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Mini review

Looking to the stars for answers: Strategies for determining how astrocytes influence neuronal activity

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ABSTRACT

Astrocytes are critical components of neural circuits positioned in close proximity to the synapse, allowing them to rapidly sense and respond to neuronal activity. One repeatedly observed biomarker of astroglial activation is an increase in intracellular Ca^{2+} levels. These astroglial Ca^{2+} signals are often observed spreading throughout various cellular compartments from perisynaptic astroglial processes, to major astrocytic branches and on to the soma or cell body. Here we review recent evidence demonstrating that astrocytic Ca^{2+} events are remarkably heterogeneous in both form and function, propagate through the astroglial syncytia, and are directly linked to the ability of astroglia to influence local neuronal activity. As many of the cellular functions of astroglia can be linked to intracellular Ca^{2+} signaling, and the diversity and heterogeneity of these events becomes more apparent, there is an increasing need for novel experimental strategies designed to better understand the how these signals evolve in parallel with neuronal activity. Here we review the recent advances that enable the characterization of both subcellular and population-wide astrocytic Ca^{2+} dynamics. Additionally, we also outline the experimental design required for simultaneous *in vivo* Ca^{2+} imaging in the context of neuronal or astroglial manipulation, highlighting new experimental strategies made possible by recent advances in viral vector, imaging, and quantification technologies. Through combined usage of these reagents and methodologies, we provide a conceptual framework to study how astrocytes functionally integrate into neural circuits and to what extent they influence and direct the synaptic activity underlying behavioral responses.

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1. Introduction

Unlike neurons, astroglia do not require rapid transmission of electrical signals in the form of action potentials to communicate with nearby cells [1], and as a result they were initially considered to be passive players in brain function with a limited influence on synaptic communication. Yet, the structural complexity and number of astroglia increase along the evolutionary timeline, reaching their apex in the human brain [2,3]. Moreover, the morphological properties of astrocytes enable just a single astrocyte to contact and monitor hundreds of dendrites and by extension hundreds of thousands of synapses [4–7]. We now know that astrocytes respond directly to neuronal neurotransmitter release [8,9], and react to bouts of synaptic activity with homeostatic regulation of ion buffering [10], clearance of neurotransmitters [11], and with the release of various neuroactive chemicals [5,12–14]. These properties allow astroglia to differentiate and uniquely respond to activity at diverse synaptic inputs [15]. Astrocytes are responsible for the vast majority of GABA and glutamate clearance from the synaptic cleft following neuronal release, a cellular function that is mediated via patterned expression of various transporters including GAT3 (GABA) and GLT-1 (glutamate), with modifications in the expression or localization of transporters serving as a means to directly influence neurotransmission and synaptic plasticity [16–18]. In addition to neurotransmitter uptake systems, astrocytes express a myriad of ionotropic and metabotropic neurotransmitter receptors which allow them to sense and respond to synaptic activity [19,20]. Moreover, the majority of astrocytes contact blood vessels, serving as an intermediary between neurons and the neurovascular network [21], both to provide metabolic support and to deliver the required precursors for the generation of GABA and glutamate, a cellular function that is required for normal synaptic communication and physiological processes [22]. Because of the large body of work detailing their role in modulating synaptic plasticity, astrocytes are now appreciated as active components of neural networks with diverse and functional roles in neuronal processing.

Among other intracellular processes [23], astroglia respond to bouts synaptic activity with intracellular calcium (Ca^{2+}) elevations, occurring across multiple cellular compartments, including the astrocytic cell body or soma, the astroglial primary branches, and the fine membranous peripheral perisynaptic astroglial processes (PAPs) [6,24]. Generally, elevated intracellular astrocytic Ca^{2+} levels are considered the cellular substrate of astrocytic action, a phenomenon often linked to a biological response in astroglia, often thought as somewhat of a proxy for the electrical excitation and action potentials observed in neurons [24,25]. Indeed, many of the cellular functions of astroglia, including aspects of the homeostatic support of neurons and their ability to influence synaptic communication, rely on intracellular Ca^{2+} signaling [26]. Consequently, a considerable body of work now demonstrates that tightly regulated astroglia Ca^{2+} dynamics and the related homeostatic function of astroglia are required for many normal physiological processes [27,28], with disruption of these cellular systems linked to the pathophysiology of neuropsychiatric diseases, including substance use disorders [22,29–45]. As we progress further in understanding the role of astroglia in shaping neural communication, there is an increasing need to evaluate astrocytic spatiotemporal Ca^{2+} dynamics to better understand the mechanistic underpinnings of how astrocytes influence local neurotransmission at both the single cell and circuit-level *in vivo*. To date, a significant amount of pioneering work in this field has been done *ex vivo*, which has provided a solid understanding of the bidirectional chemical communication between neurons and astrocytes, including the astroglial intracellular signaling pathways leading to astrocytic Ca^{2+} dynamics, and the neuroactive chemicals released by astroglia as a result of this intracellular signaling modality [25,26,46–48]. However, current strategies, such as *in vivo* two-photon calcium imaging in behaving animals [48–50], combined with advanced analysis pipelines [51,52], now enable the study of astrocytes as potential computational entities, allowing for a more complete understanding of if and how astroglia shape neuronal activity patterns during complex behaviors, including learning and motivated responses. Importantly, while astrocytic Ca^{2+} events are remarkably heterogeneous (discussed in length below) and can occur at spatially restricted subcellular domains within individual cells [53], these events can also expand across multiple cells in astroglial networks [54,55], which likely reflects their ability to influence neuronal network activity. Further, evidence suggests that the functional outcomes of these spatiotemporally distinct Ca^{2+} events are themselves functionally unique [56]. Accordingly, care must be taken to interpret Ca^{2+} signals in context of astrocyte morphology, given the complex structure–function relationship exhibited by astroglia. As an example, the spatiotemporal specifics of Ca^{2+} dynamics enable astrocytes to gate and influence neurotransmission in a variety of modalities, including through the dynamic structural remodeling of their physical synaptic presence and by extension the regulation of the literal space that neurotransmission occurs in [57]. Apart from this, Ca^{2+} dynamics have also been directly linked to the release of glial-derived neuromodulators and adaptations in ion buffering or transmitter uptake [26,47,58], underlining the need to better understand Ca^{2+} signaling in astroglia and how it is linked to alterations in synaptic plasticity.

