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Review

Behaviorally consequential astrocytic regulation of neural circuits

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SUMMARY

Astrocytes are a large and diverse population of morphologically complex cells that exist throughout nervous systems of multiple species. Progress over the last two decades has shown that astrocytes mediate developmental, physiological, and pathological processes. However, a long-standing open question is how astrocytes regulate neural circuits in ways that are behaviorally consequential. In this regard, we summarize recent studies using *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio*, and *Mus musculus*. The data reveal diverse astrocyte mechanisms operating in seconds or much longer timescales within neural circuits and shaping multiple behavioral outputs. We also refer to human diseases that have a known primary astrocytic basis. We suggest that including astrocytes in mechanistic, theoretical, and computational studies of neural circuits provides new perspectives to understand behavior, its regulation, and its disease-related manifestations.

INTRODUCTION

Understanding how the brain works is arguably one of the last frontiers of currently conceivable biology. This is an important goal with societal relevance because disorders of the nervous system are a major and increasing health burden. There is also an expectation that a deeper understanding of the brain will inspire new types of biological computing and artificial intelligence. Impressive strides have been made over several decades since the building blocks of the nervous system, neuronal and glial cells, were discovered.

Considerable effort has been devoted to the study of neurons as the excitable cells of the nervous system. Advances made using model organisms, electrophysiology, genetics, neuroanatomy, and imaging at multiple scales are now providing a mechanistic understanding of neurons, neuronal circuits, and their contributions to complex behaviors (Luo et al., 2008, 2018). In contrast, our understanding of glia and how they contribute to the functions of neural circuits and behavior is comparatively primitive, even though glia were discovered in parallel with neurons (Kettenmann and Verkhratsky, 2008). For many researchers, CNS circuits can be simplified as comprising neurons, perhaps forestalling the necessity to consider glia. However, a more useful definition of a neural microcircuit is "comprising neurons and associated cells such as glia, organized to carry out specific operations within a region of the nervous system" (Shepherd and Grillner, 2010), reflecting the anatomical and evolutionary reality that glia and neurons have coexisted since the Palaeozoic era (Freeman and Rowitch, 2013).

In this review, we explore four experimental model organisms that represent the vanguard in efforts to understand how astrocytes (or astrocyte-like cells) contribute to neural circuit function and behavior. Each section begins with a brief introduction to the key features of astrocytes in each organism (Figure 1) and then describes recently identified functions and mechanisms by which they guide behavior (Figures 2 and 3).

At this stage, there is no common set of approaches that have been used across all model organisms we consider. Instead, we

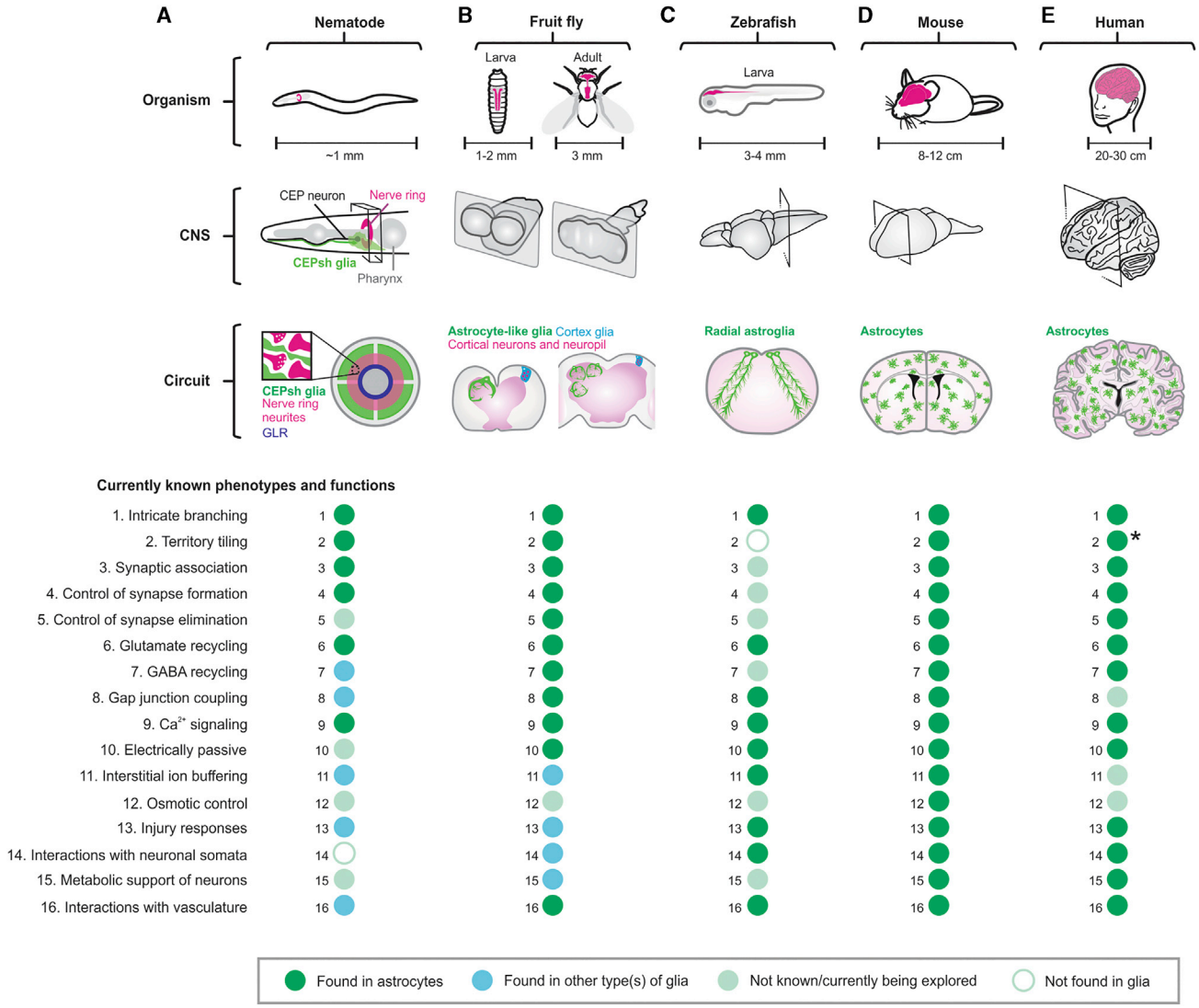

Figure 1. Phenotypes and functions of astrocytes from different species discussed here

(A–E) The schematics illustrate the locations of CNS (gray), neuropil (purple), and astrocytes (green) in nematode (A), fruit fly (B), zebrafish (C), mouse (D), and human (E) at the level of organism, CNS, and circuit. Dot plots summarize that 16 well-defined cellular phenotypes/functions of astrocytes are found in astrocytes (green) or other type(s) of glia (blue), not known or currently being explored (light green), or not found in glia (white) in the relevant organism indicated. Some human astrocytes project long unbranched processes that cross cortical laminae (asterisk).

highlight the most informative studies pertinent to the topic of this review that employ methods best suited to the organism and the question at hand. For example, in mice, many studies have employed chemogenetics, whereas in *C. elegans*, *Drosophila*, and zebrafish, additional genetic interventions and screens have been very informative. In the case of mice, we mainly focused on studies showing how acute astrocyte signaling can regulate behavior (seconds to hours) and did not focus on many studies where behavioral alterations result over longer periods, such as following deletion of a critical gene in astrocytes or during development and aging. In the case of *C. elegans*, *Drosophila*, and zebrafish, we focused only on the most informative studies related to astrocytes and behavior. We finish by briefly mentioning human disorders with a known primary astrocytic basis. In the summary comments, we draw on common themes across species, interpretations, and key open questions.

ASTROCYTES: BACKGROUND NARRATIVE AND CORE FEATURES

Rudolf Virchow proposed neuroglia as a type of ''nerve cement'' in 1858. The cellular elements comprising neuroglia were subsequently identified and called astrocytes by Michael von Lenhossek in 1893 and microglia and oligodendrocytes by Pio del Rio-Hortega in 1919 (reviewed in Kettenmann and Verkhratsky, 2008). Astrocytes, microglia, and oligodendrocytes,

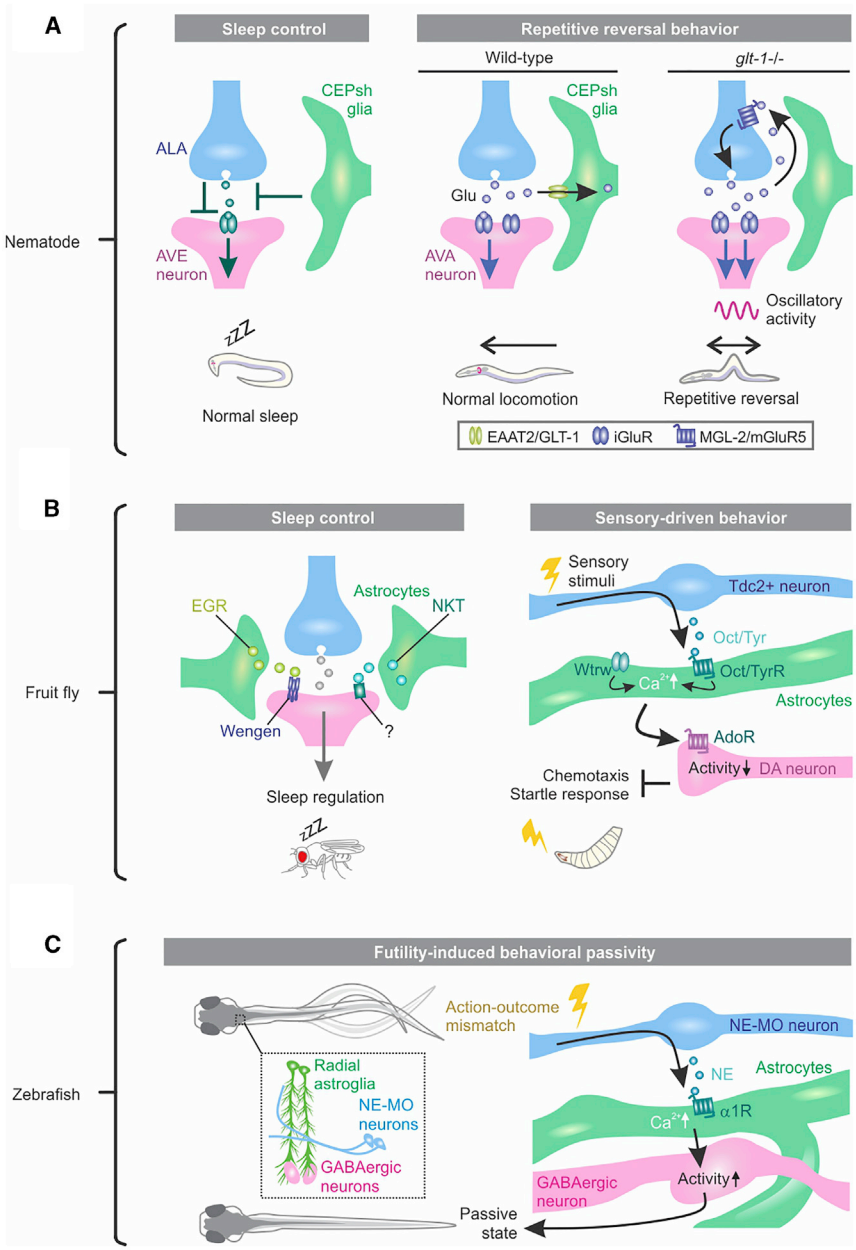

Figure 2. Neural circuit and behavioral functions of astrocyte-like cells in nematode, fruit fly, and zebrafish

