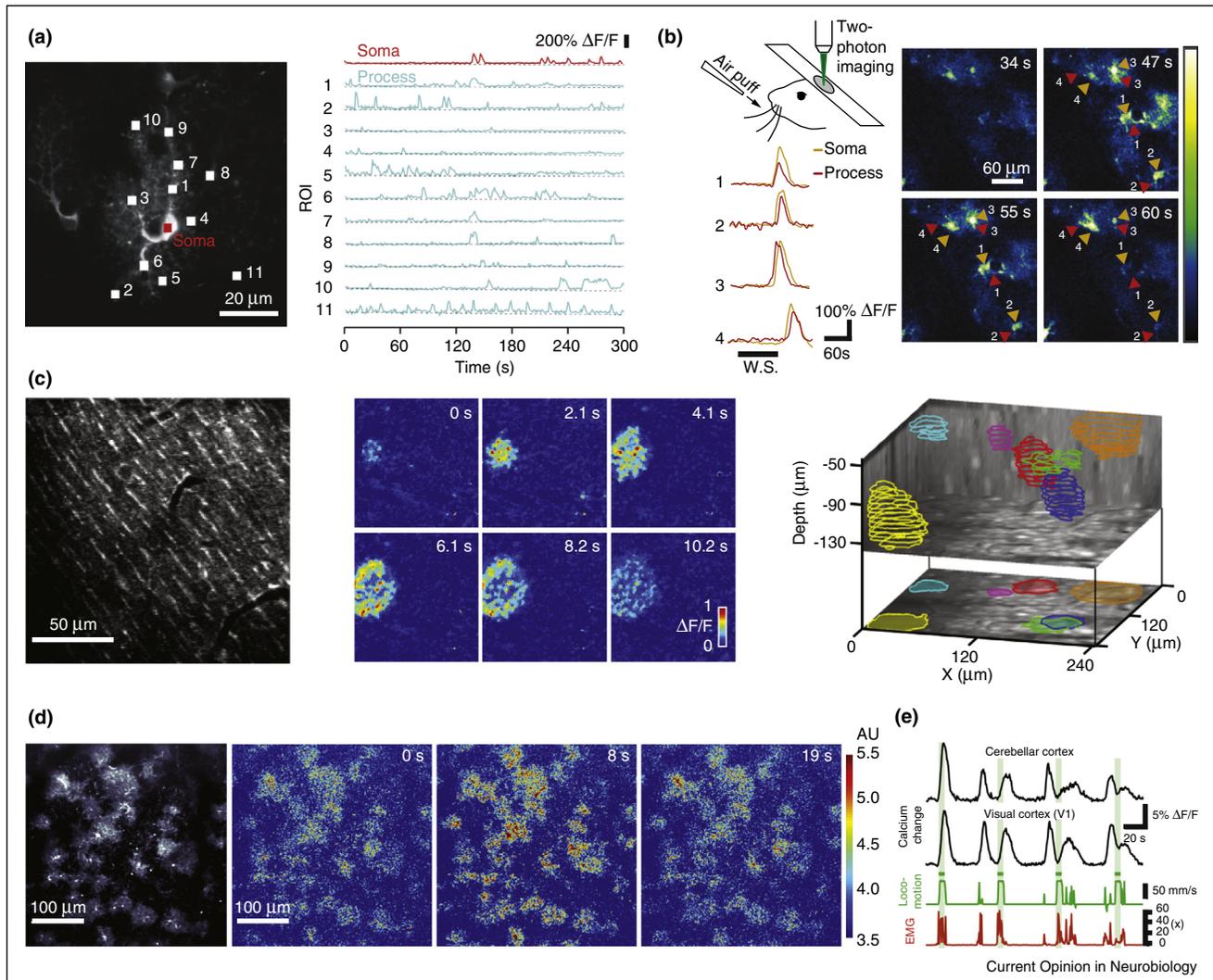
Representative forms of astrocytic calcium activity in the adult brain. **(a)** Spontaneous calcium activity in astrocytic processes and soma. *Left*, image shows a GCaMP3-expressing astrocyte in stratum lucidum of a mouse hippocampal slice. *Right*, calcium transients detected in the numbered regions of interest (ROIs) shown at the left. **(b)** Sensory-evoked calcium activity in astrocytic processes and somata *in vivo*. *Top left*, schematic of experimental setup. *Right*, time-lapse images showing GCaMP5G-expressing astrocytes in layer 2/3 of barrel cortex from an anesthetized mouse. Time after onset of whisker stimulation (W.S.; 5 Hz, 30 s duration) is shown in top right corners. Pseudocolor scale on right indicates changes in GCaMP5G fluorescence over baseline ($\Delta F/F_0$). *Bottom left*, time course comparison of evoked calcium transients in astrocytic processes (red) and somata (orange) from regions indicated on the right. **(c)** Spontaneous, localized multi-cellular calcium waves/bursts in portions of few neighboring cortical cerebellar astrocytes (Bergmann glia) *in vivo*. *Left*, fluorescence image showing optical cross-section through processes from many different astrocytes in the molecular layer of cerebellar cortex from an anesthetized rat stained with synthetic calcium indicator Fluo-5F. *Center*, time-lapse images showing spatiotemporal evolution of a multi-cellular calcium wave, displayed as $\Delta F/F_0$, at a select imaging depth. Time after event onset is indicated in top right corners. *Right*, volumetric profiles of individual calcium waves/bursts (shown in the same color) detected by 3D two-photon microscopy during an 8.2 min imaging period. **(d)** Locomotion-evoked calcium transients in astrocytes of the visual cortex (V1) *in vivo*. *Black and white image*, fluorescence image of layer 1 astrocytes in primary visual cortex (V1) of a *GLAST-CreER;R26-lsl-GCaMP3* mouse. *Color images*, spatiotemporal evolution of concerted astrocytic calcium activity. Time after onset of walking on a linear treadmill is indicated in top right corners. **(e)** Locomotion-evoked large-scale calcium activity in populations of astrocytes from two distinct brain regions, measured simultaneously using dual fiber-optic photometry. Black traces show $\Delta F/F_0$ in GCaMP3 bulk fluorescence from astrocytes in indicated brain regions. Green shows locomotor activity and rest as detected by an optical encoder coupled to the linear treadmill; periods of enforced locomotion are highlighted by the horizontal green bars and vertical green-shaded areas. Red trace shows simultaneously recorded electromyography (EMG; given as fold increase in 200 Hz–1 kHz EMG power). (a–e) Note that kinetics, amplitude and frequency of detected calcium transients may depend on employed fluorescence indicator or staining method. See text for more details. (a–e) Adapted from [31,34,35*,38**,40] with permission from Elsevier and National Academy of Sciences.