1.1. Examination of astroglial structural plasticity

As described above, astrocytic structural complexity increases across the evolutionary timeline reaching its apex in the human brain, with human astrocytes displaying a more ramified and complex overall structure that is accompanied by more efficient and rapid induction of intracellular Ca^{2+} signaling as compared to their rodent counterparts [59]. Armed with this information, the Nedergaard laboratory performed a study utilizing chimeric mice where human glial cell progenitors were engrafted into the murine forebrain, including the hippocampus and cortex. Remarkably, the human astroglial progenitors became mature astrocytes that fully integrated into the mouse brain and extant glial syncytium, forming functional gap junction connections with murine host cells, yet retaining the larger structural profile and enhanced overall complexity that typifies hominoid astroglia. Interestingly, the hominoid astrocytes displayed more rapid induction of Ca^{2+} events and functionally enhanced hippocampal long-term potentiation, resulting in enhanced behavioral performance in a variety of cognitive and conditioning tasks [60]. This study not only speaks to the structure–function relationship evident in astroglial cells, but also establishes that enhanced glial cell complexity and Ca^{2+} signaling efficacy can be directly linked to the synaptic plasticity underlying cognitive performance and learning, demonstrating that astroglia functionally influence neural communication as it pertains to learning and memory. Despite being less complex than their human counterparts, rodent astroglia still serve as an excellent model system to examine the functional influence of astroglia on neural networks. Among other complex functions, rodent astrocytes display experience-dependent structural remodeling in a Ca^{2+}-dependent manner, enabling astrocytes to regulate the extent of their interaction with neighboring active synapses and to facilitate neurotransmission [57]. Taken together, data from studies of both hominid and rodent astroglia support the general hypothesis through their structural complexity, interaction with synapses, and

their ability to chemically respond to neural activity, astrocytes actively regulate and tune neurobiological processes. These data highlight the need for the continued examination of astrocyte physiology, adaptations in transporter expression, morphology, synaptic interaction, and Ca^{2+} dynamics to better understand their role in directing and refining synaptic plasticity.

Initially, studies examining rodent and human astroglial numbers and overall morphology focused on alterations in glial fibrillary acidic protein (GFAP), a cytoskeletal protein found in the primary astrocytic branches, often used as a canonical astrocytic marker. However, this approach has significant limitations given reports that GFAP expression is not present in all astroglia [61–63] and that GFAP, when present, only constitutes about 15 % of total cell volume. Accordingly, the signal attained from GFAP staining provides a partial and largely incomplete representation of the complex membranous structure of astrocytes and vastly underestimates the extent of their presence at the tripartite synapse [64]. The use of dye filling strategies [64,65] and viral vectors utilizing GFAP promoter-driven cytosolic or membrane-targeted fluorescent probes, such as LCK-GFP, in combination with high-resolution microscopy now allows for a more complete visualization of astroglia including their perisynaptic ramifications and fine membranous peripheral processes [66]. Further, employing the highest resolution imaging strategies, including stimulation emission depletion (STED) microscopy or electron microscopy, allows for detailed visualization of astrocytes in the context of the synaptic microenvironment and the extent of PAP coverage of the pre- and post-synaptic cell [6,67–69]. Using these techniques, it is now possible for Ca^{2+} events in the astroglial leaflets to be mapped onto super-resolution micrographs to detail nodes of activity in the tripartite synapse [6]. By examining astroglia with these strategies, it has become evident that astrocytes tile the parenchyma, occupying largely non-overlapping domains or territories [64], and have an active synaptic presence [6,69]. Akin to neuronal processes, astroglial processes are also subject to experience-dependent plasticity, that occurs in combination with modulation of nearby neurotransmission [27,57,70]. In parallel with the experience-dependent synaptic plasticity and concordant morphological plasticity observed at dendritic spines, synapse-associated astroglial processes exhibit enhanced motility following induction of LTP, demonstrating that astroglia also exhibit brain-state dependent structural plasticity [57]. Interestingly, LTP-induced astroglial structural plasticity has direct consequences for excitatory neurotransmission, as LTP induction protocols can cause GLT-1-rich PAPs to retract from potentiated synapses, allowing for glutamate spillover and enhanced glutamatergic signaling, possibly extending to intrasynaptic crosstalk [70]. These data demonstrate that astroglial structural plasticity can directly impact excitatory neurotransmission. In keeping with these findings, our laboratory and others have demonstrated drug- and withdrawal-dependent reductions in the morphometric features of astrocytes and reduced synaptic interaction within the nucleus accumbens, phenomenon that has been directly linked to relapse vulnerability following drug exposure and withdrawal. Additionally, re-exposure to drug-conditioned cues also induces astrocytic structural plasticity and restores astrocytic synaptic presence in the nucleus accumbens, with these astrocytic changes critical in limiting cue-drug seeking [30,31,33,43,71]. Changes in astroglial structure and synaptic interaction are often accompanied by additional functional adaptations including downregulation of GFAP and the glutamate transporter, GLT-1, resulting in impaired glutamate homeostasis at the tripartite synapse [30,72]. Collectively, these results demonstrate that astroglial plasticity and adaptations in their synaptic presence are directly linked to the neuroplastic adaptations that underlie relapse vulnerability and cue-induced drug seeking.

Many studies, including the ones discussed above, employ viral vectors to selectively engage astrocytic expression of a membrane-targeted fluorescent molecule [lymphocyte-specific protein tyrosine kinase (LCK)-GFP] to assess adaptations in astroglial morphometric features (surface area, volume) and changes in the extent of astrocyte-synapse interactions. Inclusion of LCK allows for the fluorophore to be trafficked to the cellular membrane [73], and enables a more complete visualization of the fine astrocytic processes that would otherwise be lost using cytosolic markers [66]. Fig. 1 illustrates the level of detail gained from employing an adeno associated viral (AAV) construct packaged under the truncated GfaABC1D promoter to express the membranous LCK-GFP (AAV2/5-GFAP-LCK-GFP) in concert with a cytosolic tdTomato (AAV2/5-GFAP-tdTomato). The combination of AAV serotype 2/5 and GfaABC1D promoter is a commonly used strategy for viral transduction of mammalian astrocytes [74,75], although the AAV8 serotype and/or ALDH1 promoter have also been used to effectively and selectively transduce astroglia [76,77]. As the methodologies for labeling and manipulation of astroglia have become established and refined, expression of optogenetic constructs [27,78–80], chemogenetic constructs [28,81–85], and various genetic fluorescent indicators for Ca^{2+} [18,48,49,86,87], as well as various fluorescent neurotransmitter indicators [75,88] have become more routinely employed. As these tools are being utilized for the study of astroglial function as well as astroglial Ca^{2+} dynamics both *ex* and *in vivo*, the heterogeneity and complexity of astroglial Ca^{2+} dynamics, and the underlying functional relevance of this signaling modality, is beginning to be elucidated.