(A) Nematode CEPsh glia are required for normal sleep and locomotion. Left: the oscillatory activity of AVE neurons correlates with head retraction of the worm and regulates locomotion. ALA neurons are active during sleep and synaptically inhibit AVE. The working model from ablation experiments suggests that CEPsh glia tune the ALA-AVE synapse in proper behavioral state transition from wakefulness to sleep. Right: AVA neurons are a major class of interneurons driving backward locomotion. CEPsh glia take up glutamate (Glu) from the synaptic cleft at excitatory synapses onto AVA. Deletion of the glutamate transporter GLT-1 from CEPsh glia results in spillover of Glu from the synapses, activating presynaptic mGluR5 to cause repetitive excitation of AVA and reversal in worm locomotion.

(B) Fly astrocytes regulate sleep and sensory-driven behavior. Left: the *Drosophila* TNF-α homolog Eiger (EGR), expressed in fly astrocyte-like cells (green), acts on Wengen, a receptor of EGR on neurons to regulate normal sleep. An astrocyte-enriched small secreted immunoglobulin (Ig)-domain protein, Noktochor (NKT), exhibits reduction and fragmentation in night sleep but not in day sleep. Right: upon sensory stimuli, Tdc2-expressing neurons release the neuromodulator tyramine (Tyr) or octopamine (Oct), the invertebrate analogs of NE, to increase Ca^{2+} in fly astrocytes in the ventral nerve cord. Waterwitch (Wtrw)/TRP channel also produce Ca^{2+} in the same types of astrocytes. Astrocyte Ca^{2+} signaling inhibits dopaminergic neuron firing via ATP/adenosine and is required for olfaction-driven chemotaxis and touch-induced startle responses.

(C) Fish radial astroglia play causal roles in behavioral passivity triggered by futility. When fish recognize an accumulated unsuccessful attempt, noradrenergic neurons in the NE cluster of the *medulla oblongata* (NE-MO) become active, and released NE activates the α1-adenoceptor (AR) in radial astroglia. Radial astroglia Ca^{2+} signaling, in turn, enhances activity of GABAergic neurons in the lateral hindbrain to cause behavioral passivity.

collectively called glia, probably represent no more than 50% of CNS cells.

Astrocytes are far more complex than first envisioned in the 1890s. Astrocytes vary morphologically between species and brain areas, but one feature that sets them apart from microglia and oligodendrocytes is their highly complex anatomy, which has been described as bushy and sponge-like. The finest processes of astrocytes extensively contact synapses and other cells but seem to be relatively stable compared with those of microglia, which display spatial dynamics at the cellular and submicrometer scale over minutes (Bernier et al., 2019; Davalos et al., 2005; Nimmerjahn et al., 2005). Astrocytes do, however, undergo extensive structural remodeling during injury and display more subtle alterations during synaptic plasticity (Adams and Gallo, 2018; Henneberger et al., 2020). The complex anatomical features of astrocytes suggest that, although most neurons evolved to perform their core functions at a distance through dendritic and axonal arbors, most astrocytes evolved to perform their core functions locally through complex, compact morphological forms impinging on other cellular elements of the neuropil. Signaling of astrocytes at distances beyond their own territories can occur, however, via low-resistance diffusive pathways formed by gap junctions between neighboring astrocytes (Giaume et al., 2010).

In terms of signaling, astrocytes are mostly electrically silent (Kuffler, 1967); their resting membrane potential rarely deviates from near the K^+ equilibrium potential by more than a few millivolts, and there is no evidence of any propagated or graded electrical signals that function in a manner analogous to those in neurons (Savtchenko et al., 2018). Indeed, astrocytes seem

to be a poor substrate for propagating electrical signals because of their extremely low membrane resistance, highly branched processes, high surface area, multiple consecutive failure and divisive points for current flow, and the fact that they lack predominant voltage-dependent channels. In the absence of an obvious electrical signal, the discovery of astrocyte intracellular Ca^{2+} dynamics (Charles et al., 1991; Cornell-Bell et al., 1990) provided a great impetus to explore such slower biochemical signals as a means by which astrocytes communicate with other cells. Much *in vivo* work over that last few years has shown that astrocyte Ca^{2+} dynamics are extremely rich, occur locally in fine processes as well as globally, can be triggered by neuromodulators and neurotransmitters, occur in a behaviorally relevant manner, and are altered in disease (Bazargani and Attwell, 2016; Shigetomi et al., 2016).

Several known mechanisms allow astrocytes to regulate synapses and neurons. These include changes in neurotransmitter and ion homeostasis, release of neuromodulators and synaptogenic cues from astrocytes, movement of astrocyte processes relative to synapses to alter synaptic transmission, and contributions to multicellular neuroinflammatory responses (Allen and Lyons, 2018; Khakh and Sofroniew, 2015). We consider some of these mechanisms in the following sections in relation to behavior. Furthermore, recent discoveries show that astrocytes display diversity within and between brain areas. This work is beyond the scope of this review but has been summarized (Ben Haim and Rowitch, 2017; Khakh and Deneen, 2019).

INSIGHTS FROM *C. ELEGANS*

CEPsh glia: worm astrocytes

The nematode *C. elegans* is an important setting for glia studies. The hermaphrodite and male nervous systems are fully mapped connectomes of 302 and 391 neurons and 56 and 92 glia, respectively, mediating locomotion, sleep, mating, decision-making, memory, and other behaviors (Doroquez et al., 2014; Jarrell et al., 2012; White et al., 1986). Powerful molecular genetics tools coupled with *in vivo* imaging and optogenetics allow visualization and manipulation of any glial cell or neuron (Singhvi and Shaham, 2019). Because *C. elegans* cell survival is generally programmed by lineage (Sulston and Horvitz, 1977; Sulston et al., 1983), glia are not required for neuron viability. Thus, primary effects of glia manipulation on neuron activity can be distinguished from confounding secondary causes (Shaham, 2005).

The *C. elegans* brain, a nerve ring composed of processes and synapses of ~180 neurons, is ensheathed by two glial types (Figure 1A). Four astrocyte-like CEPsh glia cover the outer surface and penetrate the structure, and six GLR glia abut the inner border (Singhvi and Shaham, 2019; White et al., 1986). CEPsh glia development suggests homology to vertebrate astrocytes (Rapti et al., 2017). The nerve ring is anatomically similar to the vertebrate spinal cord, with neural tissue surrounding a fluid-filled space and midline-crossing commissural tracts projecting rostrocaudally. In vertebrate development, radial glia extend processes from ventricular to pial surfaces, where they branch and guide commissural axons (Dominici et al., 2017; Varadarajan et al., 2017). Likewise, embryonic *C. elegans* CEPsh glia extend a neuron-guiding process (Rapti et al., 2017). Netrin, from radial glia pial branches or from *C. elegans* CEPsh glia processes, hones axon guidance. Semaphorins and Flamingo/CELSR also do so in *C. elegans*.

When neurogenesis is complete, some vertebrate radial glia transform into astrocytes (Noctor et al., 2008; Schmechel and Rakic, 1979). CEPsh glia undergo a similar cell division-independent transformation (Rapti et al., 2017), and in both settings, glial branches contact synapses (White et al., 1986). Like astrocytes, CEPsh glia influence synaptogenesis (Allen et al., 2012; Christopherson et al., 2005; Colón-Ramos et al., 2007; Eroglu et al., 2009; Shao et al., 2013). Furthermore, astrocytes and CEPsh glia cover non-overlapping neural domains, respecting still unknown tiling rules (Bushong et al., 2002; White et al., 1986).

Gene expression profiles support the homology of CEPsh glia and mammalian astrocytes. Quantitative comparisons reveal that CEPsh glia are more similar to mouse astrocytes than any other brain cell (Katz et al., 2019). For example, CEPsh glia express homologs of astrocytic glutamate transporter GLT-1 and glial fibrillary acidic protein (GFAP). The Olig2 transcription factor, expressed by some mature vertebrate astrocytes (Tatsumi et al., 2018), is also expressed in CEPsh glia (McMiller and Johnson, 2005; Yoshimura et al., 2008). Like astrocytes, CEPsh glia are diverse, with Netrin, Pax6, and Hmx expression segregated dorsoventrally (Wadsworth et al., 1996; Yoshimura et al., 2008), affecting functional diversity and developmental potential (Mizeracka and Heiman, 2015). Finally, vertebrate astrocytes exhibit Ca^{2+} transients (Shigetomi et al., 2016; Yu et al., 2020a) and gap junctions allow Ca^{2+} flow to adjacent astrocytes. CEPsh glia exhibit similar Ca^{2+} responses (M. Katz and S.S., unpublished data) and also express gap junction proteins (Altun et al., 2015), for which functional coupling has not yet been demonstrated.

CEPsh astrocytes modulate synapses to control *C. elegans* behavior

Post-embryonic ablation of CEPsh glia does not perturb the nerve ring structure, but animals exhibit several independent locomotory defects (Katz et al., 2018, 2019). Ablated animals move slowly and follow abnormal circular paths. Ablation also affects sleep. Unlike most *C. elegans* neurons, the ALA neuron, which forms inhibitory synapses onto locomotory AVE interneurons, exhibits frequent Ca^{2+} transients during sleep (Nichols et al., 2017). In wakefulness, these synapses are inactivated by CEPsh glia (Katz et al., 2018). CEPsh glia ablation uncovers ALA-AVE inhibition and uncouples AVE firing from movement. Strikingly, CEPsh glia-ablated adults exhibit narcolepsy-like locomotory pauses and prolonged sleep bouts (Figure 2A). Importantly, astrocyte regulation of sleep is conserved in *Drosophila* and in mice, as discussed later.