influence optical recordings: insufficient indicator may lead to the erroneous conclusion of inactivity, while surplus indicator may compromise normal astrocytic function by chelating calcium [91]. In addition, transgenic approaches, particularly those involving constitutive expression of transgenes [92], may lead to whole-brain or whole-animal disturbances, making it difficult to relate local changes in astrocytic activity to altered microcircuit function or behavioral phenotypes [93,94•]. Chemogenetic approaches, such as those involving DREADDS or optogenetic approaches, such as those which utilize channelrhodopsin or light-responsive G-protein receptors [95], have been effective at delineating the contribution of different classes of neurons and neural networks to behavior. At present, it is unclear whether the collection of genetically encoded effectors currently available will be similarly useful in defining the role of astrocytes or whether further modification or development of new effectors will be required; there is concern that these proteins may only partially engage endogenous signaling cascades (e.g., through pharmacological activation of foreign receptors expressed in astrocytes), cause inadvertent shifts in ion gradients (e.g., promote proton influx through channelrhodopsin) or insufficiently mimic normal spatiotemporal forms of astrocytic excitation (e.g., by recruiting pathways only active under high levels of calcium, or by inhibiting normal cell signaling in the aftermath of concerted calcium store depletion). The mode of gene delivery can also lead to problems. In particular, viral vectors can induce inflammatory responses that may affect astrocyte morphology or function directly or indirectly (e.g., through microglia-mediated chemokine release) [96], and all Cre-ER mouse lines used to manipulate gene expression in astrocytes (e.g., *GLAST-CreER, GFAP-CreER, Cx30-CreER*) also influence radial glial cells, leading to altered gene expression in neurons within the dentate gyrus and the olfactory bulb, which are continually generated from radial glia in adulthood. Finally, imaging itself can lead to phototoxic effects that may influence the frequency and form of astrocytic excitation [37], and surgical preparation, such as thinning or removing the skull overlying the imaging site, can induce reactive gliosis [97,98].

Similar challenges exist for studying the role of astrocytes in disease. For example, optogenetic manipulation of astrocyte activity following ischemic stroke induces ion and transmitter level alterations that reduce brain damage [99]. However, due to tissue scattering and absorption, such optical approaches are currently limited to comparatively small tissue volumes. In addition, optical accessibility may change over time due to tissue swelling, neovascularization, or glial scar formation. Likewise, astrocytic gene expression and morphology may change over time [100] potentially affecting the specificity of genetic targeting and pharmacological interventions. Development of red-shifted calcium indicators [72,101] and opsins [102,103], more efficient and less inflammatory

viral vectors [96], more specific Cre/CreER mouse lines to manipulate distinct populations of astrocytes using information gained from gene expression analysis [89,104••], will alleviate some of these issues and help resolve existing controversies in the field.