1.2. Astroglial calcium dynamics and their subcellular localization

As described above, astrocytes typically respond to bouts of synaptic activity with Ca^{2+} transients in various cellular compartments including astroglial leaflets or microdomains within the cellular architecture [6,87]. These calcium events can then expand to encompass the somatic region of the cell, or the entirety of the astrocyte itself. Beyond Ca^{2+} propagation within individual cells, astrocytic Ca^{2+} dynamics can spread across individual territories to adjacent cells within the astroglial syncytium, an effect that occurs as a result of gap junction connectivity, cytoplasmic continuity, and diffusion of second messengers like inositol 1,4,5-triphosphate (IP_3), ATP, and diacylglycerol [55]. This phenomenon is often described as Ca^{2+} waves [54,89], and has been linked to synchronization of neuronal activity [46,58]. In addition to the regional diversity within the cell structure, astrocytic Ca^{2+} signals also display temporal heterogeneity, with the duration of Ca^{2+} events lasting from hundreds of milliseconds to tens of seconds [90,91], with rapid microdomain Ca^{2+} linked to vasodilatation [92], cerebral blood flow [86], as well as neuromodulation via astroglial-derived chemicals [24,93].

Early explorations into the mechanistic underpinnings of astroglial Ca^{2+} dynamics and the functional relevance of Ca^{2+} mobilization from internal stores were somewhat inconclusive. While organism-wide activation of astroglial G-protein coupled receptors (GPCRs) via chemogenetic manipulation was shown to impact autonomous nervous system function and various behaviors tied to locomotion, deletion of an IP_3 receptor subtype most prominently expressed in astroglia (IP_3R2) and subsequent blockade of GPCR action via disruption of IP_3 signaling did not prevent this behavioral phenotype [94]. Moreover, selective genetic deletion of astroglial IP_3R2 did not appear to dramatically influence neuronal activity, anxiety- or depression-like behaviors, or learning and memory [94,95]. In parallel, early means for measuring astrocytic Ca^{2+} dynamics were carried out via bulk loading of Ca^{2+} indicator dyes, such as OGB-AM or Fluro-AM, methods that have considerable limitations [96], including low signal-to-background

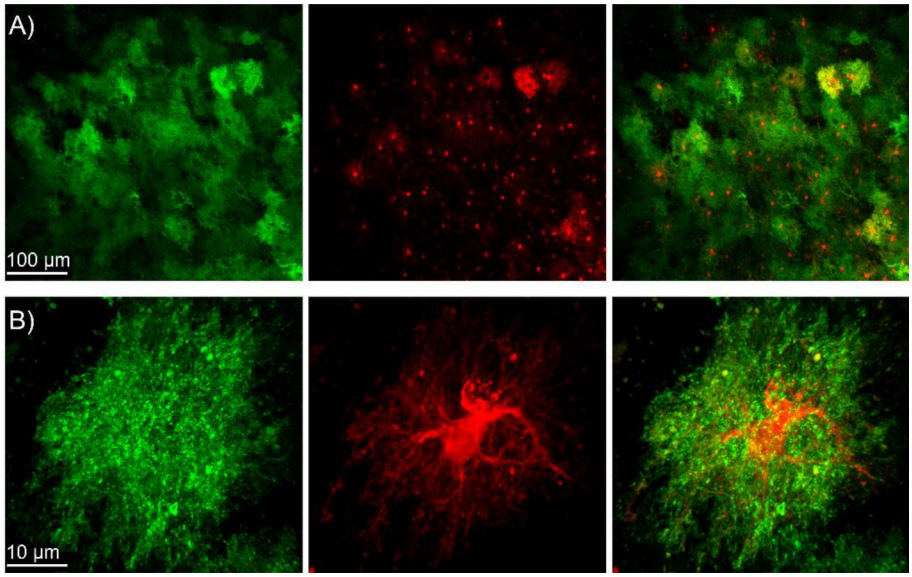

Fig. 1. Comparison of membrane targeted vs cytosolic astroglial expression of fluorescent proteins. A) Low magnification confocal images of membrane-targeted GFP expression via AAV2/5-GFAP-LCK-GFP (green) vs cytoplasmic expression of tdTomato via AAV2/5-GFAP-tdTomato (red) in a field of cortical astroglia. B) High magnification images of a single astrocyte with co-expression of membrane targeted GFP (green) and cytosolic tdTomato. Note the differences in overall appearance of these cellular compartments. Scale bars depict 100 µm in A) and 10 µm in B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

noise ratio and restricted visualization of astrocytic calcium dynamics outside of the somatic region or the larger primary branches [90]. As such, the data obtained from these early studies indicated that intracellular Ca^{2+} elevations may not directly cause astrocyte-derived neurochemical release and subsequent adaptations in neuronal activity, and thus likely did not consequently impact or direct the neural activity underlying learning or complex behaviors [97,98]. However, the advent of ultra-sensitive genetically encoded calcium indicators (GECIs) [99,100] and discovery of additional IP_3 receptor subtypes (IP_3R1 and 3) in astrocytes has substantially refined our understanding of the diversity and temporal dynamics of astrocytic Ca^{2+} responses and the cellular systems that regulate intracellular Ca^{2+} elevations in astroglia [101]. Specifically, astrocytic Ca^{2+} elevations in the somatic region and primary branches are evoked in large part through IP_3R2 stimulation [101], yet microdomain Ca^{2+} dynamics are often attributed to other IP_3 receptor subtypes, including IP_3R1 or IP_3R3, or additional non IP_3R-mediated means for Ca^{2+} flux including transient receptor potential ankyrin 1 [102], L-type voltage gated Ca^{2+} channels [103], the sodium/calcium exchanger [104], transient receptor potential canonical channels [105], and Ca^{2+} mobilization from mitochondria [87]. For a comprehensive review of this topic, including discussion of various IP_3R knock out animal models and analyses of the resulting impact on astrocytic Ca^{2+} dynamics, see [106]. Collectively, these findings demonstrate that astrocytic Ca^{2+} dynamics are more uniquely heterogenous in their magnitude and subcellular distribution than we had originally perceived, with heterogeneity also evident in the intracellular signaling required to evoke astroglial Ca^{2+} dynamics and the functional consequences derived from of each modality of intracellular Ca^{2+} events (discussed at length below) [86,87,91,93].

