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A perspective on astrocyte regulation of neural circuit function and animal behavior

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Abstract

Studies over the past two decades have demonstrated that astrocytes are tightly associated with neurons and play pivotal roles in neural circuit development, operation, and adaptation in health and disease. Nevertheless, precisely how astrocytes integrate diverse neuronal signals, modulate neural circuit structure and function at multiple temporal and spatial scales, and influence animal behavior or disease through aberrant excitation and molecular output remains unclear. This Perspective discusses how new and state-of-the-art approaches, including fluorescence indicators, opto- and chemogenetic actuators, genetic targeting tools, quantitative behavioral assays, and computational methods, might help resolve these longstanding questions. It also addresses complicating factors in interpreting astrocytes' role in neural circuit regulation and animal behavior, such as their heterogeneity, metabolism, and inter-glial communication. Research on these questions should provide a deeper mechanistic understanding of astrocyte-neuron assemblies' role in neural circuit function, complex behaviors, and disease.

Keywords

actuator; astrocytes; behavior; computational approaches; genetic targeting; indicator; neural circuit

1 | INTRODUCTION

“What is the function of glial cells in neural centers? The answer is still not known, and ... it may remain unsolved for many years to come until physiologists find direct methods to attack it.” (Ramon y Cajal, 1911).

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This prophecy turned out to be accurate. Astrocytes, glial cells of the central nervous system (CNS), are thought to regulate neural circuit formation, operation, and adaptation (Zuchero & Barres, 2015). During development, they help regulate neurogenesis, synapse number and function. In the adult CNS, they influence synaptic plasticity, neuronal excitability, network synchronization (e.g., brain state switching), rhythmic activity (e.g., in central pattern generating circuits), learning, and memory (Akther & Hirase, 2021; Allen & Eroglu, 2017; Araque et al., 2014; Di Castro & Volterra, 2021; Nimmerjahn & Bergles, 2015). Their molecular and functional heterogeneity suggests that astrocytes form regional networks, act in a neuronal cell type and circuit-specific manner, and—based on behavioral and machine learning approaches—may process information in ways complementary to neurons. Astrocytes also play essential roles in pathology. After CNS injuries and in patients with neurodegenerative disease, they adapt their structural and functional properties, thereby regulating neuronal health and survival (Pekny et al., 2016; Sofroniew, 2020). These critical advances in our understanding of astrocyte biology—from the subcellular to network level—are primarily due to recent technical progress, including new and improved fluorescent indicators, bidirectional actuators, sophisticated behavioral assays, recording techniques (e.g., wearable microscopes), computational methods, and theoretical frameworks allowing measurement, manipulation, and modeling of these cells' in vivo structure and function across CNS regions (e.g., cortical, subcortical, brainstem, spinal cord).

Despite this progress, many longstanding questions remain (Box 1), including how astrocytes integrate and (nonlinearly) respond to diverse neuronal signals in their extracellular environment at the cellular and population level, how they convert this task- and animal state-dependent information into functional outputs that modulate neural circuit dynamics and animal behavior on various temporal and spatial scales, whether this influence on neurons—however sophisticated—primarily serves homeostatic functions (e.g., transmitter recycling, restoration of ionic balance, toxic substance removal, energy substrate delivery, synaptic scaling) or augments/complements neural circuit properties (e.g., increased computational power, flexibility, or learning and memory through coincidence detection-triggered excitatory and inhibitory synaptic gain modulation, extracellular K^+ -mediated changes in network excitability/synchrony, or temporally holding information about prediction error extent to update synaptic weights accordingly), and whether they perform similar computations in various brain and spinal cord regions that differ in cytoarchitecture and functional dynamics.

Filling this knowledge gap will require a systematic approach (1) measuring relevant neuronal signals in relation to astrocyte activity and morphology, (2) manipulating astrocyte activity and structural plasticity in physiologically relevant ways, (3) relating astrocytes' functional and morphological changes to changes in neuronal dynamics and animal behavior, and (4) integrating the various data into a coherent picture using computational modeling and theoretical frameworks. Insights from these studies promise to transform our modern understanding of systems neuroscience and provide the conceptual framework for placing astrocytes in higher information processing.

In this perspective, we first provide an overview of state-of-the-art tools for measuring and manipulating astrocyte communication, including various indicators, actuators, and

genetic targeting approaches. Next, we discuss how combining these interrogations with behavioral measurements and computational methods might help uncover astrocytes' role in regulating neural circuit function and animal behavior. Finally, we consider how astrocyte heterogeneity and diversity and astrocytes' interaction with other non-neuronal cells may complicate the picture. Given the wide range of topics covered in this perspective, the cited literature merely serves to exemplify where the field stands today, the challenges it faces, and tools and approaches that might help tackle them. For a more in-depth discussion of the various topics, we refer the reader to the cited and topical reviews in this Special Issue.

2 | MEASURING, MANIPULATING, AND TARGETING ASTROCYTE FUNCTIONS

2.1 | Indicators

The potential pathways by which neurons and astrocytes communicate with one another run the gamut of signaling mechanisms in the CNS, including chemical and electrical synaptic transmission and neuromodulation (Verkhatsky & Nedergaard, 2018). Critical for elucidating these molecular interactions are reagents that allow quantitative monitoring of corresponding signaling mechanisms *in vivo*. Most functional imaging is currently centered on a small number of mechanisms, particularly those that involve calcium excitation. To develop a deeper understanding of neuron-astrocyte communication, additional signaling mechanisms need to be studied. Fortunately, an increasingly broad and powerful toolbox of biosensors to track signaling between and within cells has recently become available (Table 1). This toolbox includes indicators for the readout of astrocyte input and output signals, as well as intracellular biochemical activity. Additionally, spectral variants of these indicators are beginning to allow the study of pathway interactions.

2.1.1 | Indicators for readout of astrocyte input—Indicators for neurotransmitters, neuromodulators, and neuropeptides will be crucial for determining precisely how animal behavior engages these pathways and shapes astrocytes' excitation in a given region. The spatial and temporal heterogeneity of astrocytes' calcium transients suggests that they are exposed to a continually varying cocktail of transmitters in their environment. How these complex time-varying patterns of neuronal (and non-neuronal) signals are then integrated by astrocytes, which calcium sources contribute to this process (e.g., plasma membrane calcium channels, ion exchangers, endoplasmic reticulum (ER), mitochondria, and potentially lysosomes; Heidemann et al., 2005; Semyanov et al., 2020), and what information the various astrocyte activity patterns encode remains largely unknown. Experimental evidence suggests that astrocytes in the CNS may use multiple parameters (e.g., transient onset, amplitude, duration, frequency, and spatial pattern) to encode information (Nelson et al., 2019; Nimmerjahn & Bergles, 2015). Direct measurement of the input signals together with astrocyte excitation in the context of quantifiable behavioral assays will be critical to address this question. These assays should allow control over the type of pathways being activated (e.g., dopamine) and their timing. Reward-based assays seem particularly well suited in this context (see below). While an increasing palette of indicators is starting to allow multiplex measurements, the number of signals that can be measured simultaneously will likely remain limited (e.g., due to indicator signal-to-noise ratio [SNR] or emission spectrum overlap).

Therefore, the behavioral assays also need to be highly reproducible so that measurements with different indicator combinations obtained from different groups of animals can be compared.

2.1.2 | Indicators for readout of astrocyte intracellular signaling—Indicators for the intracellular biochemical activity will be critical in determining its relationship to receptor activation and astrocyte responses. For example, astrocytes express several subtypes or levels of G-protein coupled receptors (GPCRs; G_s -, G_i -, and G_q -coupled). Activation of these receptors (e.g., by dopamine, norepinephrine, serotonin, acetylcholine) increases or decreases intermediate signals, such as inositol-trisphosphate (IP_3), cyclic adenosine monophosphate (cAMP), reactive oxygen species (ROS), or inositol 1,4,5-triphosphate (IP_3) receptor-binding protein released with IP_3 (IRBIT) (Figure 1). These signals are then integrated spatially and temporally (e.g., at the mitochondrial or ER level), leading to calcium and protein kinase A (PKA) signals reflective of local and projection neuron activities. Elevations of calcium and PKA activity, in turn, are thought to jointly or individually modulate neuronal excitability and plasticity through several mechanisms and on various timescales (e.g., neuroactive substance release, extracellular potassium concentration changes, morphological alterations) (Dong et al., 2012; Horvat et al., 2016; Impey et al., 1998; Masmoudi-Kouki et al., 2007; Verkhratsky & Nedergaard, 2018). Nevertheless, precisely how local neural and projection neuron activities regulate these signals during animal behavior remains largely unknown. Measuring intracellular signaling in behaving animals will, therefore, provide vital clues as to how local and projection neuron activity is integrated within astrocytes, regulates astrocytes' gene expression (e.g., through level or frequency of nuclear calcium transients), or regulates astrocyte-derived extracellular ion or neuroactive factor concentrations.

2.1.3 | Indicators for readout of astrocyte output—Indicators for soluble and contact-mediated signaling will be essential to relate changes in astrocyte excitation to neural circuit modulation. For example, proximity labeling and genomic profiling have revealed distinct adhesion molecules in perisynaptic astrocyte processes (PAPs) that modulate either excitatory or inhibitory synapses (Singh et al., 2016; Stogsdill et al., 2017). Whether there is specificity in the type of synapses being enveloped within an astrocyte's territory and what the functional implications of such specificity might be (e.g., changes in excitation-inhibition balance) remains largely unknown. Indicators for molecules released by astrocytes (e.g., glutamate, GABA, ATP, D-serine, lactate) (Table 1) may also help resolve controversies and discrepancies regarding astrocyte-to-neuron signaling, including the conditions under which it occurs, its spatiotemporal dynamics, and the types of transmitters being released (Bonvento & Bolaños, 2021; Savtchouk & Volterra, 2018; Sherwood et al., 2021; Wolosker et al., 2016).

In summary, novel genetically encoded indicators are needed to shed light on the complex intra- and extracellular signaling utilized by astrocyte-neuron assemblies. This may require complementary targeting methods that restrict indicator expression to specific subcellular or intracellular compartments (Broussard et al., 2018; Henderson et al., 2015; Shemesh et al., 2020; Suzuki et al., 2014). However, close attention should be paid to

the indicator expression level, as it can interfere with normal cell function (Armbruster et al., 2020). Control experiments (e.g., expression level titration, sensors with ablated target molecule binding) should be employed to rule out measurement artifacts whenever possible. Some signaling molecules may act on and be released by astrocytes (e.g., ATP), requiring additional interventions to distinguish between cause and effect. Additionally, some signaling molecules (e.g., K^+ , IP_3) may spread and act intracellularly given astrocytes' gap junctional coupling (including with other glial cells, such as oligodendrocytes [OLs]), a route that may need to be considered when interpreting physiological or actuator-evoked indicator signals and effects (see below). Nevertheless, increased capabilities to measure extra- and intracellular signaling promise to make a substantive difference in examining astrocyte function and their roles in brain circuits (Figure 1).