Astrocytes in different species
Astrocytes have evolved with phylogeny (Figure 1a). Human astrocytes, as defined by GFAP expression, are larger, structurally more complex, and contact many (around one order of magnitude) more synapses than their rodent counterparts [105] (Figure 1b). In addition, they show differences in calcium signaling. Humans and primates also exhibit types of astrocytes not found in rodents (e.g., interlaminar astrocytes whose processes traverse several cortical layers) [3,105]. Likewise, rodent astrocytes are larger, more complex, and functionally different from their fish, fly, or worm counterparts. Some of these differences appear to be cell autonomous, as human glial progenitors transplanted into the mouse brain develop into astrocytes and retain their larger size and more complex morphology [106]. Such xenographs may provide a means to study the unique characteristics of human astrocytes in an *in vivo* context. Despite the differences, many astrocyte signaling pathways appear conserved across species [107], similar to neural circuitries and adaptive behaviors [108]. In addition to their accessibility to genetic manipulations, organisms such as *C. elegans*, *Drosophila*, zebrafish, and mice have the distinct advantage of reduced size and complexity (Figure 1b,c), making them amenable to large-scale imaging of cellular networks. For example, recent studies in head-immobilized, optically transparent zebrafish larvae demonstrated the feasibility of whole-brain imaging with single-cell spatial and up to tens of Hertz temporal resolution using light sheet [109], or light field microscopy [110], and large-scale data analysis [111]. A similar analysis of astrocyte-like/astroglial cells and networks in the zebrafish brain [112] remains to be performed.

Conclusions
Astrocytes form a complex network of highly ramified, interconnected cells that are common to all regions of the CNS. Like neurons, they express many distinct classes of cell surface receptors and exhibit a form of excitability based on changes in intracellular calcium. Understanding how astrocytes detect and respond to changes in their environment, and ultimately influence other neuronal and glial cell populations, remains an ambitious, but achievable goal. Many approaches that have initially been developed to interrogate neuronal networks have been readily adopted by glial biologists, such as *in vivo* two-photon imaging and genetically encoded calcium indicators. However, the study of astrocytes presents many additional challenges — their activity is potently inhibited by anesthesia, they undergo dramatic morphological and physiological changes following CNS injury, and they do not exhibit stereotyped electrical activity akin to

action potentials. Moreover, the simple addition of calcium indicators, which are themselves calcium buffers, and the act of illuminating tissue to visualize fluorescence changes, can markedly alter the state of astrocyte activity. Uncovering their full range of spatiotemporal activity patterns, particularly in behaving animals and animal models of human disease, will require the development and application of new imaging approaches that extend the depth of imaging, enhance the imaging area, report the activity of distinct signal transduction pathways, and enable visualization of astrocyte activity simultaneously with that of neurons, glia, and vascular cells. Relating their widely varying forms of activity to synaptic, microcircuit, and higher-order function will require combining large-scale imaging techniques with large-scale *in vivo* staining, manipulation, network anatomical, genomic, and computational approaches. Although some of these complementary tools exist, others will need further or new development for use with astrocytes. For example, while an ever-growing list of optogenetic approaches enables precise manipulation of electrical activity and protein function in genetically defined neurons and their compartments [95,113–116], a comparable list of tools for precise spatiotemporal control of astrocyte function is largely lacking [117]. Likewise, optical approaches for three-dimensional control of cellular activity patterns are in their infancy [118–121]. There is perhaps undo emphasis being placed on calcium signaling at the present time, reflecting, in part, the tools available to monitor this

behavior, but it is clear that astrocytes express receptors that couple to signaling pathways that do not directly alter intracellular calcium. Our knowledge about the role of these forms of signaling is very limited. Nevertheless, existing approaches have already begun to provide exciting new insight into how astrocytes participate in physiological functions such as sleep, breathing, feeding, and metabolism, and pathological processes associated with stroke, CNS trauma, and diseases such as epilepsy and Huntington's disease [19,21••,122,123].

Decoding how astrocytes integrate information from various cell types, adapt their behavior, and modulate brain physiology will further our understanding of how homeostasis and neuromodulation are achieved in the CNS, and reveal new therapeutic directions to treat complex diseases. Although much remains to be discovered about their diverse functions, it is clear that astrocytes are true stars in the complex CNS universe (Box 1).

Conflict of interest statement
Nothing declared.

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Box 1 Selection of unresolved biological questions

Synaptic level
- How are frequency, duration, spatial and temporal pattern of synaptic activity encoded in the time course and spatial extent of astrocytic calcium activity [20]?
- Do astrocytic microdomains contain functionally independent compartments, similar to dendritic spines [124]?
- How do astrocytes functionally respond to diverse spatiotemporal inputs from different cell types (e.g., excitatory or inhibitory neurons, oligodendrocytes or microglia)?
- To what extent do microdomain events summate to trigger somatic calcium transients?

Microcircuit level
- How functionally independent are individual astrocytes in local gap junction-coupled networks?
- What is the relationship between astrocytic calcium excitation and natural activity patterns in neuromodulatory projections from different anatomical sources in behaving animals?
- How do the distinct forms of astrocytic calcium transients influence astrocytic gene expression, their physiological properties, gap junction coupling, or morphology [125]?
- How are astrocytes' spatiotemporal activity patterns linked to regional differences in gene expression profile [126–129]?
- What role does astrocyte network activity play in regulating network function/homeostasis in healthy and diseased animals [21••]?

Systems level
- How do large-scale systemic changes (e.g., circadian rhythm/sleep or aging) influence astrocytic activity and effector function?

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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