As expected, microdomain-level Ca^{2+} events are thought to be largely dictated by activity patterns at nearby synapses, where astroglia both perform their traditional homeostatic roles [107], and act to specifically tune excitatory or inhibitory neurotransmission through release of gliotransmitters or adaptations in their physical presence at the synapse [11,53]. Specifically, microdomain Ca^{2+} dynamics enable cytoarchitecture plasticity, actin-mediated cytoskeletal dynamics, and growth towards or retraction from the synaptic cleft, a process directly linked to adaptations in neurotransmitter reuptake, gliotransmitter release, and synaptic regulation [108]. Importantly, Ca^{2+}-dependent release and clearance of neuroactive chemicals by astrocytes can bidirectionally modulate neuronal activity depending on the type of gliotransmitter released, the type of receptors present at tripartite synapse, the characteristics of the pre- and postsynaptic neurons, and the preference for and presence of astroglial physical interaction at the pre- or postsynapse [69,109–112]. Evidence now demonstrates that Ca^{2+} elevations in astrocytic microdomains as a result of neurotransmission occur on rapid timescales, comparable to neuronal calcium dynamics [49], and often propagate to the parent branch and somatic region, culminating in cell-wide Ca^{2+} elevations and subsequent glial-derived release of neuroactive chemicals, which can also influence Ca^{2+} dynamics in neighboring astroglia [108] as a means to influence neuronal network activity [58]. As described above, astrocytic gap junction hemichannels direct intracellular communication via cytoplasmic continuity [113] and as such are permeable to charged molecules (Ca^{2+}, NAD^+), second messengers (IP_3, ATP), and even glutamate [55]. Thus, Ca^{2+} dynamics that occur in the PAPs of an individual astrocyte could quickly be translated to a Ca^{2+} activity within neighboring, functionally connected cells and occur on a timescale that is consistent with an ability to influence local synaptic transmission [49]. Taken together, these data support the hypothesis that astrocytes shape the formation of neuronal ensembles and influence long-range circuit communication. The rapid nature of PAP Ca^{2+} dynamics also establishes the possibility that astrocytes themselves can store information and participate in encoding discrete environmental stimuli. These hypotheses are directly supported by work done by Poskanzer and Yuste, which established that astrocytic activation regulates circuit UP states, a phenomenon that can be described as a period of time (hundreds of milliseconds) in which neurons are depolarized and fire a multitude of action potentials [46]. These studies demonstrate that stimulation of an individual

astrocyte is sufficient to increase Ca^{2+} activity throughout the astroglial network, and that astroglial-derived glutamatergic and purinergic signals direct the formation of circuit UP states *ex vivo* [46]. Moreover, Poskanzer and Yuste also found that astrocytes control cortical circuit state switching *in vivo*, with astroglial glutamatergic signaling responsible for the shift from high-frequency neuronal firing towards synchronized, low-frequency circuit activity [58]. Taken together, it is apparent from these studies that astrocytes sense neuronal activity and respond with rapid microdomain Ca^{2+} events that can translate into long-range Ca^{2+} waves through the astroglial syncytia, ultimately culminating in the coordinated release of astroglial-derived neuroactive chemicals to gate neuronal network activity.

Despite these well-documented outcomes of astrocytic Ca^{2+} signaling and subsequent modulation of neuronal activity, the extent of Ca^{2+}-dependent astrocyte-derived neuroactive chemical release under physiological conditions remains controversial [98]. For example, experiments examining either increases or decreases in astrocytic Ca^{2+} levels through stimulation of Gq-GPCRs or deletion of components in the IP_3 signaling pathway provide evidence that these manipulations do not alter neurotransmission and synaptic plasticity *in vivo*, and that some methodologies used to manipulate astrocytic activity *in vivo* and stimulate gliotransmission can be considered not physiologically relevant [98]. Moreover, the presence of SNARE proteins in astrocytes and Ca^{2+}-dependent vesicular release of neuroactive chemicals remains disputed. While these observations support the opinion astrocytes lack the machinery for Ca^{2+}-dependent vesicular release of gliotransmitters, it is important to consider gliotransmitter release has been reported to occur through several vesicular and non-vesicular modalities. Moreover, gliotransmission likely does not exhibit 1:1 parity with analogous molecular mechanisms dictating release of neuroactive chemicals from neurons [114]. In a well-written perspective, Savtchouk and Volterra (2018) counter the argument against *in vivo* gliotransmission and outline some oversimplifications may have contributed to this perspective [114], including simply focusing on a single neurotransmitter system or calcium source to trigger gliotransmission. Accordingly, these perspectives do not provide a wholistic view to what is happening in an intact, *in vivo* system. Further, the authors argue that it is difficult to equate Ca^{2+}-dependent vesicular transmitter release in astrocytes and neurons. Evidence does indeed exist supporting astroglial expression of low levels of glutamate-containing vesicles and alternative isoforms of SNARE proteins that support vesicular release [114], implying that astrocytes are capable of Ca^{2+}-dependent mechanisms of gliotransmitter release. Furthermore, as discussed above, astrocytic regulation of synaptic activity also extends beyond vesicular or non-vesicular gliotransmission, as astrocytes are responsible for (1) the delivery of the precursors needed to generate key neurotransmitters, (2) the reuptake of neuroactive chemicals from the synaptic cleft and cessation of synaptic activity, (3) the shaping the physical space that neurotransmission occurs in, (4) the ion buffering required to support action potential generation, and (5) the metabolic support of local neurons [1,22], with the several of these processes occurring via Ca^{2+}-dependent mechanisms. As outlined throughout this review, our perspective is that Ca^{2+} dynamics are essential for astroglia to respond to and influence the synaptic environment and regulate the local neuronal activity and circuit-level communication underlying complex behavioral responses. This viewpoint is supported by the studies highlighted herein, as well as in our discussion outlining the need to study the spatiotemporal outcomes of astrocytic Ca^{2+} signaling using sophisticated methodologies.