2.2 | Actuators

Measurements with the functional and structural indicators mentioned above can reveal correlative relationships and constrain existing theoretical models of how molecular signaling, cellular dynamics, and animal behavior are linked. To corroborate these possible links and establish causal relationships, additional targeted manipulation of neuron-astrocyte communication will be necessary.

2.2.1 | Actuators for astrocyte excitation—A wide variety of tools, including opto- and chemogenetic actuators, have been developed to tightly control the activity of genetically defined sets of neurons. All-optical approaches have provided intriguing insights into how the targeted neurons and their activity patterns encode and influence sensorimotor behavior (Carrillo-Reid et al., 2019; Robinson et al., 2020). The success of these approaches has raised hopes that a similar strategy might be possible for astrocytes. Indeed, by applying some of these tools to astrocytes, studies in mice and zebrafish have provided initial evidence for a causal link between these cells' activity, neural circuit function, and animal behavior on both short and long timescales (Nagai, Yu, et al., 2021). These studies have also highlighted that astrocytes present unique challenges. Arguably, current actuators and the ways to control them (Table 2) are still a far cry from allowing us to manipulate astrocytes in ways that mimic their physiological operation. Neither the evoked intra- and extracellular dynamics (e.g., calcium, cAMP, potassium) nor their spatiotemporal pattern mimics experimentally observed astrocyte dynamics which, given their complexity, are near impossible to reproduce with current techniques (the same technical limitations apply to neuronal actuation). However, while the evoked activity may not be entirely physiological, it provides critical information regarding the aspects of neural circuit function astrocytes can control or how their targeted manipulation may be used for therapeutic applications.

2.2.2 | Actuators for astrocyte inhibition—Aberrant astrocyte activity in the brain and spinal cord has been described in various disease models (Nelson et al., 2019; Nimmerjahn & Bergles, 2015). Tools to inhibit astrocytes' excitation or transmission are, therefore, critical. For neurons, expression of tetanus toxin light chain (Kim et al., 2009), diphtheria toxin (Roselló-Díez et al., 2018), inhibitory opsins or DREADDs (Lerner et al., 2016; Rajasetupathy et al., 2016; Roth, 2016), and vesicular inhibitory amino acid transporter deletion (Hirrlinger et al., 2019; Rahman et al., 2015) have been used for

silencing. Unfortunately, silencing astrocytes appears more difficult due to these cells' distinct physiological properties. For example, opsins and DREADDs that inhibit neuronal firing (e.g., ArchT, hM4Di) typically mediate calcium excitation in astrocytes (Nagai et al., 2019; Poskanzer & Yuste, 2016). Genetically encoded chelators or pumps have been shown to attenuate intracellular signaling (e.g., calcium or cGMP increases) but currently provide limited temporal resolution or reversibility (Table 2) (Ros et al., 2019; Ros et al., 2020; Yu et al., 2018). Another challenge is compartment-specific manipulations (e.g., cell body, branches, or microdomains). Currently, only a few genetic targeting approaches exist for that purpose (e.g., H2B and Lck for nuclear and plasma membrane targeting, respectively).

Clearly, there is an urgent need for new and improved actuators that allow precise control over astrocytes' spatial and temporal dynamics and specific signaling pathways. Thorough characterization, including transcriptomic and proteomic profiling, will be necessary to develop a mechanistic understanding of precisely how their activation influences astrocytes, neural circuits, and animal behavior, either directly or indirectly (Figueiredo et al., 2014; Octeau et al., 2019; Philtjens et al., 2021; Yu et al., 2020). Such experiments should also consider how the observed effects depend on stimulation parameters and the approach itself (e.g., light-induced heating-mediated effects; Courtney et al., 2021; Owen et al., 2019; Schmidt & Oheim, 2020). Together with established genetic interventions (e.g., CRISPR/Cas9-based approaches to genetically or epigenetically modify astrocytes' ability to sense, process, or send signals), these new tools will be essential in mapping the interplay between astrocytes and neural circuits in behavior on both short and long timescales.

2.3 | Genetic targeting approaches

While various genetic tools allow astrocyte targeting, differences exist in these approaches' ability to control regional and temporal expression and target astrocyte subtypes. Astrocytes are a heterogeneous cell population. Their structural and functional properties depend on the CNS region. Even within a given area, astrocytes can show considerable heterogeneity. This suggests a complex interplay between these cells and their local environment and that astrocytes may modulate neural circuit function in a region-dependent manner. Therefore, genetic approaches for dissecting astrocytes' role in neural circuits may need to match this diversity, as more widespread interventions (e.g., global gene knockout or signaling pathway activation) could obscure region- or subtype-specific effects. Genetic targeting of astrocytes with high specificity, efficiency, and in a region-dependent manner has turned out to be challenging (Hirbec et al., 2020). While studies to identify region- and subpopulation-specific promoters are ongoing, several existing approaches (either alone or in combination) can be used to target astrocytes with varying degrees of specificity.

Numerous transgenic mouse lines allow fluorescent indicator, actuator, Cre-DNA recombinase, or tet-system component expression in astrocytes using different promoters (e.g., GFAP, GLAST/SLC1A3, Aldh11) (Casper & McCarthy, 2006; Hirrlinger et al., 2006; Mori et al., 2006; Natsubori et al., 2015; Nolte et al., 2001; Pascual et al., 2005; Pfrieger & Slezak, 2012; Slezak et al., 2007; Tanaka et al., 2010; Winchenbach et al., 2016; Zhuo et al., 2001; see also https://networkglia.eu/en/animal_models). Each of these mouse lines has its advantages and disadvantages. None of them is entirely astrocyte-specific, though

off-target expression varies by region, targets a homogenous subset of astrocytes in a given area or neural circuit, or captures all CNS astrocytes. Notably, germline recombination has been reported for several of these mouse lines (Luo et al., 2020; Zhang et al., 2013). While some of these issues can be overcome, at least in part, by temporally controlled transgene expression after postnatal development, corresponding data should be interpreted with the above limitations in mind.

Several strategies can be used to achieve more circuit-specific astrocyte targeting:

1. **Viral vectors:** Stereotactic delivery to the CNS region of interest allows spatially confined manipulations. Targeting is also achieved by leveraging viral tropism, astrocyte promoters (e.g., *gfaABC1D*), detargeting strategies (e.g., *miR124*), or injecting Cre-dependent vectors into astrocyte promoter-driven Cre-expressing mice. However, given the lack of truly pan-astrocyte or astrocyte sub-population-specific promoters (Hirbec et al., 2020), off-target expression in the investigated region should be quantified. Standard vector systems used to target astrocytes include adeno-associated viruses, adenoviruses, and lentiviruses. While it is beyond the scope of this Perspective to discuss these different systems in detail (see Delzor et al., 2013; Hirbec et al., 2020 for recent reviews), the following considerations determine which approach is most appropriate for a given application: packaging capacity, tropism, expression speed or level, spatial spread (viral particle size-dependent), immunogenicity, ability to infect dividing or nondividing cells, and genome integration. Direct stereotactic injections result in small lesions and corresponding glial responses. Some viral vectors allow systemic delivery, thereby preventing local injuries. However, these blood-tissue-barrier permeable vectors lead to off-target infection and other potential side effects (e.g., liver toxicity). In both cases, the immune response needs to be carefully considered.
2. **Combinatorial genetics:** This strategy allows targeting of cell populations not defined by the activity of a single promoter but the simultaneous or consecutive activity of two or more promoters (Table 3). However, while this approach has been successfully used for neurons, defining appropriate promoter combinations to target specific subsets of astrocytes or astrocytes in specific neural circuits is still an open question. Ongoing population-wide and single-cell sequencing studies promise to provide information about region-specific gene expression and promoters, thereby allowing future targeting of astrocyte subpopulations and interrogating their roles in regulating neural circuit function and animal behavior.

In summary, further development of genetic targeting approaches is needed to achieve astrocyte specificity, subtype, or circuit targeting.

3 | RELATING ASTROCYTE FUNCTIONS TO NEURAL CIRCUIT OPERATION AND ANIMAL BEHAVIOR

3.1 | Behavioral approaches

Evidence that astrocyte properties are modulated by sensory experience and motor behavior, and that astrocyte manipulation can affect neuronal properties and behavioral phenotype is a foundation for the hypothesis that these cells play crucial roles in regulating neural circuit function and animal behavior. Nevertheless, the diversity of experimental approaches and measurement conditions has led to a flood of data with sometimes seemingly contradictory results. Hence, despite the recent technical progress in measuring neuronal, astrocyte, and transmitter dynamics in behaving animals, precisely how the complex patterns of astrocyte excitation relate to neural circuit function and animal behavior remains unclear. Apart from the apparent model or technical differences, one possible explanation for this predicament is that task-related trials can generate unique behavioral and cellular activities, each influencing astrocytes' dynamics in a deterministic yet complex nonlinear fashion. Hence, to link astrocyte excitation to neural circuit function and behavioral changes, it will be necessary to employ quantitative behavioral assays that allow precise control or readout of sensory input, internal state, and behavioral response, with or without complementary approaches (e.g., molecular genetic interventions).

3.1.1 | Select insights from brain recordings—Studies in the brain have indicated that astrocytes' complex intracellular signaling, most prominently calcium transients, is triggered by neurotransmitter and neuromodulator receptor activation on their surface; that receptor co-activation can lead to nonlinear responses (e.g., pairing sensory stimulation with motor-behavior evoked neuromodulator signaling); and that signal integration within astrocytes influences their subsequent activity (Bazargani & Attwell, 2017; Kastanenka et al., 2020; Nimmerjahn & Bergles, 2015). As a result, astrocyte signaling spans multiple spatial and temporal scales, from sub-second transients in single astrocytes to seconds- or even minutes-long transients in astrocytic networks. This complexity suggests that astrocytes may carry out computations on various timescales related to sensory processing, brain state modulation, and memory formation (Figure 2). For example, astrocytes can influence behavioral adaptation and spatial memory, as shown by genetic approaches to modulate astrocyte G_q GPCR signaling, either locally or across brain regions (Nagai, Bellafard, et al., 2021). However, as with any approach that globally manipulates astrocyte excitation (e.g., by opsin, DREADD, calcium pump, or chelator expression), it remains unclear to what extent these interventions mimic astrocytes' varied responses and effects on neural circuits. Recent studies using reward-based behavioral assays and machine learning approaches have begun to link astrocyte excitation to information processing and behavioral variables. Using simultaneous imaging of astrocyte and neuronal calcium activity in the dorsal hippocampus (CA1 region) of head-fixed mice navigating a virtual environment, individual astrocytes' activity was found to co-vary with virtual space location. The combined information about neuronal and astrocyte activity allowed the animal's position to be reconstructed more faithfully in decoding algorithms than from neurons alone (Curreli et al., 2021). Measuring astrocyte calcium dynamics also in the hippocampal CA1 region but employing a different virtual environment, another group found that astrocytes seemingly encode reward location

in an experience-dependent manner (i.e., in a familiar but not novel environment) (Doron et al., 2021). While the two studies disagreed regarding how astrocytes encode spatial information, both suggest that non-neuronal cells may contribute to information processing. Notably, astrocytes represented the spatial environment differently than neurons. However, the extent to which motor behavior itself influenced these studies' astrocyte responses remains unclear. Astrocytes are known to exhibit widespread calcium excitation in response to locomotion (Ding et al., 2013; Nimmerjahn et al., 2009; Paukert et al., 2014). Run parameters, particularly the timing of astrocyte response onset relative to run onset and the period between runs, can strongly influence experimental results and interpretation. Addressing this concern head-on, a recent study in the mouse motor cortex showed that astrocyte population responses are linked to run onset, duration, and inter-run interval (Merten et al., 2021). Using a quantitative, reward-based visual detection task, statistical modeling, and machine learning approach, this study also showed that behavioral trial type and animal performance deterministically shaped astrocytes' spatial and temporal response characteristics. Astrocyte population responses were found to encode the animals' decision, reward, and sensory properties. Importantly, astrocyte responses seemed to carry behaviorally relevant information depending on and complementary to neuronal coding.