Animals lacking CEPsh astrocytes also exhibit defects in the balance of forward and backward locomotion. Off food, adults mostly move forward, reversing infrequently. Reversal initiation events follow a Poisson distribution with a fixed temporal probability. Animals lacking CEPsh glia, or *glt-1* mutants, instead exhibit repeated reversal initiation bouts (Katz et al., 2019; Mano et al., 2007). This repetitive behavior can be spontaneous or elicited. *In vivo* dual imaging of extracellular glutamate and

intracellular Ca^{2+} in AVA, a backward locomotion interneuron, revealed surprising dynamics in *glt-1* mutants. Although wild-type animals occasionally exhibit spontaneous glutamate release onto AVA and subsequent AVA firing, *glt-1* mutants exhibit oscillations of glutamate release near AVA and of AVA firing. Oscillation distribution matches repetitive behavior statistics, suggesting a causal role. Circuit analysis suggests that repetitive AVA firing originates in presynaptic neurons. In the absence of glial GLT-1, glutamate diffuses from AVA postsynaptic sites, engaging an extrasynaptic glutamate receptor, MGL-2, homologous to vertebrate mGluR5, on presynaptic neurons. This leads to un-evoked EGL-30/Gαq-dependent glutamate release, driving an autocrine feedforward loop causing repetitive AVA firing and repetitive reversal behavior (Katz et al., 2019). Thus, CEPsh astrocytes are critical for restricting reversal motor program initiation (Figure 2A).

GLT-1 conditional knockout in mouse astrocytes yields repetitive grooming (Aida et al., 2015), and mGluR5 inhibition prevents repetitive behaviors in mouse autism and repetitive behavior disorder models (Silverman et al., 2010). Thus, the *C. elegans* studies suggest that mammalian repetitive behavior, thought to involve inhibition defects among multiple brain regions (Nikolaus et al., 2010), may, at least in part, originate from defects in synaptic glutamate dynamics.

C. elegans glia control neuron receptive ending cell biology and physiology

Because *C. elegans* contains so few cells, individual cells often assume multiple functions. CEPsh astrocytes are a striking example, with each cell also sending a process to the nose, wrapping around sensory neuron-receptive endings (NREs) (Doroquez et al., 2014; Perkins et al., 1986; Ward et al., 1975). Sensory NREs and their associated glia/glia-like cells resemble synapses in which presynaptic signaling is replaced by environmental cues. Glia at both sites secrete thrombospondin TSP1 domain proteins, and the same receptor families (GPCRs, acetylcholine receptors [AChRs], and iGRs) engage presynaptic cues (Shaham, 2010). CEPsh glia ablation early in development results in truncated sensory neuron dendrites (Yoshimura et al., 2008). A more severe dendrite extension defect is seen in animals lacking AMsh glia, a sensory organ glial cell that does not interact with the nerve ring (Heiman and Shaham, 2009; Singhal and Shaham, 2017).

Studies of *C. elegans* sensory organ glia have unmasked how glia are specified, how glial compartments surrounding NREs are generated and how their size is determined; how glia control NRE shape, modulate NRE structural plasticity, control the NRE microenvironment, affect age-dependent changes in neuronal structure and function, and integrate associated neuron activities; and how a glial cell distinguishes among its associated neurons (Bacaj et al., 2008; Grant et al., 2015; Han et al., 2013; Huang et al., 2020b; Johnson et al., 2020; Labouesse et al., 1994; Melkman and Sengupta, 2005; Oikonomou et al., 2011, 2012; Perens and Shaham, 2005; Procko et al., 2011, 2012; Singhvi et al., 2016; Tucker et al., 2005; Wallace et al., 2016; Wang et al., 2008, 2017; Yoshimura et al., 2008; Zhang et al., 2020). *C. elegans* sensory organ glia also display Ca^{2+} signals following behaviorally relevant stimuli (Ding et al., 2015), perhaps providing a setting for understanding Ca^{2+} transients, their relevance to synaptic control, and their behavioral implications.

INSIGHTS FROM *DROSOPHILA*

The fruit fly *Drosophila melanogaster* is a well-characterized invertebrate model organism for investigating the roles of glia in the CNS. The ease of powerful genetic manipulations along with a large collection of openly available transgenic fly lines render it straightforward to specifically label small subsets of CNS cells, including astrocyte-like glia (Freeman, 2015; Yildirim et al., 2019). Using *Drosophila,* it is possible to genetically manipulate, visualize, and electrophysiologically examine the CNS in behaving individuals, including at the single-cell level.

Despite their small number (~10% of all CNS cells), glial cells display surprising morphological and functional diversity in *Drosophila* (Figure 1B). Among seven morphologically defined types (Yildirim et al., 2019; Bittern et al., 2020), two appear to share roles attributed to astrocytes in mammals; because of historical precedent, these are called astrocyte-like glia (here referred to as astrocytes) and cortex glia.

In the *Drosophila* larval brain, neuronal cell bodies lie in the outer cortical region and extend their processes into the neuropil, where all CNS synapses form. Cortex glia envelop neuronal cell bodies and proximal neurites throughout the synapse-deficient cortical regions, and there they provide trophic support for neurons, buffer extracellular ions, and monitor the extracellular environment. In contrast, astrocytes extend highly branched projections throughout the entirety of the neuropil, where they interact with neural circuits via synapses (Figure 1B). Fly astrocytes are electrically non-excitable, utilize intracellular Ca^{2+} signaling to regulate communication, form gap junction-coupled networks, establish tight associations with tracheal elements (the fly vasculature), and organize in a tiled fashion to cover the neuropil with a modest overlap at astrocyte-astrocyte boundaries (Ma et al., 2016; Stork et al., 2014; Yildirim et al., 2019; Bittern et al., 2020). As in mammals, well-studied functional roles of *Drosophila* astrocytes include modulation of synapse formation and plasticity and circuit remodeling. For instance, ablation of astrocytes reduced the numbers of synapses that formed in developing circuits (Muthukumar et al., 2014), and fly astrocytes engulf and clear pruned synapses and other neuronal debris via pathways such as Draper/MEGF10 that are conserved in vertebrates (Awasaki et al., 2006; Chung et al., 2013; Hakim et al., 2014; Tasdemir-Yilmaz and Freeman, 2014).

Electron microscopy studies show that astrocytes do not entirely ensheath synapses during the larval or adult stage, and the distance of astrocytic processes from synapses (e.g., of a looper neuron) in a third-instar larva is ~375 nm (MacNamee et al., 2016; Muthukumar et al., 2014; Stork et al., 2014). These values are comparable with the spatial interactions of rodent astrocyte processes with synaptic elements associated with dopamine release (~300 nm) but slightly longer than those associated with fast excitatory synapses (Chai et al., 2017; Haustein et al., 2014; Octeau et al., 2018). It is feasible that the differences in the spatial relationships between astrocyte processes and synapses between species affect

neuron-astrocyte interaction mechanisms, but the reality is that further detailed anatomical work is required at the synaptic scale with methods that have sufficient resolution. Such methods are becoming routine, and their use has the potential to advance our understanding of astrocyte-synapse interactions, including how the ultrastructural organization of astrocytes modulates synaptic activity.

Functions of astrocytes in the fruit fly nervous system and for behavior

Selective Gal4 drivers for astrocytes enable *in vivo* characterization and manipulations of genes to explore their effects on behavior (Li et al., 2014; Stork et al., 2014). A recent study employing translating ribosome affinity purification (TRAP) RNA sequencing has shown that in flies and mammals, astrocytes have substantially overlapping gene expression profiles (Ng et al., 2016). In this study, genetic screening using RNA interference (RNAi) for 318 targets identified multiple genes that were required for behavior (locomotive activity, circadian rhythmicity, or vibration sensitivity). Such rapid and inexpensive *in vivo* forward genetic approaches to identify genes of interest are a key advantage of this system over use of rodents. The genes identified included many transporters, metabolic support proteins, and secreted proteins. One type of secreted proteins were thrombospondins, whose contributions to synapse formation and locomotor behavior have been documented in mice (Christopherson et al., 2005; Eroglu et al., 2009; Eroglu and Barres, 2010; Nagai et al., 2019).

Secreted factors for sleep regulation

Several astrocyte-secreted factors have been shown to control sleep in fruit flies. An astrocyte-enriched small secreted immunoglobulin (Ig) domain protein, Noktochor (NKT; fly CG14141), has been identified to promote sleep (Ng et al., 2016; Sengupta et al., 2019). *Drosophila* sleep in the middle of the night and day. Adult flies lacking NKT exhibited reduced and fragmented night sleep, but day sleep was normal, consistent with the hypothesis that different pathways regulate each sleep phase. The cellular and molecular targets of NKT remain to be elucidated. Furthermore, as for vertebrates (Shoham et al., 1987; Stellwagen and Malenka, 2006), cytokine signaling affects sleep behavior. The *Drosophila* tumor necrosis factor alpha (TNF-α) homolog Eiger (EGR) is expressed in astrocytes (Ng et al., 2016) and controls sleep duration (Vanderheyden et al., 2018). Astrocytic but not neuronal EGR RNAi decreased baseline sleep during the day and night. Knockdown of Wengen, a receptor of EGR on neurons, had no effect on baseline sleep but dramatically blunted recovery sleep after deprivation. The authors suggest that the discrepancy in outcomes between the two mutants (effects on sleep amount versus sleep homeostasis) may be explained by additional TNF-α receptors for EGR (Figure 2B). Recent studies also show that increased astrocyte Ca^{2+} dynamics correlate with sleep need and contribute causally to sleep in *Drosophila* via release of Spätzle, the interleukin-1 analog. Spätzle then acts on specific neurons (R5) to regulate sleep (Blum et al., 2020).

Neurotransmitter uptake to control circuit activity

Drosophila astrocytes participate in neurotransmitter homeostasis by expressing a set of transporter proteins, such as the excitatory amino acid transporter 1 (EAAT1/GLAST) and the GABA transporter (GAT) (Muthukumar et al., 2014; Stork et al., 2014) to ensure the balance of excitation and inhibition. Loss of EAAT1 resulted in shortened lifespan, neuropil degeneration that could be suppressed by drugs used in the clinic to suppress seizure activity (Rival et al., 2004), and extended glutamatergic interneuron-evoked inhibitory postsynaptic currents in motor neurons, even in synapses that lacked astrocytic contacts (MacNamee et al., 2016). Elimination of GAT led to early embryonic lethality, whereas partial loss led to strong defects in locomotor behavior, both of which can be rescued by astrocyte-specific expression of GAT (Stork et al., 2014). It has been suggested recently that EAAT1 plays a key role in long-term memory (LTM). Fruit flies form LTM in 24-h spaced training paradigms (Matsuno et al., 2019), and during training, astrocyte EAAT1 expression is induced via the glial transcription factor Repo and the homophilic cell adhesion molecule Klingon (Klg). Age-related memory impairment in LTM (AMI-LTM) in Repo and Klg null mutants was rescued by EAAT1 overexpression. How each of these phenotypes relates to changes in extracellular glutamate levels was not measured directly. Stimulating astrocytic Ca^{2+} influx through activation of a TrpA1 channel led to rapid endocytosis of GAT from astrocytic membranes, behavioral paralysis, and termination of neuronal activity (Zhang et al., 2017), providing a potential mechanism for how astrocyte Ca^{2+} signaling might regulate neurophysiology.