1.3. Quantification of astroglial action

An increased appreciation of the diversity of Ca^{2+} signals in astrocytes has led to a parallel refinement of strategies for the quantification and analyses of how astrocytes respond to various stimuli. Importantly, the methodologies for quantification of astrocytic Ca^{2+} events are also applicable to the analysis of fluorescent signals from recently developed neurotransmitter-specific genetic indicators that, when expressed in astrocytes, allow for the detection of astoglial receipt of glutamate [115], norepinephrine [116], GABA, and dopamine [117,118]. The progression, characteristics, and advantages of the various analysis pipelines used for Ca^{2+}- and/or neurotransmitter-linked fluorescent imaging in astrocytes has recently been reviewed, for a more complete discussion on these topics see [119] and [90]. Briefly, astrocyte-specific computational imaging analysis toolkits have evolved in parallel with our increasing ability to visualize and detect localized Ca^{2+} dynamics in astrocytes, and the continued refinement of computer-aided region of interest (ROI) detection. One early open-source toolkit developed by the Khakh laboratory is GECIquant, an ImageJ-based pipeline for the analysis of 2D + time data that allows for the estimation of an individual astrocyte territory, and subsequent separation and quantification of somatic, wave-like, and microdomain-specific Ca^{2+} dynamics [120]. When combined with expression of GCaMP6f in astrocytes, the Khakh laboratory was able to use this quantification method to effectively demonstrate that while somatic Ca^{2+} responses were not present in astroglia of IP_3R2 KO mice, these IP_3R2 KO astrocytes still displayed microdomain-level Ca^{2+} oscillations both in slice and during a startle response *in vivo*, aiding in the development of a more complete understanding of the functional ramifications of somatic vs microdomain Ca^{2+} signaling in astroglia [120]. Another fundamental step forward was the development of "accurate quantification of astrocyte and neurotransmitter fluorescence dynamics for single-cell and population-level physiology" or AQuA by the Poskanzer laboratory [52]. This ROI-based methodology employs machine learning to bring a fine level of detail to the quantification, tracking, propagation and directionality of astrocytic microdomain-level Ca^{2+} events. In the initial description of AQuA, Wang et al. also mention the advantage of using an intersectional viral vector approach that employs a "dynamic" Ca^{2+} or neurotransmitter genetic indicator used in combination with a "static" cytosolic label (tdTomato is suggested as most Ca^{2+} and neurotransmitter indicators utilize GFP). This experimental strategy allows for a more direct assessment of individual astrocyte territories and the repeated tracking of Ca^{2+} activity in the same astroglia over time. For an example of a co-expressed chemical indicator and static cytosolic label in astrocytes see **(**Fig. 2**).** A more recent software package developed to assess intercellular activity within astrocytic networks was developed by Dzyubenko et al (2021), deemed Astral, and is geared at examining astrocyte-astrocyte communication at the population level [51]. The Astral software package employs pipelines for data processing and signal extraction in 3-D + time data sets and groups adjacent pixels together with their change in intensity to evaluate the existence of a calcium waves, evaluated in light of background noise, based on their standard deviation. This quantification strategy is a powerful tool geared towards examining activity patterns in the astroglial syncytia in live-imaging preparations [51], and if coupled with *in vivo* 2-photon calcium imaging, could aid in elucidating how astrocytic networks adapt during various behaviors. Through the continual advances in these toolkits and the use of intersectional viral vector strategies to define individual astrocytic territories, microdomain

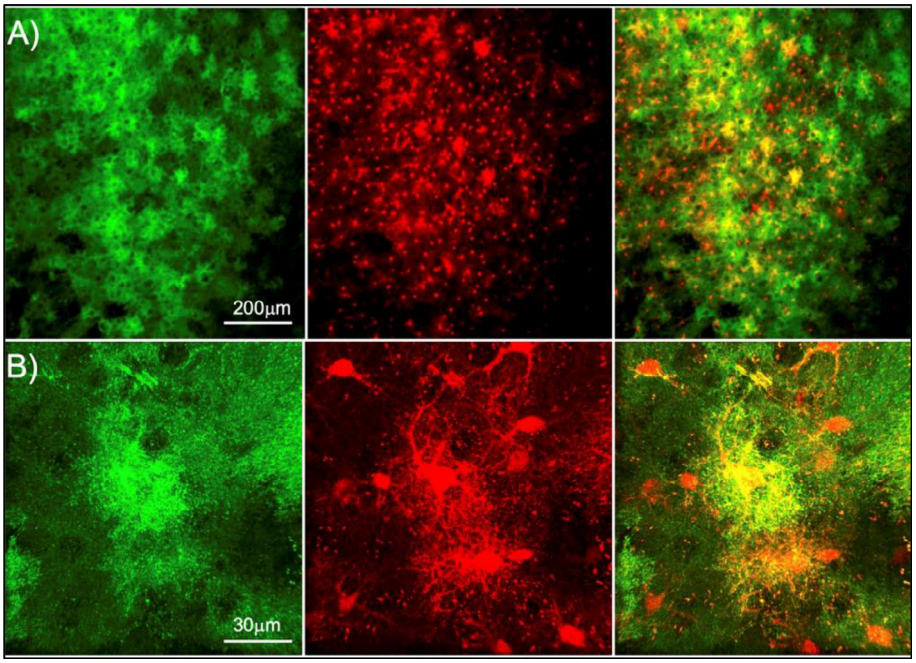

Fig. 2. Comparison of iGluSnFR vs cytosolic astroglial expression of tdTomato. A) Low magnification confocal images of the fluorescent glutamate neurotransmitter indicator iGluSnFR via AAV2/5-GFAP-iGluSnFR (green) vs cytoplasmic expression of tdTomato via AAV2/5-GFAP-tdTomato (red) in a field of cortical astroglia. B) High magnification images of a several astrocytes with co-expression of iGluSnFR (green) and cytosolic tdTomato. Note the differences in the overall appearance of these cellular compartments, which resembles Fig. 1 above. Scale bars depict 200 μm in A) and 30 μm in B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Ca^{2+} and transmitter events are now being quantified within individual cells over time, with these toolkits also allowing the quantification of how these signals are translated across the astroglial network. These advances have fundamentally improved the ability to evaluate how astroglial signaling events are refined over time and will aid in establishing their functional relevance.