3.1.2 | Select insights from spinal cord recordings—To date, most spinal cord studies involving astrocytes have been performed in reduced preparations or anesthetized animals, mainly due to the technical difficulty of obtaining stable recordings from this CNS region during animal behavior, which typically results in large amplitude and nonlinear tissue displacements (Nelson et al., 2019). The few studies that have been conducted in awake mice demonstrate that anesthesia powerfully suppresses spinal neuron and astrocyte activity and that sensorimotor-driven astrocyte excitation is complex and regionally heterogeneous (Sekiguchi et al., 2016; Shekhtmeyster, Carey, et al., 2021; Shekhtmeyster, Duarte, et al., 2021). For example, using quantitative sensory and motor assays together with in vivo calcium imaging, superficial (i.e., lamina I–II) dorsal horn astrocytes were found to respond to innocuous sensory stimuli (tail pinch) with asynchronous increases in the frequency but not amplitude or duration of their microdomain transients. In contrast, noxious stimuli evoked concerted calcium transients throughout the superficial astrocyte syncytium within and across spinal segments, both in the presence and absence of a flight response (Sekiguchi et al., 2016; Shekhtmeyster, Duarte, et al., 2021). Using the same standardized behavioral assays, local neural activity was measured and compared to astrocyte excitation using separate groups of mice (Sekiguchi et al., 2016; Shekhtmeyster, Carey, et al., 2021). As expected, innocuous sensory stimuli of increasing intensity elevated the number and amplitude of responding excitatory neurons, suggesting that astrocytes' microdomain transients are driven primarily by synaptic activity. Excitatory neural activity continued to rise monotonically for more intense and noxious stimuli, implying that the spatially and temporally distinct astrocyte syncytium responses in this stimulus regime are mediated not only by synaptic but also volume transmission (e.g., neuromodulator release from descending projection fibers).

Recent studies have also indicated that spinal cord astrocytes are regionally heterogeneous (Kronschläger et al., 2021; Molofsky et al., 2014; Shekhtmeyster, Carey, et al., 2021), that

this heterogeneity is functionally relevant (Kelley et al., 2018; Kohro et al., 2020) and associated with distinct spatiotemporal excitation patterns. For example, using translaminal imaging in behaving mice with chronically implanted microprisms, astrocytes in sensory and premotor areas of the spinal dorsal horn were found to respond to noxious mechanical stimuli and locomotion in a lamina-specific manner (Shekhtmeyster, Carey, et al., 2021). In contrast, Tac1-expressing neurons, involved in pain processing, responded to the same noxious stimuli with calcium spiking in sensory regions but did not respond to locomotion, suggesting that lamina-specific astrocyte activity depends on the neuronal cell type. How, in turn, this astrocyte excitation may control neuronal activity on single-cell, circuit, and behavioral levels remains to be determined.

Studies focused on pain signaling also showed that Hes5-positive astrocytes in superficial spinal laminae gate descending noradrenergic commands that control mechanosensory behavior (Kohro et al., 2020). Additionally, dorsal horn astrocytes were shown to gate peripheral nociceptive signals. A β -fiber activation induced calcium excitation in dorsal horn astrocytes, in turn mediating long-term depression in neurokinin 1 receptor-positive projection neurons in an adenosine A1 receptor-dependent manner, thereby resulting in pain inhibition (Xu et al., 2021). Furthermore, dorsal horn astrocytes were shown to play essential roles in amplifying chronic itching through STAT3- and lipocalin-2-dependent mechanisms following their transition to a reactive state (Shiratori-Hayashi et al., 2015).

Similarly, ventral astrocytes seem to play essential roles in controlling motor behavior. Studies on various central pattern generator (CPG) circuits suggest that bi-directional communication between astrocytes and neurons constrains CPG output within an optimal operating range and in a state-dependent manner (Broadhead & Miles, 2021; Nelson et al., 2019). Multiple mechanisms (e.g., ATP, S100 β , K⁺, transporter expression/activity) have been proposed to mediate this inhibitory feedback enabling motor actions to be tailored to the animal's needs.

In summary, a deeper and more complete understanding of astrocytes' contribution to neural circuit function and animal behavior may be achieved by systematically measuring, manipulating, quantifying, and modeling astrocytes' functional status in the context of standardized and quantitative behavioral tasks. The use of such behavioral assays for relating molecular to cellular dynamics and animal behavior is all the more important given that the various dynamics may be challenging to measure simultaneously (e.g., due to a lack of appropriate indicator color variants). Nevertheless, stochastic changes in experimental conditions and behavioral state can create inherent variability across recordings and sessions, complicating the identification of relevant cellular and molecular mechanisms. Outwardly, identical behavioral states can differ in the internal state (e.g., attention), leading to differences in how local microcircuits and their constituents respond to sensory input (e.g., differences in tonic neuromodulator levels). One approach to deal with this intrinsic variability is to gather as much data as possible, including the animal's internal state, external environment, and performance on the task, such that unbiased computational approaches can be used to identify similar trials that can then be clustered and compared. Another approach is to turn to statistical modeling and machine learning approaches capable of identifying or accounting for dependent and hidden variables.

3.2 | Computational approaches

How do astrocytes integrate neuronal (and non-neuronal) signals? How do they regulate neural circuits on both short and long timescales? Computational analysis and modeling approaches are essential in answering these questions.

3.2.1 | Computational analysis—Astrocyte signal (e.g., calcium) fluctuations occur in cell bodies, major branches, and distal processes/microdomains. Multiple types of events can occur within a single astrocyte due to intracellular signal compartmentalization. Different events can have diverse footprints and heterogeneous time courses. Signals can also propagate within or between astrocytes. This propagation can be heterogeneous in terms of initiating position, speed, direction, and spread, and the propagation patterns can be distinct for different events in the same region. Accurately quantifying events that change size or location across time, propagate within or across cells, and spatially overlap with other signals is difficult with the traditional region of interest (ROI)-based methods. Recently, probabilistically principled, unbiased, and data-driven frameworks, such as Astrocyte Quantification and Analysis (AQuA), have been developed (Table 4). These frameworks use advanced machine learning approaches and consider the specific properties of astrocyte signals, promising to more accurately and comprehensively quantify the full spectrum of astrocyte activity patterns in an unbiased manner. Similar approaches may allow quantification of extracellular signals in relation to astrocyte activity, ideally in all spatial dimensions. Recent studies have also suggested that ionic (e.g., calcium) baseline changes are physiologically relevant (King et al., 2020; Tufail et al., 2017). Quantifying and modeling these changes may be particularly important in various disease settings.

Complementary to the analysis of astrocytes' complex spatiotemporal activity will be the accurate quantification of their three-dimensional structure (e.g., territory, arborization, microdomain structure) and intracellular organelle distribution (e.g., mitochondria, ER) with respect to their surrounding environment (e.g., excitatory and inhibitory synapses, neuromodulatory fibers, neuronal cell types). For example, astrocytes are in close contact with various types of neurons or neuronal structures and exhibit diverse morphologies across layers and tissue regions (Eilam et al., 2016; Lanjakornsiripan et al., 2018; Refaeli et al., 2021). The complex morphology and cellular environment place fundamental constraints on what kinds of signals astrocytes can sense, how they can be integrated locally and globally, and which types of commands they can transmit to neurons. The restricted intracellular space of astrocyte leaflets, for example, limits intracellular signal diffusion, enabling them to exert localized effects. Therefore, accurate anatomical data is crucial for model development as it provides fundamental geometric constraints on astrocytes' functional dynamics and output.

3.2.2 | Predictive modeling—Studies over the past decades have indicated that astrocytes actively respond to local and projection neuron activity, first by modulating cytosolic ion concentrations (e.g., calcium, sodium), then secreting signals, and ultimately modifying their gene expression pattern and morphology. Thus, while neurons are unarguably essential players in information processing, astrocytes need to be accounted for and integrated into circuit models to understand better how a given network operates

or dysfunctions. Indeed, it is appealing to consider astrocytes and neurons as a unified circuit (“astrocyte-neuron assembly”) since they participate in information processing in complementary manners in terms of both temporal and spatial domains. Neurons communicate on the millisecond timescale locally and over long distances and at defined release sites. Astrocytes operate on the seconds or even longer timescale (Figure 2), transmitting signals more locally and diffusely. In silico approaches provide an opportunity to incorporate these multilevel and multiscale data into a coherent framework and make testable predictions. Among computational models that include astrocytes, four general types can be discerned (Manninen et al., 2018): single astrocyte models, astrocyte network models, neuron-astrocyte synapse models, and neuron-astrocyte network models. Over time, these models have become increasingly complex, some incorporating astrocytes’ intricate three-dimensional architecture and multiscale dynamics (Savtchenko et al., 2018).

In silico approaches offer many advantages. They allow investigation of questions difficult if not impossible to address in live animals in a tightly controlled manner. For example, guided by omic approaches, they can help assess how (patho-)physiological astrocyte variations affect various dynamic properties (e.g., how regional differences in IP₃ receptor expression/distribution influence astrocyte calcium transients, or how disease-associated changes in K⁺ channel expression alter spatial buffering and neuronal excitability) (Denizot et al., 2019). Modeling also allows critical evaluation of experimental interventions (e.g., how different calcium indicator/exogenous buffer concentrations alter astrocyte compartment or network transients and, thereby, downstream signaling such as transmitter release) (Semyanov et al., 2020). Additionally, plausible mechanisms contributing to experimental observations can be explored (e.g., whether and how different astrocyte signaling pathways contribute to neuronal synchronization during attentional shifts or epileptic discharges). In silico approaches are also essential for generating new hypotheses or making experimentally testable predictions about astrocytes’ supportive or augmenting role in neural circuit function (e.g., their calcium elevations transiently store neuronal activity traces allowing them to determine optimal energy resource distribution or modulate specific synaptic connections upon recurrence or recall) (Gordleeva et al., 2019; Gordleeva et al., 2021; Kastanenko et al., 2020).