Ca^{2+} signaling, circuits, and behavior

Using a forward genetic approach to identify Ca^{2+} signaling-related genes that function in astrocytes to regulate a simple olfaction-driven behavior, the transient receptor potential (TRP) channel Water witch (Wtrw) has been identified as an *in vivo* regulator of whole-cell changes in astrocytic Ca^{2+} in *Drosophila* larval astrocytes (Ma et al., 2016). Whole-cell astrocyte Ca^{2+} signaling in the larval CNS was elicited by activity of Tdc2 neurons, which release octopamine and tyramine, the invertebrate analogs of norepinephrine (NE) that evokes similar whole-cell astrocyte Ca^{2+} increases in mice (Ding et al., 2013; Paukert et al., 2014). Dual-color Ca^{2+} imaging of Tdc2 neurons and astrocytes revealed that Tdc2 neurons show oscillatory Ca^{2+} signals followed by astrocyte activities with a delay of tens of seconds, which could be blocked by silencing Tdc2 neurons or by genetic elimination of octopamine and tyramine. Astrocytes sensed octopamine/tyramine through the dual-specificity Oct-TyrR receptor expressed on astrocytes, which, through the PLCβ NorpA, has been proposed to activate the Trp channel Wtrw and drive Ca^{2+} influx. Strikingly, cell-specific manipulations and pharmacological experiments revealed that olfactory-driven chemotaxis and touch-induced startle responses require this astrocyte signaling pathway and that it likely acts by inhibiting dopaminergic neuron firing by increasing extracellular ATP/adenosine (Ma et al., 2016; Figure 2B).

INSIGHTS FROM ZEBRAFISH

The zebrafish is a powerful vertebrate model system that offers unique experimental advantages for glial physiology and behavior. In particular, the small size and near transparency of

zebrafish embryos and young larvae permit brain-wide cellular-resolution imaging of activity while the organism displays relevant naturalistic behaviors (Ahrens et al., 2012; Vladimirov et al., 2014). Importantly, zebrafish brains contain conserved regions associated with cognition in mammals (Jurisch-Yaksi et al., 2020), so there has been an awareness that zebrafish may provide a quantitative handle on higher-order functions not readily assessed in worms and flies.

On radial astroglia, radial astrocytes, and astrocytes

Until recently, one striking difference between the zebrafish and mammalian CNS or, more specifically, between anamniotes (including fish) and amniotes (including mammals), was thought to be the absence of stellate (protoplasmic) astrocytes in anamniotes (Lyons and Talbot, 2014). In the developing and mature zebrafish CNS, GFAP labels a prominent type of glial cell that has a radial morphology, typically spanning the entire width from the ependymal coating of the ventricles to the pial surface of the brain (Figure 1C). These GFAP+ radial glial cells serve as progenitor cells throughout life (Goldshmit et al., 2012; Kroehne et al., 2011; Kyritsis et al., 2012), whereas in mammals, radial glial cells serve as progenitor cells during development of the CNS and functionally and morphologically transform mainly into astrocytes at the end of embryonic development (Malatesta et al., 2008), except for a few locations such as the retina and the cerebellum, where the radial morphology of glia persists throughout life.

In zebrafish, GFAP+ cells have also often been referred to as radial astroglia (Cuoghi and Mola, 2009) when they express astrocyte markers; e.g., glutamine synthetase (GS), aquaporin-4 (AQP4), EAAT2b/GLT-1, S100β (Grupp et al., 2010; Lange et al., 2020; McKeown et al., 2012; Raj et al., 2018). They also display intricate branching of processes associated with neurons (Freifeld et al., 2017; Jurisch-Yaksi et al., 2020) and/or form glial networks through gap junctions (Diaz Verdugo et al., 2019). Recent single-cell RNA sequencing (RNA-seq) analyses of the zebrafish brain (Cosacak et al., 2019; Lange et al., 2020; Raj et al., 2018) revealed that zebrafish GFAP+ cells have molecular diversity and that a subset of those cells share close transcriptomic signatures with murine astrocytes. Therefore, studies have raised the possibility that GFAP+ radial glia and/or radial astroglia in zebrafish may perform and/or sub-serve many tasks ascribed to mammalian astrocytes (Lyons and Talbot, 2014).

In addition to radial glia and radial astroglia, a zebrafish cell type remarkably similar to mammalian astrocytes has been described recently (Chen et al., 2020). The authors generated transgenic lines to label Glast+ cells and found cells with dense cellular processes in the developing zebrafish CNS. With single-cell resolution imaging, these cells were shown to transform from radial glia into astrocyte-like cells that elaborated a dense meshwork of fine cellular processes morphologically similar to astrocytes in *Drosophila* and mammals. These cells exhibited additional defining features of mammalian astrocytes, including expression of GS, close association with synapses, astrocyte tiling, and spontaneous microdomain Ca^{2+} transients that respond to NE (Chen et al., 2020). Finally, a cell-specific CRISPR-Cas9 approach demonstrated a functional role for Fgf receptors 3 and 4 in vertebrate astrocyte morphogenesis, as was found to be the case in *Drosophila* (Stork et al., 2014).

Functions of radial astrocytes and astroglia in the zebrafish nervous system and behavior

Following extensive research of radial glia and radial astroglia in regenerative responses after tissue injury, their physiological roles in neural circuit function have begun to be revealed recently. A recent study identified a subset of GFAP+ cells called "radial astrocytes" in the zebrafish *medulla oblongata* with long processes that ramify at distal ends and suggested that these cells play causal roles for information processing in failure of intended actions and triggering behavioral passivity (Mu et al., 2019). In the study, the authors designed a virtual reality environment where zebrafish larvae fictively swam with realistic visual feedback that was given during attempted swimming (closed loop). When such feedback was suddenly withheld to render swim efforts ineffective (open loop), fish increased their swim vigor for tens of seconds but then abruptly stopped swimming and became passive. This futility-induced passivity appears to be caused by decoupling of motor action and sensory feedback, reminiscent of highly conserved adaptive behaviors such as passive coping and learned helplessness in mammals. Combining this behavioral paradigm, whole-brain dual-color Ca^{2+} imaging using light sheet microscopy and cell-specific perturbations, Mu et al., 2019 discovered bi-directional interactions between neurons and radial astrocytes. The noradrenergic system, known to encode action-outcome mismatching, initially became activated ~10 s before onset of the passive state. Within a few seconds of activation of the noradrenergic system, radial astrocyte Ca^{2+} signaling via α1-adrenergic receptors ramped up and activated GABAergic neurons in the brain stem to trigger behavioral passivity. These findings suggest that radial astrocytes convert information that actions are futile and thus accumulate evidence for behaviorally relevant decision-making (Figure 2C).

INSIGHTS FROM MICE

A typical gray matter mouse astrocyte comprises a cell body, one or two endfeet bearing processes that contact blood vessels, six or seven thick primary branches that split into secondary and tertiary branches, and thousands of branchlets and leaflets that form highly complex sponge-like territories throughout the CNS (Sun and Jakobs, 2012). The finest astrocyte leaflets extensively contact synapses and perhaps all other CNS cell types. The processes of one astrocyte do not encroach onto that of its neighbor, causing astrocytes to evenly tile the CNS in non-overlapping territories (Bushong et al., 2002). Exploration of astrocytes and how they regulate neuronal circuits is advanced in mice (Figure 1D). There is a huge amount of data, but as stated at the outset, we restrict our summary mainly to acute astrocytic regulation of circuits and behavior (Figure 3). We did not consider in depth the wealth of studies where behavioral alterations result over longer periods, such as following deletion of critical genes within astrocytes, except for studies of circadian and sleep/wake behaviors, which occur over a timescale of days by definition. Because astrocytes express a rich variety of GPCRs, DREADDs (designer receptors exclusively

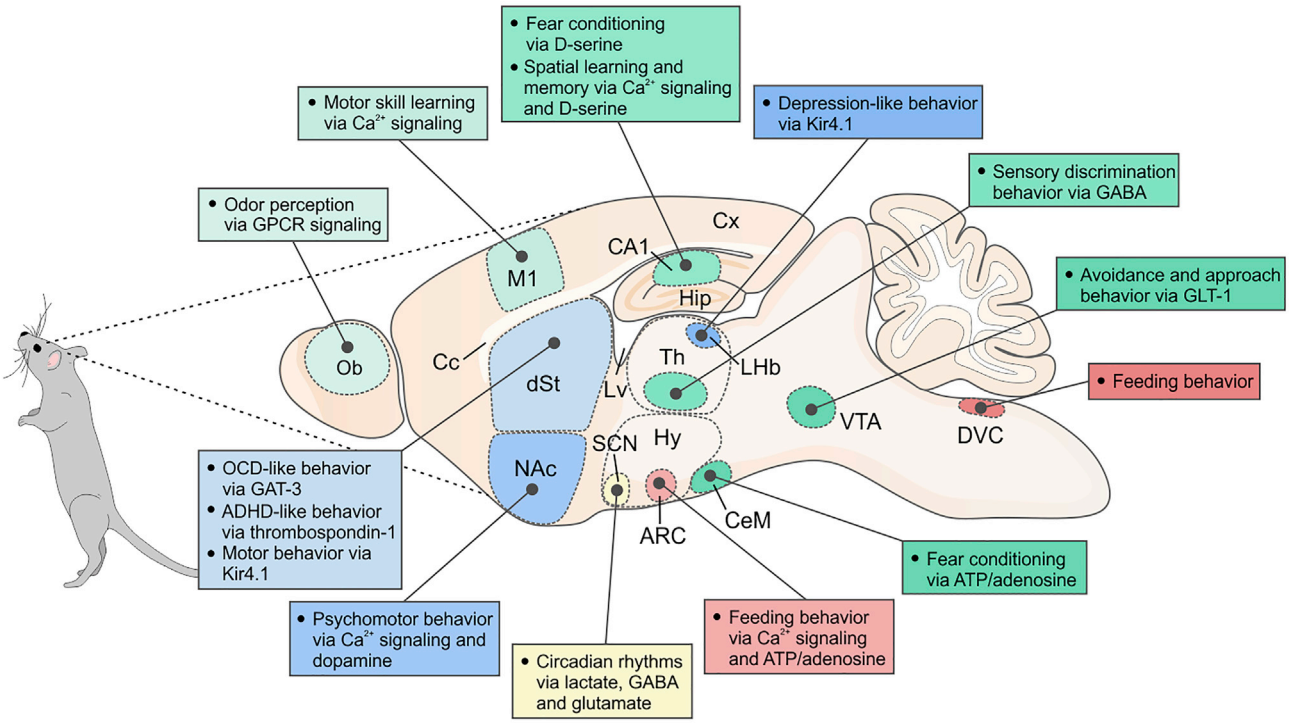

Figure 3. Summary illustrating acute astrocytic regulation of neuronal circuits and behaviors relevant to different regions of the mouse brain
Schematic of a sagittal section of a mouse brain, depicting various regions and nuclei as well as associated behaviors that have been shown to be regulated by acute astrocytic mechanisms. Ob, olfactory bulb; Cx, cerebral cortex; M1, primary motor cortex; Lv, lateral ventricle; Cc, *corpus callosum*; dSt, dorsal striatum; NAc, *nucleus accumbens*; Hip, hippocampus; Th, thalamus; LHb, lateral habenula; Hy, hypothalamus; ARC, arcuate nucleus; SCN, suprachiasmatic nucleus; CeM, central amygdala; VTA, ventral tegmental area; DVC, dorsal vagal complex. In the text, we also consider sleep, but this is not illustrated here because it involves multiple brain nuclei. Furthermore, the cartoon does not include studies where behavioral alterations result over longer periods, such as following deletion of a critical gene within astrocytes or during development and aging.