1.4. How do astrocytes respond to and influence neuronal circuit activity?

Astrocytic response to and influence on synaptic activity provides regulation of the neuronal circuit dynamics related to memory, cognition, and behavior. As an example, chemogenetic disruption of calcium dynamics in hippocampal astrocytes can lead to reduced output-selective neuronal activity patterns, reduced downstream neuronal recruitment, and impaired memory recall [28]. Additionally, astrocytes differentiate and decode information from afferent neuronal projections in a Ca^{2+}-dependent manner, and subsequently release gliotransmitters to influence local synaptic activity [121]. Yet, precisely how astrocytes interrogate incoming information, influence local neurotransmission, and gate outgoing projections in a synapse-specific manner remains to be completely understood. Further, it also remains to be determined if and how these cells influence formation of neuronal ensembles that encode information related to salient environmental stimuli and/or behavioral output. Fortunately, this can now be addressed as astrocytic and neuronal dynamics can be simultaneously visualized and manipulated using cutting-edge techniques in awake, behaving animals.

1.4.1. In vivo calcium imaging

The continued refinement of viral-mediated *in vivo* imaging and manipulation strategies now allows for the concomitant measurement and control of cell-type specific activity, an advance that is required for an improved understanding of how astrocytes shape local and circuit-level neuronal dynamics during behavior. Towards this end, it is necessary to employ strategies that allow visualization and longitudinal tracking of astrocytic activity at both the single cell and population level during complex behavioral tasks. Many laboratories routinely employ strategies such as head-mounted miniscope imaging [122–124] and head-fixed 2-photon imaging [125–130] with neuronal GECIs to characterize Ca^{2+} activity patterns in different cellular compartments (soma or cell body, dendrite, axon) in awake behaving animals. While miniscopes are light-weight and allow for visualization of deep brain neuronal dynamics in freely moving animals [131], head-fixed 2-photon imaging provides higher resolution imaging that can be used to capture subcellular Ca^{2+} events [128,132]. As there is a need to design experiments that incorporate astrocytes into systems neuroscience [133], 2-photon microscopy can be used to visualize and quantify diverse subcellular astrocytic Ca^{2+} dynamics and relate astrocytic Ca^{2+} events to neuronal activity patterns *in vivo* [49,58]. While it is important to consider that the dimensionality of astrocytic Ca^{2+} events, including the spatial and temporal profile, may be more difficult to assess *in vivo* given resolution limitations and Ca^{2+} buffering in GECI-expressing astrocytes [134], this is a necessary trade off as repeated measurement of concomitant astrocytic and neuronal activity patterns will be required to understand how activity patterns in each cell type relate to learning, cognitive function, and complicated behaviors like reward seeking.

As *in vivo* two-photon microscopy with concurrent mouse behavioral assays becomes a more common means to measure and longitudinally track neuronal Ca^{2+} dynamics, methods for analyzing these data have also become more sophisticated [125–129,135]. These methods include principal components analysis (PCA) and clustering algorithms, which are used to identify unique neuronal ensembles that differentially encode information related to environmental stimuli and behavioral output [125,135]. For example, our laboratory has identified unique ***neuronal ensembles***

that emerge during training and stabilize after learning in a Pavlovian sucrose conditioning task, with each cluster encoding specialized information related to the sucrose reward, reward-predictive stimuli, and the behavioral response to the stimuli [135]. Importantly, these neuronal ensembles are not present early in learning, but emerge across training to encode learned information. This method could be extended to define ***astroglial ensembles*** that may act to shape neuronal activity and complex behaviors, such as learning and reward seeking. Specifically, by combining astrocytic GECIs with 2-photon imaging, it is possible to longitudinally track Ca^{2+} dynamics in individual astrocytes and time-lock astrocytic Ca^{2+} dynamics to behaviorally relevant events. Given the evidence supporting the role of astrocytic Ca^{2+} signaling and concordant gliotransmission in altered neuronal activity and state-switching *in vivo* [58], it is reasonable to hypothesize astrocytes are engaged during learning, influence neuronal activity (through excitation and/or inhibition), and coordinate ensemble formation. When combined with means to manipulate neuronal and astroglial activity, future experimentation will allow direct testing of the hypothesis that astroglia function as computational entities that encode discrete stimuli to shape neuronal activity.

The advent of non-GFP based genetic Ca^{2+} indicators now allow for concomitant expression of GECIs in astrocytes and neurons, which provides an experimental platform to simultaneously evaluate neuronal and astroglial Ca^{2+} dynamics *in vivo*. An example of this strategy would be to employ an astrocytic GCaMP alongside a neuronal RCaMP (red wavelength GECIs; [136]), which would allow for examination and tracking of activity dynamics in both cellular populations, simultaneously, in repeated behavioral sessions [49,137]. It was through this strategy that Stobart et al. identified that Ca^{2+} transients in the astroglial leaflets occurred on a rapid timescale (~120 ms) following neuronal activity [49], indicating astrocytic activity is indeed quick enough to respond to and modulate neurotransmission and linked behavioral responses. Moreover, similar strategies have been used to identify Ca^{2+} dynamics in astroglia that encode spatial information related to navigation through a virtual environment [137]. These astrocytic Ca^{2+} events were topographically organized in subcellular compartments, including the processes and soma, and complimented activity patterns of neighboring neurons during navigation [137]. The combination of multiphoton imaging during various behavioral paradigms in concert with a multiplexed GECI approach will