However, the power and accuracy of in silico models critically depend on information about dependent variables and experimental data, and while incorporating more variables can increase model flexibility, it also bears the risk of overfitting. Variables to consider may include:

- a. The types and concentrations of neurotransmitters, neuromodulators, and neuropeptides (co-)released into the extracellular space (e.g., dopamine during rewarded behavioral task trials),
- b. Their spatiotemporal characteristics (e.g., synaptic or volume transmission),
- c. Their concentration-dependent action on relevant receptors (e.g., α_1 or α_2 receptor activation during high- or low-extracellular norepinephrine concentrations, respectively),

- d. Nonlinear interactions between signaling pathways (e.g., glutamate and norepinephrine),
- e. Constraints on astrocyte excitation (e.g., refractory duration to replenish intracellular stores following calcium depletion),
- f. Astrocytes' dynamic morphology and coupling (e.g., activity-dependent alterations),
- g. Astrocyte heterogeneity (e.g., layer-specific differences in functional properties),
- h. The potential influence of environmental or blood-borne factors (e.g., pO₂ and pH),
- i. Metabolic and circadian factors, and
- j. Network state (e.g., asleep, awake, attentive).

Modeling these multilevel, multiscale interactions is, without a doubt, a daunting task and necessarily phenomenological at early stages. Perhaps the first step to tackle this challenge is to construct (or build on existing) simplified models that recapitulate defined sets of *in vivo* observations (e.g., astrocytes' nonlinear calcium responses to neurotransmitter and neuromodulator receptor co-activation). As more *in vivo* data becomes available (e.g., from direct neuromodulator signaling measurements), these models will need to be iteratively updated. Models that recapitulate different aspects of neuron-astrocyte interaction will then need to be integrated and extended to larger cellular networks. Gradually, these models are expected to become more predictive and identify potential hidden factors that influence astrocyte excitation (e.g., blood-borne factors), thereby guiding future experiments. Measurements from different CNS areas or using behavioral task variations to interrogate a given circuit will help determine conserved or regionally unique mechanisms. Behavioral assays should allow control of signaling pathway recruitment (e.g., dopamine release) and quantitative readout of animal state, behavior, or the effect of targeted pathway alterations (e.g., by AAV-mediated interventions).

In summary, new and improved data analysis tools and mathematical models promise to provide the sorely needed quantitative description of how astrocytes spatially and temporally integrate molecular signals in their environment and how the intracellular dynamics that underlies this integration leads to functional outputs that modulate synaptic function or network state on various timescales.

4 | CHALLENGES IN INTERPRETING ASTROCYTE FUNCTION

4.1 | Astrocyte heterogeneity, interspecies diversity, and their relevance to neural circuit function and animal behavior

Astrocytes are a heterogeneous cell population. They differ in their structural and functional properties between, but also within CNS regions (Clarke et al., 2021; Emsley & Macklis, 2006; Farmer & Murai, 2017; Khakh & Deneen, 2019; Matias et al., 2019; Matyash & Kettenmann, 2010; Oberheim et al., 2012; Zhang & Barres, 2010). Various metrics have been defined to characterize cellular heterogeneity. Metrics commonly used to define

neuronal subpopulations include the location (e.g., brain region or cell layer), morphology (e.g., local or projection neuron, axonal or dendritic arborization), gene expression (e.g., parvalbumin, somatostatin, vasoactive intestinal peptide), biochemical (e.g., neurotransmitter usage), and physiological properties (e.g., action potential firing properties). Similar metrics have been used to describe astrocyte heterogeneity (Table 5). Some overlap exists between the resulting cell populations.

Astrocyte heterogeneity is functionally relevant for the control of neural circuit function. For instance, gene expression differences across cortical layers include genes involved in synaptic regulation (e.g., *Sparc*, *Mertk*), neurotransmitter uptake (e.g., *Slc1a3/GLAST*), and astrocyte coupling (e.g., *Gja1/Cx43*) (Lanjakornsiripan et al., 2018). Intriguingly, these gene expression-defined “astrocyte layers” are distinct from cortical neuronal layers and differ between cortical areas (Bayraktar et al., 2020). Astrocyte populations support synaptogenesis in ways that depend on their gene expression profiles (John Lin et al., 2017). Hippocampal and striatal astrocytes differ in their K^+ channel, gap junction protein (e.g., *Gja1/Cx43*), glutamine synthetase, calcium channel, and pump expression (Chai et al., 2017). The latter contributes to differences in calcium signaling (Chai et al., 2017). Given the central role of calcium excitation in astrocyte physiology, these disparities may be partly responsible for regional differences in gliotransmission, neuromodulation, structural dynamics, and blood flow regulation (Clarke et al., 2021; Khakh & Deneen, 2019; Semyanov et al., 2020). This heterogeneity may extend down to the subcellular level, with each astrocyte microdomain potentially performing different functions depending on its proximity to surrounding neurons or non-neuronal cells. Regional heterogeneity may also cause differences in injury responses, with the injury itself promoting astrocytes’ functional differentiation (Sofroniew, 2020). In summary, astrocyte heterogeneity will likely need to be considered in models of neural circuit function. Given the existing tools, determining exactly how local and regional astrocyte heterogeneity contributes to neural circuit function will be a significant experimental challenge.

The observation of astrocyte heterogeneity on different spatial scales raises the question about its origin. White matter astrocytes in the spinal cord are generated by distinct developmental domains and express specific transcription factor combinations that establish their positional identity (Hochstim et al., 2008). The regional identity of astrocytes in the brain is defined by region-specific transcriptional and epigenetic signatures, suggesting that they are derived from nucleus-specific progenitors (Herrero-Navarro et al., 2021). Additionally, the interaction of astrocytes with neighboring neurons contributes to their heterogeneity, for example, through sonic hedgehog signaling (Farmer et al., 2016; Lanjakornsiripan et al., 2018; Stogsdill et al., 2017). Neuronal activity (e.g., glutamatergic signaling) promotes astrocyte functional and morphological maturation (Morel et al., 2014). An instructive role for neuronal cues on astrocyte properties was also shown in *Satb2* and *Reeler* mutant mice, in which changes in neuronal layering are accompanied by alterations in astrocyte layering (Bayraktar et al., 2020). Astrocyte heterogeneity, therefore, appears to result from developmental programs and local environmental adaptations (see Clarke et al., 2021 for an in-depth review) and may be relevant to disease.

In addition to astrocyte heterogeneity within a given organism's CNS, astrocytes show diversity across species (see Nagai, Yu et al., 2021 for a comparison between *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio*, and *Mus musculus*). This raises the crucial yet experimentally challenging question of how astrocytes modulate neural circuit function and behavioral characteristics in humans. Human and experimental animal model astrocytes differ in many properties (Bedner et al., 2020; De Majo et al., 2020). Astrocyte processes cover larger volumes in the human brain and contact ~10–20 times as many synapses as in rodents (Oberheim et al., 2006). The astrocytic domain overlap is also more extensive in humans, and two unique types of astrocytes are present in the cortex (interlaminar and varicose projection astrocytes, potentially involved in long-distance communication) (Colombo & Reisin, 2004; Oberheim et al., 2009). Human astrocytes also show unique gene expression profiles in health and disease (Nagai, Yu, et al., 2021; Zhang et al., 2016). How these differences affect human neural circuit function, behavior, and disease remains to be determined.

One approach to studying human astrocyte diversity and its impact on brain circuit physiology has been to transplant stem or progenitor cell-derived human astrocytes into the rodent brain (Chen et al., 2015; de Majo et al., 2020; Goldman et al., 2015). A similar approach has been used to study primate-specific interlaminar astrocytes in mice (Padmashri et al., 2021). Strikingly, grafting human astrocytes into the mouse forebrain enhanced synaptic plasticity and learning (Han et al., 2013). Transferring human induced pluripotent stem cell (iPSC)-derived glial cells from schizophrenia patients into the mouse brain resulted in astrocytes with abnormal morphology, hypomyelination, and behavioral deficits resembling a schizophrenia phenotype. These findings suggest that human glial cells contribute to disease-specific circuit alterations (Windrem et al., 2017). Models that closely recapitulate the human tissue environment will be essential to understand better how astrocytes contribute to human brain and spinal cord function.

These examples of astrocyte heterogeneity and interspecies diversity highlight that we are just beginning to understand how the CNS' complex cytoarchitecture and signaling control animal behavior. Vital questions that will likely drive research in the coming years include:

1. To what extent do current astrocyte classifications capture physiologically relevant sub-populations? Which other or additional parameters might help define functionally homogeneous populations to study their impact on neural circuits?
2. Which differences between astrocytes are crucial for controlling neural circuit function?
3. How does astrocyte heterogeneity due to cell-intrinsic properties or neuronal heterogeneity influence neural circuit function?
4. To what extent does astrocyte heterogeneity and diversity support neuronal function or serve a complementary role (e.g., control neural circuit activity or network state during animal behavior)?

5. How functionally heterogeneous are astrocytic microdomains? How does this heterogeneity relate to local neural circuits?
6. What are the conserved functions between human and model organism astrocytes? How are potential functional differences behaviorally relevant?

Tackling these questions will likely require developing novel tools and experimental approaches (see above). While it is generally accepted that brain or spinal cord function cannot be understood without taking neuronal diversity into account, this most likely applies to astrocytes as well.

4.2 | Astrocyte metabolism and its relationship to animal behavior

Metabolic cooperation between neurons and astrocytes is perhaps one of the most intuitive examples of the functional interdependency of these cell types in health and disease. Various kinds of metabolic interactions have been identified, including the glutamate-glutamine cycle (Bak et al., 2006; Martinez-Hernandez et al., 1977), glutathione metabolism (Dringen et al., 2000; Hirrlinger & Dringen, 2010), and the astrocyte-neuron-lactate shuttle (ANLS) (Pellerin & Magistretti, 1994; see Bak & Walls, 2018; Barros & Weber, 2018 for a critical discussion of the ANLS). Recent evidence suggests that astrocyte metabolism does not only contribute to metabolic and energetic homeostasis of the tissue they reside in but plays a crucial role in behavioral regulation (Bonvento & Bolaños, 2021). Astrocyte glycogen metabolism, for example, is essential for memory consolidation in chickens (Gibbs, 2016; Gibbs et al., 2006). Cortical activity upon arousal induces astrocyte lactate release via adrenergic signaling in mice (Zuend et al., 2020). Astrocyte glycogenolysis and lactate transport from astrocytes to neurons, induced by stimulation of astrocytic β 2-adrenergic receptors, is necessary for memory formation and consolidation in the rat hippocampus (Gao et al., 2016; Netzahualcoyotzi & Pellerin, 2020; Newman et al., 2011; Suzuki et al., 2011). Lactate modulates NMDA receptor-mediated signaling, plasticity-related gene expression, and learning-induced mRNA transcription in neurons (Descalzi et al., 2019; Yang et al., 2014). Notably, lactate can fulfill diverse roles in the target cells, including as an energy substrate or modulating cellular redox state, by directly activating (e.g., GPR81/HCAR1) or modulating receptors (e.g., NMDARs) and affecting various signaling (e.g., calcium) (Barros, 2013). Neuronal activity-related and animal stress-induced protein lactylation were also recently shown to depend on lactate availability (Hagihara et al., 2021), extending the range of lactate's functions to epigenetic regulation. Additional roles for lactate (and other metabolites) are likely.