activated by designer drugs) have been used widely to explore astrocyte biology in brain preparations such as slices and *in vivo*. DREADDs are engineered GPCRs that have attenuated responses to their endogenous ligands and have been engineered to respond to synthetic, biologically inert chemical ligands delivered in the animal's water or food supply or by systemic injection (Roth, 2016). DREADDs enable relatively non-invasive stimulation of GPCR pathways in a genetically targeted manner *in vivo*. Several types of DREADDs have been developed to target major Gα protein signaling pathways; Gq-coupled hM3D, Gi-coupled hM4D, and Gs-coupled rM3D are used most in astrocyte studies (Yu et al., 2020a). As discussed in the finishing section of this review, such stimulations, although revealing in a causal sense, do not indicate that the pathways are activated physiologically.

Feeding behavior

Increased firing of hypothalamic arcuate nucleus agouti-related peptide (AGRP) neurons evokes food intake, whereas firing of pro-opiomelanocortin (POMC) neurons suppresses feeding. As with all other areas of the brain, astrocytes are intermingled with AGRP and POMC neurons and form close spatial interactions with them (Fuente-Martín et al., 2012). In light of the well-established roles of AGRP and POMC neurons in regulation of feeding, it was natural to ask how astrocytes regulate AGRP and POMC neurons as well as feeding. We summarize two studies that used hM3Dq DREADDs in astrocytes. In one study, activation of hM3Dq in arcuate astrocytes resulted in decreased firing of AGRP neurons through astrocytic ATP/adenosine release and also decreased basal and ghrelin-evoked food intake (Yang et al., 2015). Opposing effects observed because of astrocyte hM4Di activation were attributed to a decrease in astrocyte Ca^{2+} activity, leading to the suggestion that astrocyte signaling controls feeding bidirectionally. It should be noted, however, that several studies have shown that Gi-GPCR activation in astrocytes actually elevates intracellular Ca^{2+} levels (Yu et al., 2020a), which echoes early pharmacological studies (Porter and McCarthy, 1997). Subsequently, activation of astrocyte hM3Dq DREADDs in arcuate astrocytes has been shown to increase the activity of AGRP neurons and increased food intake during the dark phase (Chen et al., 2016). In this study, a causal role of Ca^{2+} was also explored by using a genetically encoded inositol triphosphate (IP_3) sponge that resulted in decreased Ca^{2+} signaling and food intake (Chen et al., 2016). Thus, these studies concluded that astrocytes in the arcuate nucleus bidirectionally control neuronal activity that regulates feeding but with effects that are somewhat discordant. Technicalities such as the concentration of CNO used to activate the DREADDs may explain these differences, as could specificity of hM3Dq delivery to astrocytes within the arcuate nucleus relative to surrounding tissue. Furthermore, because AGRP and POMC neurons exert

opposing effects on feeding and are interspersed, it may be possible that the genetic strategies targeted subpopulations of astrocytes that preferentially affected AGRP or POMC neurons.

Olfactory behavior

It has been recently reported that activation of hM3Dq DREADD in olfactory bulb (Ob) astrocytes *in vivo* decreased neuronal Ca^{2+} responses to odor stimulation and improved performance in an olfactory learning task (Ung et al., 2020). In contrast, stimulation of hM4Di DREADD in Ob astrocytes caused an increase in neuronal Ca^{2+} odor responses but resulted in less accurate odor detection performance (Ung et al., 2020). Ob astrocytes have been reported to respond to the neurotransmitters glutamate, GABA, as well as dopamine (De Saint Jan and Westbrook, 2005; Doengi et al., 2009; Fischer et al., 2020), but it remains unknown which mechanisms are physiologically exploited by Ob astrocytes for fine-tuning neuronal activity and odor perception. Furthermore, transcriptomic analyses have revealed distinct molecular profiles of Ob astrocytes in relation to other brain regions (John Lin et al., 2017; Lozzi et al., 2020).

Circadian behavior

Circadian rhythms are molecular, physiological, and behavioral changes that synchronize living organisms to daily environmental cycles. Dysfunctions of circadian rhythms are associated with aging and frequently present as comorbidities with neurodegenerative diseases. Although elucidation of the molecular and neural circuit basis of circadian rhythms is the pinnacle of modern physiology and neuroscience (Sehgal, 2017), recent accumulating evidence has uncovered new and previously unappreciated roles of astrocytes that function autonomously as a central circadian clock in mice.

The transcription-translation negative feedback loops that drive circadian rhythms in mammals exist in neurons and astrocytes of the suprachiasmatic nucleus (SCN) of the hypothalamus, the master circadian pacemaker. In accordance with this, SCN neurons and astrocytes exhibit oscillations of clock gene expression and intracellular Ca^{2+} levels (Brancaccio et al., 2017, 2019; Tso et al., 2017). Importantly, the two cell populations are active at opposite phases, with neuronal activity during circadian day and astrocyte activity at night. Deletion of the clock gene *Bmal1* or the *CK1*ε^{Tau} mutant allele specifically in SCN astrocytes significantly lengthens the period of wheel-running locomotor activity during darkness; wheel running is a readily observable behavior that faithfully reflects circadian biology (Brancaccio et al., 2017; Tso et al., 2017). Restored expression of another clock gene, *Cry1*, in SCN astrocytes alone is sufficient to initiate and sustain circadian patterns of clock gene expression and locomotor activity in otherwise arrhythmic mice that lack *Cry1/2* (Brancaccio et al., 2019). This local astrocytic control of the circadian activity of SCN neurons is mediated by circadian changes in extracellular glutamate. Specifically, astrocytes release glutamate, which is mediated by Cx43 hemichannels and acts on presynaptic neuronal NMDA receptors (NMDARs) to regulate GABAergic tone across the SCN circuit (Brancaccio et al., 2017, 2019). Interestingly, deletion of *Bmal1* in GLAST+ astrocytes throughout the brain using the GLAST-Cre/ERT2 mouse line (rather than local manipulations in the SCN) has been found to alter neuronal GABA signaling, but with only a mild effect on rhythmic locomotor activity (Barca-Mayo et al., 2017). Nevertheless, cognition and lifespan of mice are reduced (Barca-Mayo et al., 2017, 2020). These differences between SCN and global effects may highlight circuit-specific roles of astrocytes in the SCN or possibly differences between types of astrocytes that are variably targeted with existing genetic strategies. Besides key clock genes, astrocyte signature genes have also been suggested to contribute to circadian behaviors. For example, expression of the astrocyte intermediate filament protein GFAP is changed significantly under constant lighting conditions or in the absence of *Bmal1* because of altered glutathionylation (Lananna et al., 2018; Moriya et al., 2000). Furthermore, mice lacking GFAP display altered circadian activity rhythms in constant light (Moriya et al., 2000). These studies suggest that astrocytes are potent regulators and determinants of physiological and behavioral rhythms mediated by the SCN. More broadly, the SCN is an important nucleus for systematically exploring astrocyte biology and its relevance to behavior in a manner that has clear relevance to human biology.

Sleep/wake behavior

Accumulating data have suggested that astrocytes play an essential regulatory role in the physiology of sleep and wakefulness (Haydon, 2017). Sleep and wakefulness are conserved behaviors across species and are regulated by coordinated interactions from multiple neural circuits. There are three well-characterized vigilance states in mammals, consisting of non-rapid eye movement (NREM) sleep, rapid eye movement (REM) sleep, and wakefulness. Different sleep/wakefulness states are identified by distinct characteristics of the brain's electrical activity and muscle movement measured by electroencephalogram (EEG) and electromyogram (EMG) recordings, which have been utilized in studies involving astrocytes.

In one approach, dnSNARE was used to abrogate astrocyte release of gliotransmitters by expressing the dominant-negative construct selectively in astrocytes using a *GFAP*-tTA mouse line. This prevented exocytotic release of ATP, which reduced accumulation of extracellular adenosine, a degradation product of ATP and known neuromodulator that activates adenosine A1 receptors to regulate sleep homeostasis (Halassa et al., 2009). Different lines of dnSNARE mice are available, but in these astrocyte-specific mice, the authors found significantly reduced EEG slow wave activity (with a frequency range of 0.5–4.0 Hz) during NREM sleep, which represented decreased sleep pressure following wakefulness periods (Halassa et al., 2009). Furthermore, in normal mice, sleep deprivation is well known to impair recognition memory (for example, in the novel object recognition task), but in dnSNARE mice, this response was significantly attenuated, likely because of reduced adenosine levels and consequently reduced activation of adenosine A1 receptors (Halassa et al., 2009). Roles of adenosine in such responses were supported by the finding that selective A1 adenosine receptor antagonists recapitulated the findings in wild-type mice; i.e., impairment of recognition memory mediated by sleep deprivation was observed with A1 receptor antagonists. This study represents initial evidence supporting astrocytic regulation of sleep homeostasis and its related cognitive functions via purinergic

signaling *in vivo*. Subsequently, additional studies have supported the importance of astrocytes in adenosine-regulated sleep homeostasis in different settings (Clasadonte et al., 2014; Florian et al., 2011; Schmitt et al., 2012).