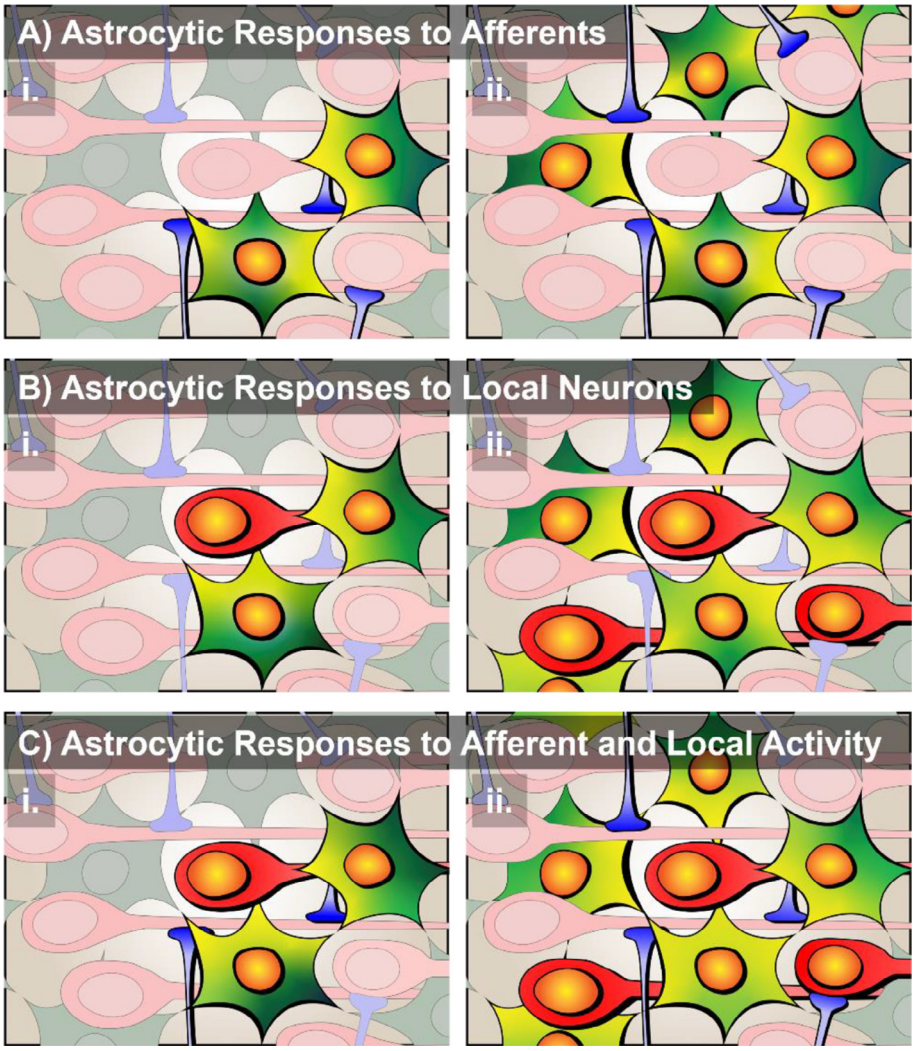

Fig. 3. Astrocytes coordinate neuronal network activity. A) Astrocytes respond to afferent projections (blue) with microdomain-level calcium events (yellow) early in a behavioral task. Following training, as more afferents are active astrocytes can differentiate between inputs and decode information in a Ca^{2+}-dependent manner. B) Peripheral astroglial processes engage because of nearby activated neurons (red) elicit microdomain Ca^{2+} events (yellow) that spread to neighboring astrocytes early in learning. Late in learning, astrocyte activity is refined and synchronized Ca^{2+} signaling coordinates local ensemble dynamics. C) Following training, astrocytes can integrate afferent information to influence local neuronal activity and formation of the neuronal ensemble dynamics that encode behaviorally relevant stimuli. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

enable researchers to longitudinally track activity patterns of both neurons and astrocytes with single-cell resolution, as they adapt throughout behavior, and relate to each other. In this way, astrocytes could be integrated into a variant of PCA with multidimensional modeling that includes both adaptations in activity of neurons and astrocytes. A multidimensional PCA coupled with clustering algorithms could aid in elucidating how activity in the astroglial syncytia relates to synchronization of neuronal activity and recruitment of nearby neurons into functional neuronal ensembles that orchestrate behavioral outputs.

1.4.2. Viral strategies for manipulation of cellular responses and concomitant in vivo imaging

By combining *in vivo* Ca^{2+} imaging in astrocytes with viral constructs that allow for the manipulation of neurons, experimentation aimed at understanding how astrocytes respond to incoming neurotransmitter release or local neuronal activity have become possible in awake, behaving animals. These data will allow for a better mechanistic understanding of precisely how Ca^{2+} signals are evoked in astroglia and if they are generated predominantly via afferent activity (Fig. 3**A**), are generated in response to local neuron activity (Fig. 3**B**), or through some mixture of both (Fig. 3**C**). Additionally, the inverse experimental paradigm is also possible, utilizing *in vivo* 2-photon Ca^{2+} imaging in neurons combined with viral constructs that allow for the manipulation of astrocytic activity. Such tools include those that direct activation of astroglia including optogenetics [27,58,78], chemogenetics [28,81–83,138], as well as those used to decrease astroglial activity including CalEx [18,139], iβark [138], as well as manipulation of intracellular IP_3 signaling pathways (for an in-depth review of current tools to manipulate astrocytic Ca^{2+} see [75]). Furthermore, genetic strategies for selective knockdown of genes controlling astrocytic-neuronal interaction or astroglial structural plasticity, such as with the CRISPR/Cas9 system [140,141], can be employed with 2-photon microscopy to examine the mechanisms by which astroglia respond to and direct synaptic transmission *in vivo*. These types of experiments will allow for a better understanding of how astrocytic activity shapes neuronal responses in awake, behaving animals.

Viral strategies often used in neuronal circuits, such as anterograde tracing, now enable investigation of input-specific astrocytic populations. The unique serotype-specific properties of adenoviral vectors enable transduction of neurons by virtue of their projection targets or their inputs. As an example, AAV1-mediated anterograde transsynaptic activity can be used to interrogate input-specific populations of neurons [71,142–145]. Recently, the Kuhn laboratory established AAV1-mediated anterograde vectors also allow for the axo-astrocytic delivery of Cre at the tripartite synapse [146], making it possible to genetically tag astrocytes at anatomically-defined synapses and evaluate if these astrocytes uniquely respond to and modulate circuit activity. When combined with Cre-dependent astrocytic expression of GECIs, this strategy can be used to probe the spatiotemporal activity patterns of astrocytes in a synapse-specific manner [146].

These studies have already uncovered ultrafast astrocytic microdomain Ca^{2+} dynamics with synapse-specific resolution as it pertains to locomotion and vibrissa stimulation [146], reinforcing the idea that astrocytes uniquely respond to circuit-specific neuronal activity. Given that astrocytes display regional heterogeneity, with specialized gene expression profiles, Ca^{2+} responses, and morphometric profiles observed across and even within various brain regions [7,147,148], experiments can now directly characterize and manipulate astrocytic Ca^{2+} dynamics at various input-defined synapses using an AAV1-based experimental design. Axo-astrocytic viral transfer is an innovative strategy that can be used to create activity maps of astrocytes within distinct neuronal circuits during specific behavioral tasks and will prove crucial for evaluating how astrocytes gate circuit-level communication. The data depicted in Fig. 4 provides a glimpse into the remarkable segregation of subsets of astroglia, within a single brain region, depen-