Mitochondrial reactive oxygen species (mtROS) also play critical roles in astrocyte metabolism and metabolic cooperation with neurons. Reduction of mtROS specifically in astrocytes leads to mouse behavioral changes in the open-field test and cognitive deficits in a novel object recognition test (Vicente-Gutierrez et al., 2019). Activation of type I cannabinoid receptors (CB₁Rs) on astrocyte mitochondria results in reduced mtROS production, glycolysis, and lactate release, neuronal energy stress, and, consequently, impaired social behavior (Jimenez-Blasco et al., 2020), highlighting the powerful effects of these receptors on cell-type-specific metabolism, astrocyte-neuron assembly function, and animal behavior.

Another essential pathway involves the amino acid L-serine. This precursor of the NMDA receptor co-agonists D-serine and glycine is synthesized in astrocytes from the glycolysis intermediate 3-phosphoglycerate via the phosphorylated pathway. Inhibition of this pathway alters NMDA receptor signaling and LTP in the mouse hippocampus and spatial memory (Le Douce et al., 2020; Neame et al., 2019). Notably, L-serine and D-serine levels are reduced in a mouse model of Alzheimer's disease, and deficits in synaptic plasticity and spatial memory were rescued by D-serine application (Le Douce et al., 2020). Astrocytic CB₁Rs contribute to regulating the extracellular D-serine concentration, and deletion of these receptors in astrocytes results in impaired LTP and object recognition memory (Robin et al., 2018).

Recent evidence also suggests that astrocytes metabolize fatty acids via β -oxidation and that this pathway is involved in metabolic cooperation between astrocytes and neurons (Eraso-Pichot et al., 2018; Fecher et al., 2019; Ioannou et al., 2019; Kontinen et al., 2019; Schirmeier et al., 2021; Timper et al., 2020). Furthermore, saturated lipids have been identified as a neurotoxic factor released from astrocytes under pathological conditions (Guttenplan et al., 2021). Whether and how astrocytes' fatty acid metabolism contributes to regulating neural circuit function and animal behavior remains to be investigated.

Together, these examples demonstrate that astrocyte metabolism and animal behavior mutually affect one another (Alberini et al., 2018; Bonvento & Bolaños, 2021). Astrocyte metabolism plays a vital role in learning, memory, and behavior. Its regulation occurs on many different and likely interconnected levels. Elucidating metabolic processes in astrocyte-neuron assemblies will be necessary for a deeper understanding of how they regulate neural circuit function and animal behavior.

4.3 | Beyond astrocytes: The contribution of other glial cells to neural circuit function and animal behavior

Accumulating evidence indicates that astrocytes are not the only resident non-neuronal cells capable of modulating neural circuit function and animal behavior on various timescales. Microglia and oligodendroglial lineage cells (OLCs), either alone or in close coordination with astrocytes, can also modulate the structural and functional plasticity of neurons.

Microglia are the intrinsic immune sentinels of the CNS. Their highly motile processes continually survey the environment, allowing them to detect membrane-bound signals (e.g., phosphatidylserine) (Davalos et al., 2005; Fourgeaud et al., 2016; Huang et al., 2021; Nimmerjahn et al., 2005; Tufail et al., 2017). These physical cell-cell interactions are critical for shaping neural circuits and connections during development and disease (e.g., pruning immature, inactive, or dysfunctional synapses) (Hong et al., 2016; Li & Barres, 2018; Paolicelli et al., 2011; Wilton et al., 2019). They also contribute to learning-dependent synapse formation in the mature brain, thereby modulating animal behavior on a longer timescale (Parkhurst et al., 2013). Additionally, microglia communicate with neurons (and astrocytes) through soluble factors (e.g., purinergic signaling), allowing more rapid communication (Cserép et al., 2021). For example, microglia have recently been shown to control neuronal firing frequency and synchrony via adenosine, providing an important

negative feedback loop for neural circuits (Badimon et al., 2020). Thus, microglial actions may need to be considered when investigating glial control of animal behavior.

Similarly, OLCs—which include oligodendroglia precursor cells (OPCs; also referred to as NG2 cells) and oligodendrocytes (OLs)—dynamically interact with neurons, regulating their fate, myelination, and circuit properties (Bonetto et al., 2021; Foster et al., 2019; Moore et al., 2020; Suminaite et al., 2019; see also the recent GLIA Special Issue on “Plasticity of Myelinating Glia”: Fields & Richardson, 2019). OPCs receive direct synaptic input from neighboring axons (Bergles et al., 2000), and glutamatergic signaling (synaptic and non-synaptic) affects OLCs on different levels (Kula et al., 2019). Developing OLs regulate myelin sheath elongation and stability in an activity-dependent manner (Baraban et al., 2018; Krasnow et al., 2018). Myelination of axons in the adult brain is also controlled by neuronal activity and has been shown to modulate motor learning, motor performance, sound localization, and memory persistence (Bacmeister et al., 2020; Ford et al., 2015; Gibson et al., 2014; McKenzie et al., 2014; Pan et al., 2020; Wang et al., 2020; Xiao et al., 2016). OLs adapt their metabolite supply according to axonal activity (Saab et al., 2016). Conversely, nutritional signals regulate OL proliferation, myelin regulatory factor-dependent perineuronal net remodeling, and tuning of the circuit that controls food intake and weight gain in the mediobasal hypothalamus (Kohnke et al., 2021). Adaptive myelination is also critical for spike-timing-dependent plasticity in neural circuits (Monje, 2018; Suminaite et al., 2019), and activity-dependent, brain region-specific oligodendrogenesis may be essential for episodic memory formation (Barboza et al., 2021).

While these examples show that microglia and OLCs dynamically modulate specific neural properties, the close interaction between non-neuronal cell types suggests an even more complex scenario (Figure 3). For example, astrocytes communicate with OLs (e.g., via gap junction-coupled pan-glial networks) (Griemsmann et al., 2015; Theis & Giaume, 2012) and contribute to myelination through lipids (e.g., cholesterol) and other signaling molecules (Camargo et al., 2017; Kiray et al., 2016; Molina-Gonzalez & Miron, 2019). Perinodal astrocytes control myelin thickness, nodal gap length, axonal excitability, and conduction velocity (Dutta et al., 2018; Lezmy et al., 2021). Microglia, too, communicate with OLs to affect myelination (Bar & Barak, 2019). Astrocytes closely interact and coordinate with microglia (e.g., synaptic pruning), and disruption of this signaling has been linked to various disease phenotypes (Jha et al., 2019; Liddel et al., 2020). Microglia can modulate astrocyte gliotransmission by regulating VAMP2 proteins (Takata-Tsuji et al., 2021). Therefore, current evidence suggests that neurons, astrocytes, microglia, and OLCs co-operate multi-laterally to regulate neural circuit function. If and how other cell types (e.g., tanycytes, ependymal cells, and pericytes) also contribute to this process remains to be investigated. Establishing a comprehensive view of these complex interactions underlying precise control of neural circuit function on various timescales in health and disease will be a significant challenge for future studies.

5 | CONCLUDING REMARKS

Astrocytes play essential roles in neural circuit function, including the control of extracellular ion homeostasis, transmitter recycling, metabolic supply, and synaptic

plasticity. Yet, fundamental questions regarding their role in behavior-related neural circuit regulation remain unresolved. How do they integrate the diverse neuronal signals associated with animal behavior? How do they modulate neural circuit function and structure on behaviorally relevant timescales in response to these signals (Figure 1)? How does impairment of these functions contribute to disease? How can astrocytes' beneficial roles be boosted in disease to protect CNS cells from damage and promote regeneration (Pekny et al., 2016)? Addressing these and other questions (Box 1) will likely require a multiscale approach, including functional, anatomical, molecular, and omic analyses and theoretical modeling. Novel indicators, actuators, genetic targeting approaches, quantitative behavioral assays, and computational methods are beginning to provide much-needed answers to these questions (Tables 1–4). However, integrating astrocytes into existing neural circuit models will not be easy. Astrocytes have unique properties presenting unique challenges. They regulate neural circuits on various spatial and temporal scales (from sub-micron to millimeters and from sub-seconds to days or more) (Figure 2), are functionally heterogeneous (Table 5), and closely coordinate with other non-neuronal cells (Figure 3). Tools to contend with astrocytes' functional complexity and 3D arrangement are still in their infancy but will be critical for uncovering these cells' sensitively orchestrated actions and adequately integrating them into predictive models.

Elucidating the full spectrum of astrocytes' dynamic functions will also be vital for optimal disease treatment. Fundamental questions in this area include: How do astrocytes initiate or perpetuate pathophysiological CNS changes? How can these changes be controlled to minimize CNS damage and promote regeneration? How does astrocyte heterogeneity contribute to region-specific disease characteristics? How does the dynamic interplay of astrocytes with other non-neuronal cells, such as microglia, OLs, and circulating or infiltrating nonresident immune cells, influence various disease stages (Liddel et al., 2020; Nutma et al., 2020)? Answering these fundamental questions will likely require a multidisciplinary approach involving researchers from neuroscience, immunology, pharmacology, clinical, and engineering disciplines.

In conclusion, uncovering the molecular, cellular, and circuit mechanisms by which astrocytes influence neural circuit dynamics promises to transform our understanding of how astrocyte-neuron assemblies shape brain and spinal cord computations and complex animal behaviors. Additionally, they will provide the basis for conceptually novel approaches to combat inflammatory and neurodegenerative conditions for which only limited or suboptimal treatment options exist.