Microarray-based gene expression analysis has revealed molecular changes in cortical astrocytes associated with sleep and wakefulness (Bellesi et al., 2015). Sleep deprivation has been found to alter astrocytic pathways, including those related to purine nucleotide binding, phagocytosis, and lactate metabolism (Bellesi et al., 2015, 2017; Petit et al., 2013). In accordance with this, deletion of the gap junction subunit Cx43 in astrocytes using the *hGFAP*-Cre mouse line limits lactate diffusion in the astrocytic network and results in reduced excitability of orexin neurons in the lateral hypothalamus (LHA) (Clasadonte et al., 2017). Mice lacking Cx43 in all astrocytes or predominantly in LHA astrocytes specifically display increased sleep time and fragmented wakefulness during the active (dark) phase, effects that are ameliorated by lactate administration *in vivo* (Clasadonte et al., 2017). Whether this astrocytic lactate-dependent regulation of neuronal excitability is a region-specific mechanism remains to be determined. However, one study has suggested that the anticonvulsive effects of a ketogenic diet, which limits glucose and lactate supply, result from reduced pyramidal neuron excitability because of limited astrocytic supply of lactate (Sada et al., 2015). Furthermore, an anti-convulsant, stiripentol, has as an off-target action, the inhibition of lactate dehydrogenase, and one of the known side effects of the drug is drowsiness (Sada et al., 2015).

Additional mechanisms regulating sleep and wakefulness that are mediated by astrocytes continue to be discovered. For instance, a mutation of the *Fabp7* gene, which encodes a fatty acid binding protein expressed in astrocytes, has been shown to be associated with fragmented sleep in humans (Gerstner et al., 2017). Moreover, mice with *Fabp7* deficiency and *Drosophila* expressing mutant human *FABP7* selectively in astrocytes both exhibit similar sleep alterations (Gerstner et al., 2017), suggesting an evolutionarily conserved mechanism by astrocyte lipid signaling in sleep. Astrocyte intracellular Ca^{2+} signaling has also been found to be critical in modulating sleep and neuronal synchronization. It has been found that intracellular Ca^{2+} signals of cortical astrocytes are less active during sleep but enhanced preceding transition to wakefulness *in vivo* (Bojarskaite et al., 2020; Ingiosi et al., 2020). Furthermore, attenuation of Ca^{2+} signaling in astrocytes using IP_3 receptor knockout (KO) mice or astrocyte-specific conditional STIM1 KO mice alters the architecture of NREM and REM sleep as well as associated brain rhythms (Bojarskaite et al., 2020; Foley et al., 2017) and impaired the homeostatic response to sleep deprivation (Ingiosi et al., 2020). These studies highlight astrocytes as an integrative component in sleep/wake behavior. Such responses may be reliant on extensive astrocytic networks and their ability to coordinate neuronal activity in large volumes of tissue. Several brain regions have emerged to be critical in induction and maintenance of different vigilance states (Chowdhury et al., 2019; Liu et al., 2017; Oishi et al., 2017; Yu et al., 2019), and it will be interesting to find out whether astrocytes have ubiquitous or diverse regulatory roles in different sleep circuitry.

Learning and memory-related behaviors

Activation of Gi or Gq-GPCR signaling with hM4Di and hM3Dq DREADDs or Gq-coupled melanopsin is commonly used to stimulate astrocyte Ca^{2+} dynamics and probe downstream effects on learning and memory. For instance, a melanopsin-based method to temporally trigger Gq activation in astrocytes provides evidence of a role of hippocampal astrocytes in spatial episodic memory (Mederos et al., 2019), and use of hM3Dq DREADD suggests that enhancing astrocyte Ca^{2+} signaling augments spatial and contextual memory formation in T maze and fear conditioning assays (Adamsky et al., 2018). This latter finding coincided with *de novo* synaptic plasticity *in vitro* and involves control of NMDAR function at CA3-CA1 synapses by D-serine, an obligatory co-agonist (Papouin et al., 2012, 2017). The authors concluded that, in this circuit, astrocyte activation enables a local and task-specific increase in neuronal activity restricted to the ensemble active during memory allocation (Adamsky et al., 2018). Conversely, stimulating the Gq-GPCR pathway in central amygdala (CeM) astrocytes using the same hM3Dq approach reduces neuronal firing through astrocyte-derived ATP/adenosine release accompanied by decreased fear responses in a cued fear conditioning task of associative learning (Martin-Fernandez et al., 2017). Although these studies may appear to contradict each other, they may point to the diverse nature of astrocytes where the same stimulus delivered to two distinct networks of astrocytes (CA1 versus CeM) is transduced differently onto the local circuit, yielding region-specific effects on behavior. Furthermore, such discrepancies may be resolved by identifying and then manipulating critical endogenous signaling pathways and genes.

Selective activation of astrocytic Gi-coupled μ-opioid receptors (MORs) in the hippocampus elicits conditioned place preference, suggesting a possible role of astrocytes in positive emotional valence (Nam et al., 2019). The authors suggest that this pathway triggers astrocyte-derived glutamate release, which acts on presynaptic mGluR1 to facilitate LTP induction at CA3-CA1 synapses, and activation of hM4Di in astrocytes mimics the behavioral effect of MOR activation and facilitation of LTP. Although in this and other reports hM4Di activation in astrocytes increased intracellular Ca^{2+} signaling (Chai et al., 2017; Durkee et al., 2019; Nagai et al., 2019), a more complex bimodal response where initial elevation is followed by modest taming of astrocyte Ca^{2+} events has also been reported (Kol et al., 2020). Exploiting this indirect reduction of astrocyte signaling, it has been suggested that activation of hM4Di during learning impairs retrieval of remote but not recent memories (Kol et al., 2020), in line with earlier work (Pinto-Duarte et al., 2019). Mechanistically, Gi-GPCR activation in CA1 astrocytes has been found to inhibit neuronal activity in the anterior cingulate cortex (ACC) and attenuated excitatory postsynaptic potentials, suggesting selective astrocytic modulation of CA1-to-ACC-projecting pyramidal neurons that support formation of remote memories. How astrocytes distinguish distant neuronal projections at a local level in the hippocampus remains unclear, as does the causal link by which acute activation of astrocyte Gi-GPCR signaling affects LTM storage.

Suppression of function approaches have also provided strong grounds to support astrocyte contributions to cognitive

behavior. Deletion of cannabinoid receptor 1 (CB1R) selectively from astrocytes, for instance, impairs object recognition memory (Robin et al., 2018), and deleting the transcription factor NF1A in adult astrocytes reduces astrocyte Ca^{2+} signaling, D-serine levels, and synaptic plasticity in the hippocampal CA1 (Huang et al., 2020a). Suppressing Ca^{2+} dynamics to probe the role of astrocytes in behavior proved to be deceptive at first because mice lacking IP_3R2 receptors (IP_3R2 KO), thought previously to be pivotal for astrocyte Ca^{2+} signaling, exhibit normal behavior (Agulhon et al., 2010; Petravicz et al., 2014). However, NMDAR/D-serine-dependent remote memory deficits have been reported in these mice (Pinto-Duarte et al., 2019), whereas mice with conditional deletion of IP_3R2 in GLAST+ astrocytes show partial reduction of astrocyte Ca^{2+} signaling in the primary motor cortex and mild impairment in a forelimb motor learning task (Padmashri et al., 2015). Similarly, mice expressing the glutathione S-transferase (GST)-IP_3 sponge in astrocytes showed modest memory impairments in the Morris water maze and in a contextual fear memory task (Tanaka et al., 2013). The fact that altering IP_3R2 signaling has modest effects on behavior seems consistent with the notion that some astrocyte Ca^{2+} dynamics are independent of IP_3R2s (Srinivasan et al., 2015). The dnSNARE mouse line (Pascual et al., 2005) has also been used to show the role of astrocyte-derived transmitter release in spatial learning, recognition memory, and working memory (Sardinha et al., 2017). In this latter study, the range of behavioral alterations coincided with desynchronization of neural theta oscillations from the dorsal hippocampus to the prefrontal cortex, both of which are normalized by D-serine administration. In a similar mouse model in which tetanus toxin is conditionally expressed in astrocytes, object recognition memory is disabled (Lee et al., 2014). These findings illustrate diverse pathways and mechanisms through which astrocytes dynamically optimize synaptic properties and functional connectivity in local circuits responsible for learning and memory.

The contribution of astrocyte Gs-GPCR signaling to learning and memory has also been explored in the context of Alzheimer's disease (AD) and aging in mice. Individuals with AD and AD model mice have been found to exhibit increased levels of adenosine receptor A_{2A} in hippocampal astrocytes accompanied by memory deficits (Orr et al., 2015). When the human Gs-coupled 5-HT_{4b} serotonin receptor Rs1 is expressed in astrocytes and activated, LTM is impaired without affecting learning in young and aging mice. In contrast, genetic reduction of astrocytic A_{2A} receptors improves memory in adult wild-type mice as well as in aged AD mice (Orr et al., 2015). This finding suggests astrocyte Gs-GPCR signaling as a mechanism and potential therapeutic target for memory loss in AD.

Tactile sensory acuity

Via the actions of diamine oxidase (DAO) and aldehyde dehydrogenase 1a1 (Aldh1a1), thalamic astrocytes convert putrescine into GABA, which is then released via Best1 channels (Kwak et al., 2020). Use of DAO to synthesize GABA is distinct from astrocytes in other brain regions, such as the hippocampus and cerebellum (Jo et al., 2014), suggesting regional diversity of astrocytic mechanisms. When released, such tonic GABA activates high-affinity extrasynaptic $GABA_A$ receptors containing δ subunits to mediate tonic inhibition in thalamocortical (TC) neurons (Kwak et al., 2020). The immediate action of astrocytic GABA is to inhibit the synaptically evoked action potential probability of TC neurons via postsynaptic shunting inhibition. Shunting inhibition by tonic GABA reduces the membrane input resistance and the time constant of TC neurons, reducing the amplitude and width of excitatory postsynaptic potentials (EPSPs). The decrease in EPSP amplitude renders synaptic integration less saturable, leading to enhanced dynamic range; i.e., greater linearity of the stimulus-response relations of TC neurons. Faster EPSP kinetics narrow the time window of synaptic integration, resulting in high temporal fidelity of TC neurons, which are capable of distinguishing two independent inputs to them. Next, the authors explore *in vivo* consequences by using the touch-based novel object recognition test (Wu et al., 2013). Low tonic GABA conditions following astrocytic knockdown of Best1, DAO, or Aldh1a1 lower discrimination indices, whereas high tonic GABA conditions improve tactile discriminability. By showing that NE-induce Ca^{2+} transients in thalamic astrocytes enhance tonic GABA, this study proposes a model where astrocyte-derived tonic GABA tunes sensory acuity following activity of *locus coeruleus* projections during attentive states. This model supports previous studies reporting that NE from the *locus coeruleus* modulates feature selectivity and sensory discrimination in mice (Hirata et al., 2006; Rodenkirch et al., 2019).