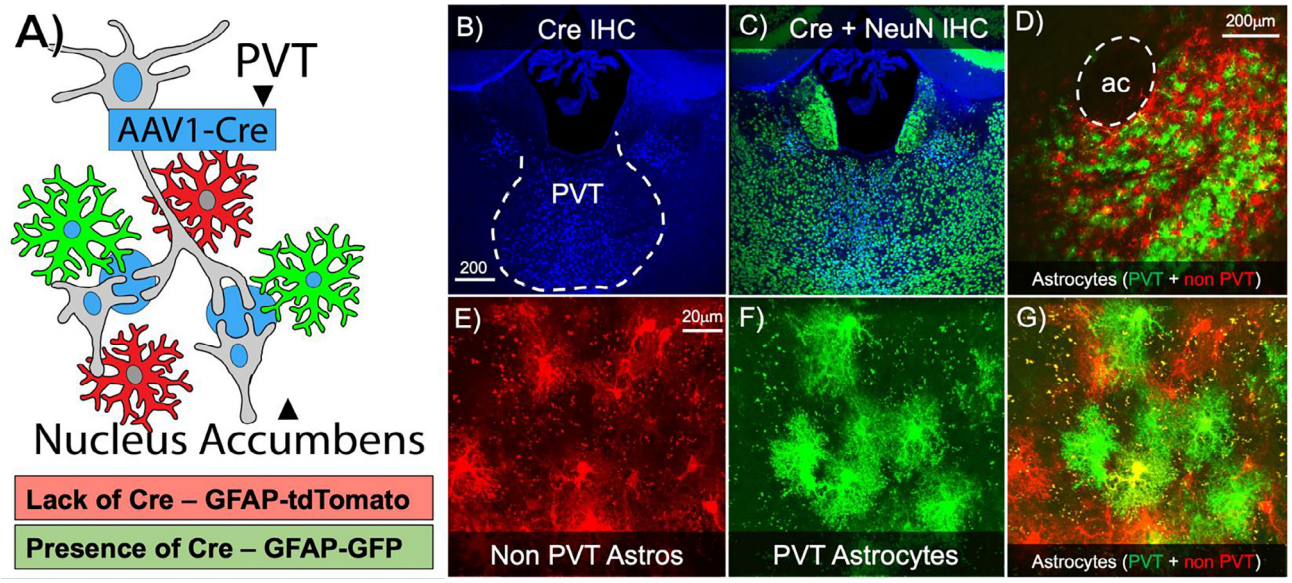

Fig. 4. Axonal-Astrocyte anterograde transfer of CRE particles can be used to label astrocytes at distinct synapses. A) Schematic of surgical strategy: AAV1-CMV-CRE was microinjected into the paraventricular thalamus (PVT) and AAV5-GFAP-dlox-tdTomato-EGFP(rev)-dlox was microinjected into the nucleus accumbens (NAc). In the presence of CRE astrocytes will express GFP, in the absence they will express tdTomato. A representative 10x Z-stack of the PVT CRE expression (B; pseudocolored blue) in neurons (C; CRE depicted as blue, NeuN shown in green). D) A representative 10x Z-stack of the NAc illustrating anatomical segregation of astrocytes at PVT and non-PVT synapses in the core and the shell. In the absence of PVT input and anterograde CRE astrocytes express tdTomato (E), and when astrocytes are present at PVT synapses and CRE is delivered, they express GFP (F). G) A representative merged 63x Z-stack of NAc astrocytes demonstrating grouping of astrocytes at anatomically-defined synapses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dent on the synapses that they are present at, hinting at potential functional subdomains within the astroglial syncytia. This approach can be coupled with CRE-dependent constructs to manipulate astrocytic activity or CRE-dependent genome editing tools to investigate the function of input-defined subgroupings of astrocytes and the mechanisms by which bidirectional chemical communication occurs between astrocytes and neurons.

Through the combination of these new tools, the field is now poised to more precisely evaluate the hypothesis that subsets of astrocytes direct plasticity at key synapses, and that astrocytes themselves are functional components of neural circuits that integrate incoming information, participate in neuronal ensemble formation, and coordinate neuronal network activity to drive behavioral responses. For example, stimulation of neuronal terminals using red-shifted optogenetic constructs [149,150] coupled with *in vivo* astrocytic Ca^{2+} imaging can be used to test how astrocytes respond to afferent information with subcellular and population-level resolution. This experimental design could also accomodate specific promoter- or CRE-driven constructs to target distinct neuronal populations and/or astrocytes at input-defined synapses. Moreover, single-cell optogenetic approaches exist for use in neurons, with stimulation of just two neurons sufficient to initiate ensemble dynamics and resulting licking behavior in mice [151]. As it has been demonstrated that stimulation of a single astrocyte is sufficient to generate an astrocytic calcium wave *ex vivo* and dictate neuronal circuit activity [46], future lines of research can employ single-cell optogenetic strategies to target individual astrocytes and quantify resulting activity patterns in the astroglial syncytia and local neuronal populations. These approaches can be coupled with genome editing tools, such as the CRISPR/Cas9 system, in astrocytes to selectively target receptors or gliotransmitter production/release pathways and determine the mechanism by which astrocytes integrate information from afferent neuronal projections and influence local neuronal activity, including ensemble formation, in behaving animals. Using the innovative approaches described above, researchers will be able to functionally tie afferent driven astrocytic Ca^{2+} signaling to recruitment of adjacent astrocytic nodes and local neuronal ensemble formation in awake, behaving animals.

2. Summary and outlook

With the continued refinement of neuroscience tools and analysis pipelines, we will soon be able to probe how astrocytes influences circuit communication *in vivo*. Despite the challenges that still exist while measuring astrocytic activity in awake behaving animals [134], investigation of astroglial Ca^{2+} signaling in an intact system during complex behavioral tasks is critical to assess how mammalian astrocytes encode environmental stimuli, relay the information to neighboring astroglia and neurons, and modulate neuronal communication from the synapse to the circuit. In summary, examining astroglial Ca^{2+} events in the context of their morphology have revealed complex signaling dynamics that coordinate neuronal activity patterns and orchestrate a range of behavioral outputs. As the field continues to develop, researchers will be able to place astrocytes as computational entities within neural networks and better understand their functional role in various behavioral responses.

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CRediT authorship contribution statement

Jacqueline E. Paniccia: Conceptualization, Writing - original draft, Writing - review & editing. **James M. Otis:** Conceptualization, Writing - original draft, Writing - review & editing. **Michael D. Scofield:** Conceptualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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