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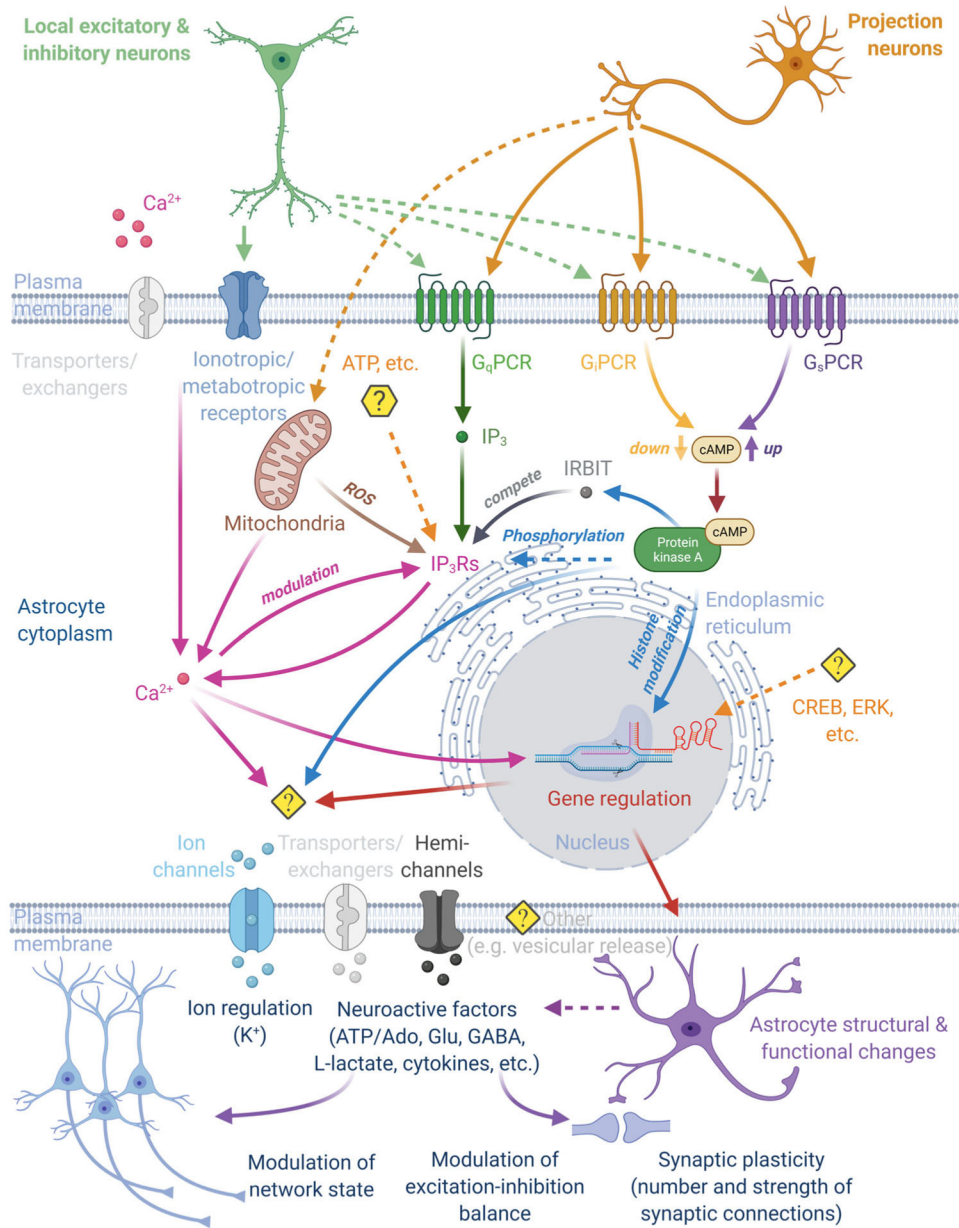


FIGURE 1. Astrocytes regulate and are controlled by neural circuits and animal behavior. Animal behavior activates a subset of local excitatory, inhibitory, and projection neurons, leading to the release of diverse molecular signals. Astrocytes are thought to spatially and temporally integrate these time-varying signals in their environment, with calcium and/or PKA playing central roles in this process. Signal integration also involves intermediate signals, such as reactive oxygen species (ROS), IP₃, cAMP, and IRBIT. Astrocyte excitation, in turn, is thought to modulate neural circuit function (e.g., network state, excitation-inhibition balance, synaptic strength, or number) through different mechanisms (e.g., extracellular ion regulation, neuroactive factor release, perisynaptic process structure) and on various timescales (second to minutes) (see also Figures 2–3). Abbreviations: Ado, adenosine;

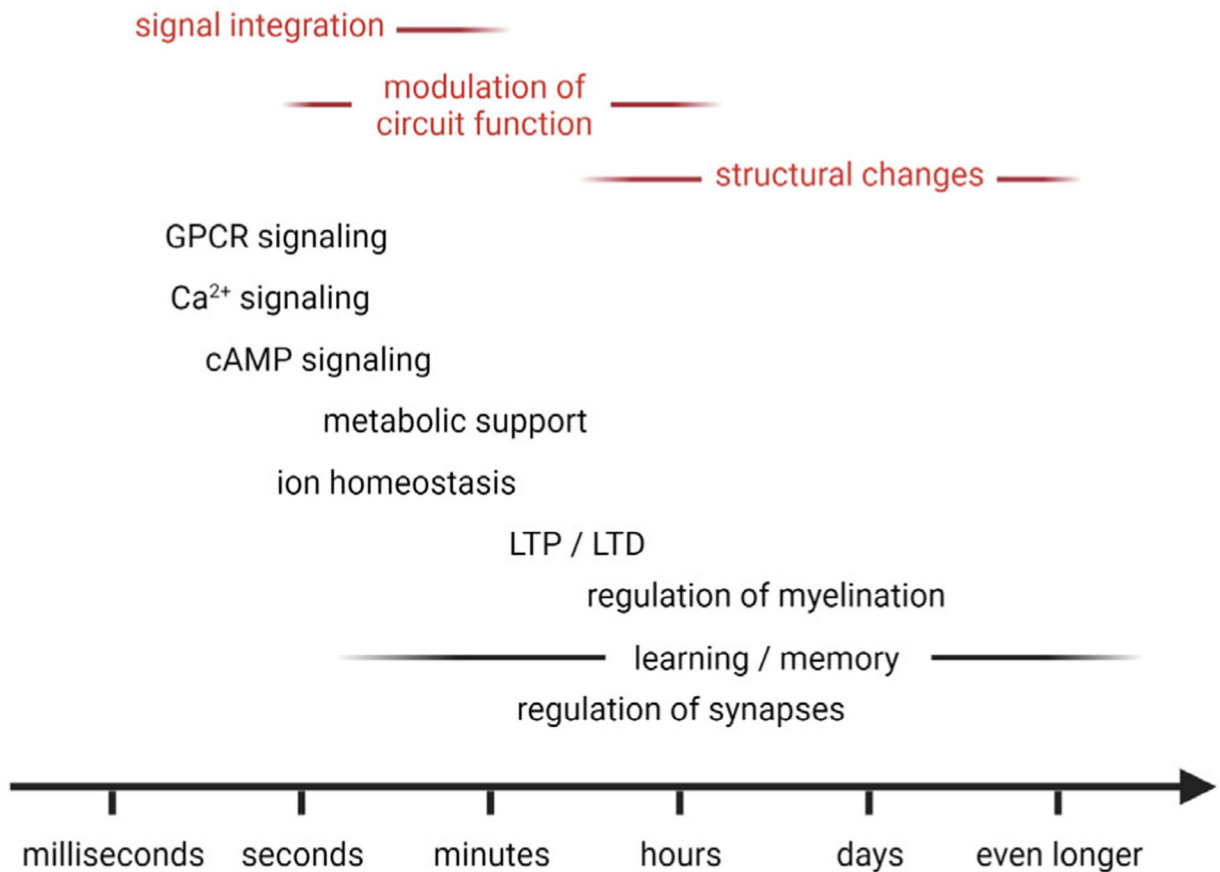
cAMP, cyclic adenosine monophosphate; Glu, glutamate; IP₃, inositol-trisphosphate; IRBIT, IP₃ receptor-binding protein released with IP₃; PKA, protein kinase A

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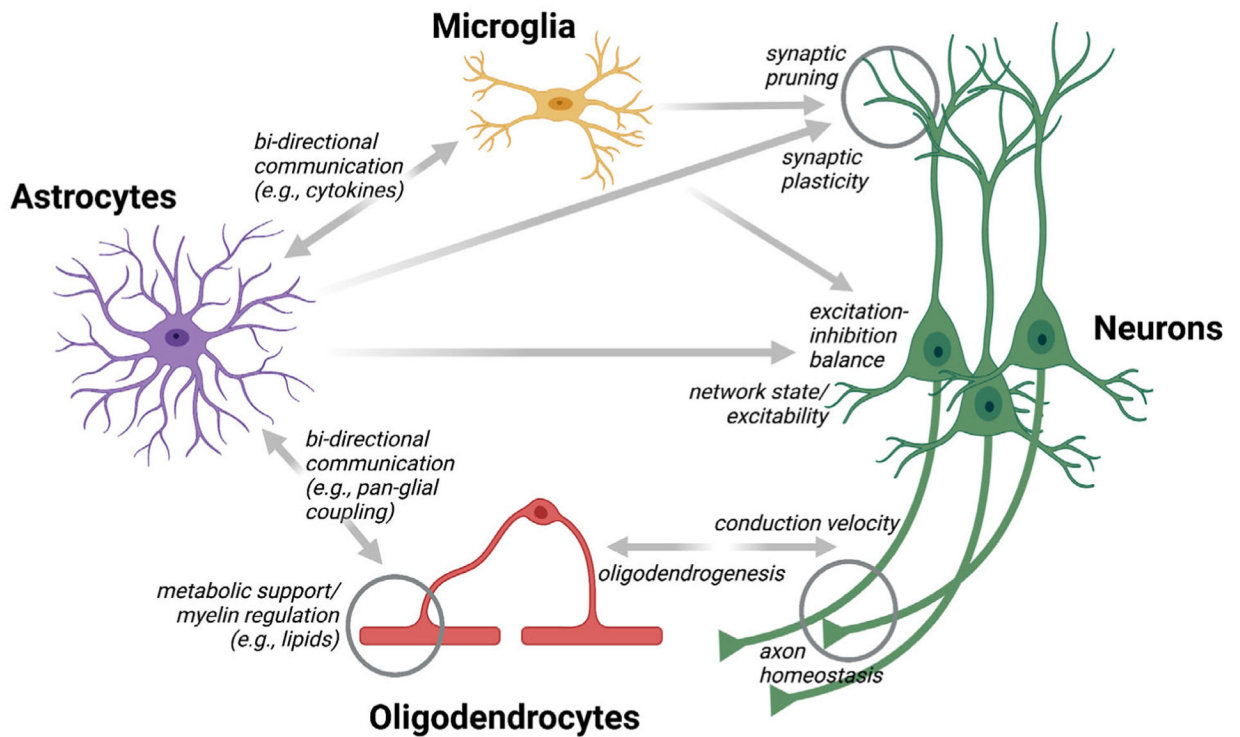
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**FIGURE 2.**