Striatum-dependent behaviors

Several studies have provided links between astrocyte function and dysfunction to animal behavioral alterations reminiscent of phenotypes seen in mouse models of human psychiatric disorders. In the striatum, astrocytes sit in a brain area where ~95% of the neurons are GABAergic medium spiny neurons (MSNs), which they contact extensively (Chai et al., 2017; Octeau et al., 2018). Astrocytes also express G-protein-coupled $GABA_B$ receptors, which elevate Ca^{2+} levels even though they couple to G_i proteins (Chai et al., 2017; Porter and McCarthy, 1997). When Ca^{2+} signaling of astrocytes in the dorsal striatum is attenuated by heterologously overexpressing a Ca^{2+} pump, mice exhibit an obsessive-compulsive disorder-like behavior (excessive self-grooming) via a mechanism modulated by astrocyte GAT-3 (Yu et al., 2018). Furthermore, activation of G_i-GPCR signaling in astrocytes from the dorsal striatum by hM4Di DREADDs triggers upregulation of the gene for a synaptogenic cue, t-hrombospondin-1, which results in enhanced excitatory synaptic transmission onto MSNs and behavioral phenotypes related to hyperactivity with disrupted attention (Nagai et al., 2019). Astrocytes from the *nucleus accumbens* (NAc) in the ventral striatum respond to dopamine release from ventral tegmental area (VTA) neurons by elevating intracellular Ca^{2+} levels (Corkrum et al., 2020). When dopamine D1Rs or intracellular IP_3R2s are deleted from astrocytes in the NAc, the mice display reduced locomotor responses to amphetamine, suggesting involvement of NAc astrocytes in reward signaling and addiction behaviors. Furthermore, cocaine increases NAc astrocyte Ca^{2+} signaling, whereas attenuating astrocyte Ca^{2+} signaling decreases the number of silent synapses in the NAc shell in response to cocaine. Such mechanisms, operating via thrombospondin 2, contribute to cue-induced cocaine seeking after withdrawal

and cue-induced reinstatement of cocaine seeking after extinction (Wang et al., 2020). Furthermore, careful analyses of gene expression changes in striatal astrocytes following multiple experimental perturbations suggest that astrocytes respond in a highly context-specific manner and that such responses could be teased apart and understood in molecular terms to devise astrocyte GPCR-based strategies to modify disease-related responses (Yu et al., 2020b).

Lateral habenula-regulated behavior

Detailed experiments implicate astrocytes in depression-like behaviors in rodents. Increased Kir4.1 in astrocytes of the lateral habenula (LHb) increases LHb neuronal firing and results in depression-like behaviors (Cui et al., 2018). Moreover, several genetic strategies that reduce Kir4.1 function in the LHb reduce depression-like behaviors in rodents. In this instance, astrocyte-mediated K^+ buffering has been proposed to regulate the intrinsic excitability and action potential firing properties of LHb neurons, which, because of their synaptic connectivity, regulate behaviors such as anhedonia and giving up (immobility) in the forced swim test (Cui et al., 2018). More specifically, increased Kir4.1 has been proposed to decrease extracellular K^+ levels around LHb neuron somata, leading to hyperpolarization and de-inactivation of a T-type voltage-gated Ca^{2+} channel that subsequently evokes LHb neuron burst firing. If this is true in humans, then the implication of this study is that a partial blocker of Kir4.1 may display anti-depressive properties. In accordance with this, the expression levels of Kir4.1 are upregulated in the parietal cortex of individuals with major depressive disorder but not in those with schizophrenia (SCZ) or bipolar disorder (Xiong et al., 2019), and several antidepressants are known to interact with Kir4.1 channel pore residues and inhibit Kir4.1 K^+ currents (Furutani et al., 2009).

HUMAN DISEASES WITH A PRIMARY ASTROCYTIC BASIS

Extensively ramified human astrocytes display unique morphological features, such as varicosities and processes that cross between cortical layers (Figure 1E). Gene expression analyses show that human astrocytes differ from those in rodents, in accordance with expectations of human-mouse differences (Zhang et al., 2016). It has been suggested that greater astrocyte complexity may contribute to the higher cognitive abilities of hominids (Oberheim Bush and Nedergaard, 2017; Oberheim et al., 2009), and chimeric mice harboring human glial cell progenitors (many of which become astrocytes) exhibit improved performance in learning and memory tasks relative to control immunocompromised mice (Han et al., 2013). However, hypothesis testing and detailed physiology of human astrocytes are in their infancy, and emerging evidence indicates that human astrocytes also exhibit heterogeneous regional gene expression (Kelley et al., 2018). Although it is not possible to link dynamic astrocyte signaling to specific human behaviors, we briefly mention disorders that have a primary basis in astrocyte-enriched proteins and result in profound behavioral alterations (Table 1).

Mutation of *GFAP* causes a rare progressive form of leukodystrophy called Alexander disease (AxD). GFAP is an intermediate filament protein expressed by astrocytes and a prototypical astrocytic marker (Messing and Brenner, 2020). Patients with AxD display seizures, ataxia, and psychomotor retardation. The pathogenic variants of the *GFAP* gene are considered to cause a gain-of-function phenotype that leads to abnormal protein aggregates in astrocytes called Rosenthal fibers as well as altered astrocyte physiology (Saito et al., 2018).

Kir4.1 weakly inwardly rectifying potassium channels contribute to passive electrical properties and extracellular K^+ homeostasis by astrocytes and oligodendrocytes (Chever et al., 2010; Djukic et al., 2007). Kir4.1 also facilitates glutamate transport and regulates cell volume and water content. Loss-of-function mutations in *KCNJ10* are linked to autosomal recessive disorders called EAST syndrome (epilepsy, ataxia, sensorineural deafness, and [a renal salt-wasting] tubulopathy) and SeSAME syndrome (seizures, sensorineural deafness, ataxia [lack of muscle coordination], intellectual [mental] disability, and electrolyte imbalance) (Bockenhauer et al., 2009; Scholl et al., 2009). Both syndromes include epilepsy, ataxia, sensorineural deafness, and tubulopathy. SeSAME EAST mutations result in reduced or abolished Kir4.1 K^+ currents (Williams et al., 2010). In contrast, gain-of-function mutations in *KCNJ10* are correlated with an autism and epilepsy phenotype and with sleep alterations in children (Cucchiara et al., 2020; Reichold et al., 2010; Sicca et al., 2011, 2016).

EAAT1 and EAAT2 are predominantly expressed in astrocytes and are responsible for removing excessive glutamate from the synaptic cleft. Heterozygous mutations in the *SLC1A3* gene cause episodic ataxia (Choi et al., 2017; de Vries et al., 2009; Jen et al., 2005; Pyle et al., 2015). Recently, another loss-of-function mutation of the *SLC1A3* gene was identified in a patient with migraine but without ataxia (Kovermann et al., 2017). This mutation impairs K^+ binding to EAAT1 and diminishes glutamate uptake. Mutations in *SLC1A2* (encoding EAAT2/GLT-1) have been linked to epileptic encephalopathies, a group of early-onset epilepsies with severe cognitive and behavioral impairments (Epi4K Consortium, 2016).

The transmembrane Na^+/K^+-ATPase pump is crucial for maintaining ionic gradients as well as for regulating cell volume and signaling pathways. Of the four α isoforms, the α_2 subunit is encoded by the *ATP1A2* gene and is mainly expressed by astrocytes. Autosomal-dominant mutations in the *ATP1A2* gene cause an inherited form of migraine called familial hemiplegic migraine type 2 (FHM2), characterized by aura, hemiparesis, and dysphasia (Bottger et al., 2012; Carreno et al., 2013; Hiekkala et al., 2018). FHM2 patients can also display seizures, cognitive impairments, and rare manifestations of psychiatric symptoms. The underlying mechanisms of how *ATP1A2* mutations lead to FHM remain mysterious. However, dysfunctions of Na^+/K^+-ATPase and its coupled proteins result in cortical spreading depression that triggers migraine aura (Friedrich et al., 2016). Mice carrying a missense mutation in *Atp1a2* display increased cortical dendritic excitability and sensitivity to head pain induction (Romanos et al., 2020).

AQP4 is a water channel located at the peri-vascular and peri-ventricular processes of astrocytes. Serum IgG autoantibodies against AQP4 (AQP4-IgG) have been described in patients with the rare idiopathic inflammatory demyelinating disease

Table 1. Examples of astrocyte-enriched genes associated with CNS disorders in humans

Disease or clinical presentation	Gene *HUMAN*, *mouse*	Protein	Mutation Type[a]	Astrocyte Enrichment versus [Other Brain Cell Type] Human; Mouse[b]
AxD	*GFAP*, *Gfap*	glial fibrillary acidic protein (GFAP)	gain of function	[neurons]: 19; 22 [oligodendrocytes]: 8; 7 [microglia/macrophages]: 70; 18 [endothelia]: 27; 36
Autism-epilepsy, sleep disorder, EAST syndrome, SeSAME syndrome	*KCNJ10*, *Kcnj10*	inwardly-rectifying potassium channel: Kir4.1	gain or loss of function	[neurons]: 14; 23 [oligodendrocytes]: 4; 3 [microglia/macrophages]: 207; 73 [endothelia]: 48; 395
Ataxia, migraine	*SLC1A3*, *Slc1a3*	glutamate transporter: EAAT1	loss	[neuron]: 12; 25 [oligodendrocytes]: 22; 61 [microglia/macrophages]: 7; 65 [endothelia]: 55; 296
Epilepsy, bipolar disorder, SCZ	*SLC1A2*, *Slc1a2*	glutamate transporter: EAAT2	probably loss of function	[neuron]: 12; 25 [oligodendrocytes]: 39; 32 [microglia/macrophages]: 452; 93 [endothelia]: 62; 423
Familial hemiplegic migraine type 2	*ATP1A2*, *Atp1a2*	Na^+/K^+-ATPase α_2 subunit	loss of function and gain of function	[neuron]: 17; 29 [oligodendrocytes]: 33; 31 [microglia/macrophages]: 598; 51 [endothelia]: 4; 30
NMO spectrum disorder (Devic's disease)	*AQP4*, *Aqp4*	water channel:aquaporin 4	relapsing-remitting autoimmune disease producing antibodies against aquaporin 4	[neurons]: 13; 27 [oligodendrocytes]: 54; 224 [microglia/macrophages]: 395; 374 [endothelial]: 71; 265
Vanishing white matter disease	*EIF2B1-5*, *Eif2b1-5*	guanine nucleotide exchange factor: eIF2B	probably loss of function	[neurons]: 0.9, 1.2, 0.5, 0.9, 0.5; 0.7, 0.8, 0.8, 0.8, 0.6[c] [oligodendrocytes]: 1.1, 1.6, 1.0, 1.0, 0.9; 0.5, 0.7, 0.6, 1.0, 0.5 [microglia/macrophages]: 1.3, 3.0, 1.0, 1.8, 0.7; 1.1, 0.9, 1.2, 0.7, 0.4 [endothelial]: 1.4, 4.0, 2.4, 1.8, 2.2; 0.6, 0.4, 0.4, 0.9, 0.3

[a]In each case, multiple mutations have been identified that cannot be listed because of space limits.
[b]The astrocyte enrichment score was estimated from https://www.brainrnaseq.org/ and represents the (astrocyte/other cell type) gene expression (FPKM) ratio for human and mouse cells.
[c]Values are shown in the order of *EIF2B1*, *EIF2B2*, *EIF2B3*, *EIF2B4*, and *EIF2B5* for each cell type.

neuromyelitis optica (NMO) (Lennon et al., 2004, 2005). NMO primarily affects the optic nerves and spinal cord of individuals and causes clinical manifestations involving visual impairment, weakness, paralysis, numbness or increased sensitivity in the legs or arms, painful spasms, uncontrollable vomiting and hiccups, and bladder or bowel problems. AQP4-IgG binding to AQP4 on astrocytes causes complement-dependent multicellular cytotoxicity without altering AQP4 water permeability (Papadopoulos and Verkman, 2012; Saadoun et al., 2010).

We restricted our discussion to causal molecular changes in astrocyte-enriched genes that lead to neurological or psychiatric behavioral readouts (Table 1). It is noteworthy, however, that vanishing white matter disease, the result of mutations in eukaryotic translation initiation factor 2B (eIF2B), appears to have a predominantly astrocytic basis even though eIF2B is expressed ubiquitously (Bugiani et al., 2018; Dooves et al., 2016; van der Knaap et al., 2006). Hence, it is possible that mechanistic studies will reveal that astrocytes are critical drivers of pathology in other diseases even when the underlying mutation exists in multiple cell types.

SUMMARY COMMENTS

Exploring how astrocytes regulate neuronal circuits *in vivo* was the logical extension of anatomical reality and several years of pioneering studies that showed that astrocytes displayed physiological responses *in vivo* during diverse types of sensory and behavioral paradigms. In this review, we summarized recent studies from diverse species suggesting that astrocytes also contribute consequentially to the functions of neural circuits and the behaviors they encode. These data reveal an underrecognized richness in nervous system function and suggest neurons and astrocytes as determinants of complex brain functions and, therefore, also as targets to be exploited to correct behavioral dysfunctions associated with disease.

How does one interpret the behaviorally relevant responses ascribed to astrocytes and discussed in this review? We suggest that three types of interpretation are possible based on the available data and that each should be considered in relation to future work.

Type 1 interpretations

In this category are examples suggesting that astrocytes integrate incoming neuronal signals over seconds and then switch to a mode resulting in altered neuronal function. Zebrafish radial astrocytes in a specific subregion of the brain stem temporally integrate neuromodulator encoded behavioral failures to accumulate evidence of futility over seconds before inducing a state of behavioral passivity (Mu et al., 2019). The ability of *Drosophila* astrocytes to regulate sleep via Spätzle and olfaction-driven chemotaxis and touch-induced startle via ATP/adenosine also require type 1 interpretations (Blum et al., 2020; Ma et al., 2016). Furthermore, genetically impairing Ca^{2+} signaling in striatal astrocytes and restoring expression of a clock gene, *Cry1*, in SCN astrocytes alone were sufficient to guide very specific behaviors: highly stereotyped self-grooming (Yu et al., 2018) and circadian patterns of locomotor activity in otherwise arrhythmic mice (Brancaccio et al., 2019), respectively. Although the behavioral relevance is unclear, in the mouse hippocampus, long bouts of action potential firing in NPY-positive interneurons results in emergence of barrage firing (Deemyad et al., 2018). This switch to barrage firing may be explained by astrocytes functioning as leaky integrators of ongoing activity and then abruptly changing to allow interneurons to switch mode. These studies suggest that astrocyte signaling performs specific functions that result in specific behavioral outcomes.

For type 1 interpretations, understanding how astrocytes integrate information at a biophysical level requires quantitative measurements and modeling. In this regard, understanding the details of astrocyte Ca^{2+} signaling in subcellular compartments will be important. For example, recent studies suggest that basal Ca^{2+} levels determine the properties of diverse types of Ca^{2+} events in brain slices and *in vivo* (King et al., 2020). If basal Ca^{2+} levels are regulated, then this could represent a substrate for a leaky integrator that shapes subsequent responses. Another possibility is that integration occurs as signals in distinct spatial locations, which also have different basal Ca^{2+} levels, summate during ongoing activity (Zheng et al., 2015). Further studies to quantify and model the potential ability of astrocytes to integrate or process activity within realistic cellular geometries or compartments are critical (Savtchenko et al., 2018). There are clear reasons to study Ca^{2+} signaling, but we ought to consider other intracellular signaling mechanisms as well.

Type 2 interpretations

One interpretation of some studies we considered is that astrocytes are broadly important for brain homeostasis/function and that therefore it is natural that some behaviors will be altered when astrocytes are changed. This type of explanation may be applicable in several cases and may be of particular interest in the context of how astrocytes contribute to disease phenotypes. An example of type 2 explanations is Kir4.1 upregulation in the LHb, which, through altered K^+ homeostasis, drives behaviors associated with depression (Cui et al., 2018). Another example is how altered astrocyte-mediated glutamate homeostasis affects multiple aspects of brain function (Danbolt, 2001). In several cases, use of DREADDs and other actuators could fall into responses requiring type 2 interpretations when there is a lack of additional evidence of a role for endogenous GPCR mechanisms that the actuator is designed to mimic. Responses requiring type 2 interpretations may be particularly meaningful in disease-related behaviors to understand the phenotypes or to modulate them for beneficial effect (Cui et al., 2018; Yu et al., 2020b).

Type 3 interpretations

For some astrocyte responses, we consider a third explanation. In this case, a neural circuit or behavioral alteration may be best explained by a coincident effect unrelated to normal astrocyte biology. One example of responses needing type 3 interpretations may be use of Channelrhodopsin in astrocytes, which elevates extracellular K^+ levels when activated for seconds or more (Octeau et al., 2019). In cases where this occurs, alterations in neural activity and behavior are expected, but these are no more insightful than saying that K^+ depolarizes neurons. Such responses tell us what astrocytes are capable of doing under artificial experimental settings but not necessarily about what they actually do in normal settings. This distinction is important to recognize for the field to grow and capture new researchers. Another example of type 3 responses is the outcome of use of promoters and driver lines that are meant to be astrocyte selective but result in alteration of neurons with resultant coincident changes in circuits and behavior that confound interpretation of astrocytic effects that may occur in parallel. An example of this category may include use of some mouse lines that target populations of neurons as well as astrocytes, making it problematic to interpret behavioral effects ascribed to astrocytes. Such issues have been widely discussed in the literature (Hirbec et al., 2020; Xie et al., 2015; Yu et al., 2020a).

More generally, although revealing in a causal sense, optogenetic and chemogenetic approaches nonetheless have general limitations. First, they often lack holistic context for the cellular effects, making it problematic to separate direct and secondary astrocyte contributions to behavior. Second, exogenous stimulation may not faithfully recapitulate endogenous pathways used *in vivo*. In the future, such explorations could be aided by suppression of defined types of ongoing physiological activity as a complementary interventional approach. Moreover, the roles of striatal astrocyte Kir4.1 in the context of HD and in the LHb in the context of depression are illustrative of a general and important point. In the LHb, overexpression of Kir4.1 led to increased excitability of LHb neurons, whereas in the striatum, downregulation of Kir4.1 led to increased MSN excitability (Cui et al., 2018; Tong et al., 2014). These data suggest that the circuit consequences of astrocyte mechanisms will be dictated by the biophysical properties of neurons in specific brain regions. Along with the evidence of region-specific astrocyte properties, there are likely to exist astrocyte mechanisms with differential effects between brain areas even when the underlying molecular change is related or the same (Huang et al., 2020a). In this regard, it will be important to expand the palette of tools available

to measure diverse astrocyte activity in parallel with neuronal function between brain areas. Improved tools to image ATP, metabolism, spatial dynamics, glycolysis, and ions such as K^+ would be a valuable start.

It will also be necessary to explore astrocyte functions in circuits from diverse species at a mechanistic level based on data-driven insights regarding molecular causation. Conservation of glial cell types, anatomical simplicity, experimental accessibility, and new tools will continue to allow model organisms such as worms, flies, and zebrafish to unveil how astrocytes regulate neural circuit function and animal behavior at cellular and molecular resolution. In parallel, it will be critical to explore astrocytic contributions to ethologically natural behaviors and to those that accompany neurological and psychiatric disease states. Both types of exploration are necessary, and a comprehensive body of such studies should reveal whether astrocytes contribute to normal or disease phenotypes or possibly to both. Another parallel direction is to combine behavioral paradigms with computational approaches to decipher the underlying logic of circuit mechanisms in relation to cognition, which relates to earlier discussions of how astrocytes integrate information. The fruit fly has contributed enormously to our understanding of many basic principles in animal development and physiology, and it seems poised to help us understand astrocytes. The powerful array of molecular-genetic approaches available for *Drosophila* holds great promise for rapid identification of genes that are required to specify, build, and operate astrocytes *in vivo* in neural circuits. In zebrafish, it is expected that taxonomic analysis (Lange et al., 2020; Raj et al., 2018) will reveal the molecular identities of astrocytes and help design novel animal lines and/or reagents that are more specific to them (Chen et al., 2020). Employing rapidly evolving imaging tools for neurotransmitters, biomolecules, and ions will also likely broaden the questions one can ask because zebrafish provide unique opportunities for monitoring neuron-glia interactions, especially at a level of scale and detail that currently would be difficult in studies using mammals. Naturally, there will likely be some differences in the precise operational mechanisms employed in any one model organism, but there is ample reason to believe that they will have many more things in common, and, irrespectively, comparisons between species will prove to be fruitful and likely teach us important aspects of biology.

With recent progress, it now seems clear that although electrophysiology and the oscilloscope were powerful early means to study fast electrical events in neurons and foreshadowed decades of insight and progress, experimental explorations of astrocytes have had to await the advent of genetic and imaging methods that capture the slower dynamics of these cells. Reassuringly, these approaches are now beginning to provide new and unexpected insights concerning astrocyte biology, nervous system function, and regulation of behavior.

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J.N. and X.Y. wrote first drafts of the species sections; all other authors expanded and revised them along lines of expertise. B.S.K. wrote the summary, opening, and closing sections and assembled comments from all authors. B.S.K. wrote the final version; all others commented.

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