Astrocytes regulate neural circuits on various timescales. Astrocyte transients (e.g., calcium, cAMP) tend to be slow (sub-seconds to minutes), resulting from molecular signal integration in their environment. Similarly, astrocyte modulation of neural circuit function (e.g., through regulation of extracellular ion homeostasis, neuroactive factor release, or metabolic support) typically occurs on the seconds to minutes timescale, whereas morphological and gene expression-dependent changes (e.g., perisynaptic process structure, myelin regulation) influence neural circuit plasticity and function on the minutes to days or even longer timescale. Notably, the spatiotemporal dynamics of astrocytes' functional signals, their spatial arrangement, gene expression, and coupling suggest that these cells serve complementary roles to neurons (see text for more details). Abbreviations: cAMP, cyclic adenosine monophosphate; GPCR, G-protein coupled receptor; LTD, long-term depression; LTP, long-term potentiation

**FIGURE 3.**

Astrocytes regulate neural circuits directly and indirectly. Astrocytes can modulate neural circuit function directly through different mechanisms (see Figure 1). Microglia have also been shown to control neuronal synapse number and activity on various timescales. Similarly, oligodendrocytes can regulate neurons' axonal properties (e.g., conduction velocity) in an activity- and behavior-dependent manner. Astrocytes bidirectionally communicate with and regulate both microglia and oligodendrocytes (e.g., through diffusible messengers and physical interactions), allowing them to influence neural circuits indirectly and in a spatially and temporally distinct manner than their direct routes

TABLE 1

Genetically encoded indicators for measuring astrocyte function^a

Available indicators for readout of neuron-astrocyte signaling		
Indicator type	Indicator name(s)	Available color variants
Acetylcholine	iAChSnFR, GRAB _{ACh}	Green
Adenosine	GRAB _{Ado}	Green
ATP	iATPSnFR, GRAB _{ATP}	Green
Dopamine	dLight, GRAB _{DA}	Green, yellow, red
D-Serine	D-serFS ^b	Cyan-yellow ^b
Endocannabinoid	GRAB _{cCB}	Green
GABA	iGABASnFR	Green
Glutamate	iGluSnFR	Blue, cyan, green, yellow
Glycine	GlyFS ^b	Cyan-yellow ^b
Nicotine	iNicSnFR	Green
Norepinephrine	nLight, GRAB _{NE}	Green
Serotonin	sLight, iSeroSnFR, GRAB _{5-HT}	Green
Available indicators for readout of intracellular signaling		
ATP/ADP	Perceval	Green
ATP	ATeams ^b	Cyan-yellow ^b
Calcium	X-CaMP series, X-GECO series	Blue, green, yellow, red, near-infrared
cAMP	Flamingo series, R-FlincA, cAMPt	Yellow, red
Chloride	SuperClomeleon ^b , ClopHensorN ^b	Cyan-yellow ^b , green-red ^b
ERK	RAB-EKARev ^b	Green-red ^b
Glucose	iGlucoSnFR, FLIPglu ^b , Green Glifons	Green, cyan-yellow ^b
IP ₃	IRIS-1 ^b	Cyan-yellow ^b
Lactate	eLACCO, Laconic ^b	Green, cyan-yellow ^b
NADH/NAD ⁺	Peredox ^b	Green-red ^b
References		
		Borden et al., 2020; Jing et al., 2020
		Peng et al., 2020
		Lobas et al., 2019; Wu, He, et al., 2021
		Patriarchi et al., 2018; Patriarchi et al., 2020; Sun et al., 2018
		Vongsouthi et al., 2021
		Dong, He, et al., 2021
		Marvin et al., 2019
		Marvin et al., 2018
		Zhang et al., 2018
		Shivange et al., 2019
		Feng et al., 2019; Oe et al., 2020
		Patriarchi et al., 2018; Unger et al., 2020; Wan et al., 2021
		Berg et al., 2009
		Inamura et al., 2009
		Dalangin et al., 2020; Dana et al., 2016; Dana et al., 2019; Inoue et al., 2019; Qian et al., 2020; Shemetov et al., 2021; Shen et al., 2018
		Hackley et al., 2018; Harada et al., 2017; Kim, Shin, & Bae, 2021; Odaka et al., 2014; Ohta et al., 2018
		Grimley et al., 2013; Raimondo et al., 2013
		Ding et al., 2015
		Díaz-García et al., 2019; Fehr et al., 2003; Keller et al., 2021; Mita et al., 2019
		Matsu-ura et al., 2006
		Nasu et al., 2021; San Martín et al., 2013
		Hung et al., 2011

NADPH	iNap ^b	Cyan-yellow ^b	Tao et al., 2017
pH	pHRed ^b , ClopHensorN ^b	Red, green-red ^b	Raimondo et al., 2013; Taniama et al., 2011
PKA	ExRai-AKAR	Green	Zhang et al., 2021
Potassium (K ⁺)	GEPII ^b , KIRIN ^b , GINKO2, KRaiON	Cyan-yellow ^b , green-red ^b , green	Bischof et al., 2017; Torres Cabán et al., 2021; Wu, Wen, et al., 2021
ROS (H ₂ O ₂)	FROG/B ^b , HyPer7 ^b , HyPerRed	Blue-green ^b , green ^b , red	Ermakova et al., 2014; Pak et al., 2020; Sugiura et al., 2020
Available indicators for readout of astrocyte-neuron signaling			
ATP, GABA, glutamate, etc.	See above	See above	See above

Abbreviations: cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; IP₃, inositol-trisphosphate; PKA, protein kinase A; ROS, reactive oxygen species.

^aThis table does not cover existing synthetic indicators (e.g., CoroNa Green AM for sodium), which may be helpful in acute experiments. For additional genetically encoded indicators (e.g., for cAMP), see the cited reviews. For an overview of additional genetically encoded metabolite sensors, see Koveal et al., 2020; San Martin et al., 2014; Zhang et al., 2020; San Martin et al., 2022.

^bRatiometric indicator.

TABLE 2

Genetically encoded actuators for interrogating astrocyte function^a

Available actuators for astrocyte “excitation”

Actuator type	Actuator name(s)	Mechanism	References
Calcium	Chr2, CatCh	Cation channel	Beppu et al., 2014; Figueiredo et al., 2014
	ArchT	Proton pump	Beppu et al., 2014; Poskanzer & Yuste, 2016
	Opto-XRs	G-protein signaling ^b	Figueiredo et al., 2014
	DREADDs	G-protein signaling ^b	Chat et al., 2017; Durkee et al., 2019
cAMP/cGMP	YFP-CaRhAC/YFP-CaRhGC	Enzyme activity (adenylyl/guanylyl cyclase)	Scheib et al., 2018

Available actuators for astrocyte “inhibition”

Actuator type	Actuator name(s)	Mechanism	References
Calcium	CalEx	Reduces calcium signaling after expression of the plasma membrane calcium pump PMCA2	Yu et al., 2018; Yu et al., 2021
	SpiCee	Reduces calcium signaling by calcium binding to a chimeric calmodulin- and α -parvalbumin-based calcium buffer	Ros et al., 2020
	β ARK	Attenuates G_q -GPCR-evoked calcium signaling by sequestering $G_{\alpha q}$ -GTP	Nagai, Bellafard, et al., 2021
cGMP	SponGee	Reduces cGMP signaling by cGMP binding to a chimeric PKG1 α /PKG1 β cGMP buffer	Ros et al., 2019
IP ₃	IP ₃ sponge	Competes with the endogenous IP ₃ Rs for IP ₃ binding in a dose-dependent manner	Miyamoto & Mikoshiba, 2017; Uchiyama et al., 2002

Abbreviations: cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; GPCR, G-protein coupled receptor; IP₃, inositol-trisphosphate.

^aWhile these actuators may target a specific signaling pathway, it is important to note that their effects can be broad, indirect, and divergent. For example, CalEx expression leads to numerous gene expression changes affecting multiple astrocyte functions at once (Nagai, Bellafard, et al., 2021). Chr2 and ArchT both elevate intracellular calcium levels but have opposing effects on glutamate release (Beppu et al., 2014). Calcium elevations can be partly due to autocrine signaling following stimulated transmitter release (e.g., ATP) (Figueiredo et al., 2014).

^bUnlike in neurons, “inhibitory” opsins and DREADDs typically lead to calcium excitation in astrocytes (Durkee et al., 2019; Poskanzer & Yuste, 2016).

TABLE 3

Genetic approaches for astrocyte targeting

Approach	Concept	Properties	References
Split-Cre	<ul style="list-style-type: none"> The open reading frame (ORF) of Cre is divided into two parts Each part is expressed by a different promoter 	<ul style="list-style-type: none"> Compatible with all LoxP-flanked alleles Enables cell-type-specific labeling (e.g., with standard reporter alleles), knockout, and analyses (e.g., using RiboTag mice) 	Beckervordersandforth et al., 2010; Hirrlinger et al., 2019; Hirrlinger, Scheller, et al., 2009; Jullien et al., 2003; Kim, Kolesnikov, et al., 2021
Split-Cre-ERT2	<ul style="list-style-type: none"> Tamoxifen inducible version of Split-Cre 	<ul style="list-style-type: none"> Temporal control of Split-Cre mediated recombination 	Hirrlinger et al., 2019; Hirrlinger, Requardt, et al., 2009
Split-Cre-Intein	<ul style="list-style-type: none"> Intein mediated interaction of Cre parts 	<ul style="list-style-type: none"> High-recombination efficiency Works with three promoters ("SpaRCLIN") 	Luan et al., 2020; Salwig et al., 2019; Wang et al., 2012
Combination of different DNA recombinases (e.g., Cre, Dre, Flp)	<ul style="list-style-type: none"> Each recombinase is driven by a different promoter 	<ul style="list-style-type: none"> Use of full-length recombinases Special reporter alleles necessary Allows cell type targeting by the consecutive activity of promoters or Boolean logic (e.g., promoter 1 active, but not promoter 2) 	Jensen & Dymecki, 2014; Liu et al., 2020; Madisen et al., 2015
Light-activated DNA recombinases	<ul style="list-style-type: none"> Light-induced dimerization of split-DNA recombinases 	<ul style="list-style-type: none"> Precise control of DNA recombination in space and time through light application 	Weinberg et al., 2019; Yao et al., 2020
Combination of DNA recombinases and the Tet-system	<ul style="list-style-type: none"> Two promoters driving Cre and tTA 	<ul style="list-style-type: none"> Combinatorial targeting by Cre and tTA Temporal control by doxycycline application 	Ahmadzadeh et al., 2020; Madisen et al., 2015; Roselló-Diez et al., 2018

TABLE 4Computational tools for quantifying astrocyte or neuronal activity^a

Select tools for quantifying astrocyte excitation		
Name	Properties	References
AQuA	<ul style="list-style-type: none"> • Java- and MATLAB-based • Probabilistically principled, unbiased, data-driven framework 	Wang et al., 2019
CaSCaDe	<ul style="list-style-type: none"> • MATLAB-based • Developed for multi-photon data 	Agarwal et al., 2017
GECIquant	<ul style="list-style-type: none"> • Java-based • Semi-automated, region of interest (ROI)-based framework 	Venugopal et al., 2019
N/A	<ul style="list-style-type: none"> • MATLAB-based • Automated region of activity (ROA)-based analysis algorithm 	Bojarskaite et al., 2020
Select tools for quantifying neuronal activity		
CalmAn	<ul style="list-style-type: none"> • Python-based • Suitable for one- and multi-photon imaging data • Enables real-time analysis on streaming data 	Giovannucci et al., 2019
CASCADE	<ul style="list-style-type: none"> • Python-based • Developed for multi-photon data • Enables spike inference from calcium imaging data 	Rupprecht et al., 2021
EXTRACT	<ul style="list-style-type: none"> • MATLAB-based • Estimation theory-based 	Inan et al., 2021
EZcalcium	<ul style="list-style-type: none"> • MATLAB-based • Developed for multi-photon data 	Cantu et al., 2020
Minian	<ul style="list-style-type: none"> • Python-based • Developed for one-photon data 	Dong, Mau, et al., 2021
MIN1PIPE	<ul style="list-style-type: none"> • MATLAB-based • Developed for one-photon data 	Lu et al., 2018
OnACID-E	<ul style="list-style-type: none"> • Python-based • Designed for online analysis of one-photon data 	Friedrich et al., 2021
Suite2p	<ul style="list-style-type: none"> • Python-based • Developed for multi-photon data 	Pachitariu et al., 2017

^aThis table does not cover denoising algorithms (e.g., Deep Interpolation) (Lecoq et al., 2021), which might enhance signal extraction from noisy data.

TABLE 5

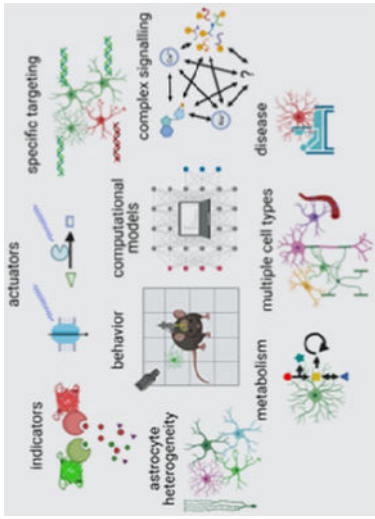
Examples of astrocyte heterogeneity and diversity

Heterogeneity/diversity metric	Example references
Location and morphology	
<ul style="list-style-type: none"> CNS region (brain, spinal cord, retina; e.g., Bergmann glia, Müller glia) Tissue region (e.g., neocortex vs. cerebellar cortex, cervical vs. lumbar spinal cord) Gray versus white matter (protoplasmic vs. fibrous astrocytes) Layer/lamina (e.g., Bergmann glia vs. velate astrocytes in the cerebellar cortex) 	Chai et al., 2017; Köhler et al., 2021; Lanjakornsiripan et al., 2018; Matyash & Kettenmann, 2010; Reichenbach & Wolburg, 2012; Somjen, 1988
Gene expression profile^a	
<ul style="list-style-type: none"> Brain: At least six groups Cortex: Layer-specific; at least five subtypes Hippocampus: At least five subtypes Striatum: Continuous transcriptional gradient 	Batiuk et al., 2020; Chai et al., 2017; Doyle et al., 2008; Gokce et al., 2016; John Lin et al., 2017; Lanjakornsiripan et al., 2018; Morel et al., 2017; Zeisel et al., 2015
Biochemical and physiological properties	
<ul style="list-style-type: none"> Transporter expression (e.g., GLAST) Ion channel expression (e.g., Kir4.1) Gap junctional coupling (e.g., Cx43) Ca²⁺ signaling (e.g., hippocampus vs. striatum) Metabolism Structural properties (e.g., GFAP expression, spine coverage) 	Chai et al., 2017; Fernández-Moncada et al., 2021; Herde et al., 2020; Hirrlinger et al., 2008; Kelley et al., 2018; Köhler et al., 2021; Köhler et al., 2018; Kronschläger et al., 2021; Miller et al., 2019; Oheim et al., 2018; Olsen et al., 2007; Theis & Giaume, 2012
Species	
<ul style="list-style-type: none"> Worms, flies, and fish (e.g., injury-responsive CNS glia) Mammals (e.g., reactive astrocytes) Humans (e.g., interlaminar astrocytes) 	Freeman & Rowitch, 2013; Nagai, Yu, et al., 2021

^aSee Hirbec et al., 2020 for a recent overview on scRNAseq studies on glial cells.

BOX 1

Select unresolved questions

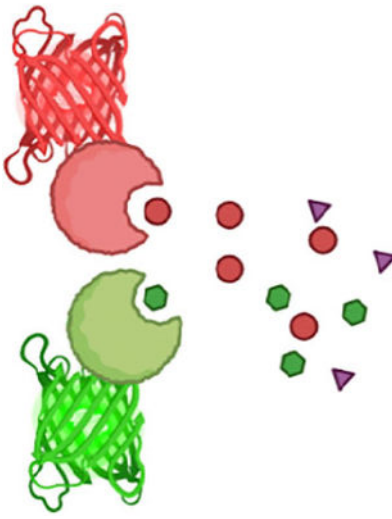


Overall

- How do astrocytes integrate and respond to diverse molecular signals in their extracellular environment at the cellular and population level?
- How do they convert this task- and animal state-dependent information into functional outputs that modulate neural circuit structure or function on various temporal and spatial scales?
- Does this influence on neurons serve homeostatic functions, augment/complement neural circuit properties, or both?
- Do astrocytes perform similar computations in different brain and spinal cord regions?
- Do astrocytes temporally hold/store information and, if so, for what purpose?

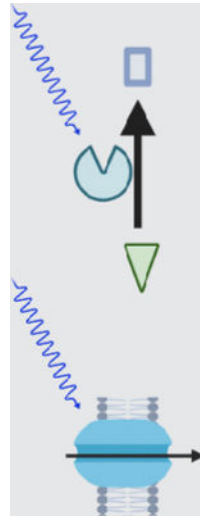
Indicators

- What new or improved indicators are needed to address the above questions (e.g., for neuropeptides, neuroactive substances, ions, transcriptional processes, synapse interactions)?
- How can current limitations to multiplex measurements be overcome (e.g., indicator signal-to-noise ratio, spectral variants, interference with endogenous signaling, buffering effects)?



Actuators

- Given astrocytes' complex spatiotemporal signals, how can physiologically meaningful regulation be achieved? Which approaches allow astrocyte "inhibition" at high spatial and temporal resolution?
- Which astrocyte signaling mechanisms other than calcium control neural circuit function and animal behavior on different timescales (e.g., K^+ , Na^+ , cAMP)?



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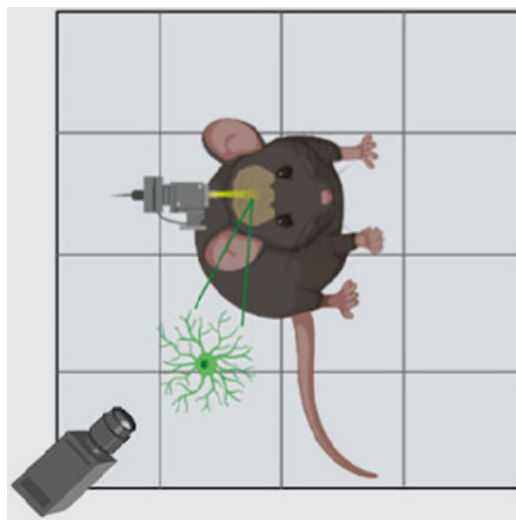
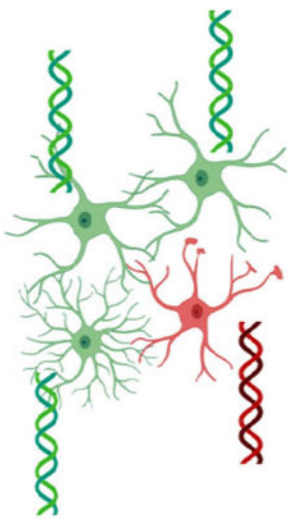
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Genetic targeting approaches

- What genetic (or other) approaches allow targeting functionally homogeneous astrocyte populations for interrogating their function?
- What approaches allow circuit-specific astrocyte targeting?



Behavioral assays

- How can quantitative behavioral assays be used to understand better astrocyte signal integration in vivo (e.g., noradrenergic, cholinergic, and dopaminergic signalling)?
- How can these assays be used to uncover astrocyte effects on neural circuit function in vivo (e.g., leveraging astrocytes' refractory period-dependent excitation)?

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Computational approaches

- What molecular, structural, or gene expression changes should be measured to understand better astrocytes' signal integration (with existing or novel indicators)?
- How do astrocytes control (directly or indirectly) neural circuit properties on various spatial and temporal scales (e.g., spike frequency, synchrony, oscillations, excitation-inhibition balance)?
- How is astrocytes' structural and functional heterogeneity relevant to neural circuit operation?
- How can modeling help predict circuit functions of astrocytes unique to humans?



Astrocyte heterogeneity

- How does astrocyte heterogeneity at the synaptic, cellular, circuit, and systems levels influence neural circuit function?
- What intrinsic and environmental factors determine this heterogeneity in health and disease?

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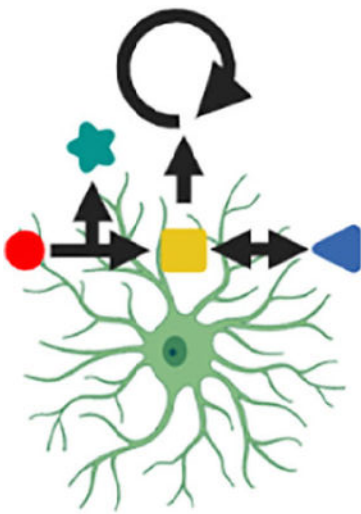
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Astrocyte metabolism

- How do astrocyte-derived metabolites (e.g., lactate) regulate neural circuit function and animal behavior?
- How does altered metabolism in astrocyte-neuron assemblies influence neural circuit function and animal behavior?



Contribution of other non-neuronal cells

- To what extent does astrocyte modulation of neural circuit function rely on other non-neuronal cells?
- How do these cells modulate astrocyte-neuron communication in health and disease?
- How do these cells influence neuronal circuit function directly?



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- How does disruption of astrocyte-neuron communication contribute to disease onset or progression?
- How does astrocyte heterogeneity or diversity relate to disease phenotype?

Disease

