ANNUAL REVIEWS

Annual Review of Neuroscience

The Glial Perspective on Sleep and Circadian Rhythms

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Annu. Rev. Neurosci. 2020. 43:119–40

First published as a Review in Advance on February 19, 2020

The *Annual Review of Neuroscience* is online at neuro.annualreviews.org

https://doi.org/10.1146/annurev-neuro-091819-094557

Keywords

glia, sleep, circadian rhythms

Abstract

While neurons and circuits are almost unequivocally considered to be the computational units and actuators of behavior, a complete understanding of the nervous system must incorporate glial cells. Far beyond a copious but passive substrate, glial influence is inextricable from neuronal physiology, whether during developmental guidance and synaptic shaping or through the trophic support, neurotransmitter and ion homeostasis, cytokine signaling and immune function, and debris engulfment contributions that this class provides throughout an organism's life. With such essential functions, among a growing literature of nuanced roles, it follows that glia are consequential to behavior in adult animals, with novel genetic tools allowing for the investigation of these phenomena in living organisms. We discuss here the relevance of glia for maintaining circadian rhythms and also for serving functions of sleep.

Contents

GLIAL CLASSES IN MAMMALS AND FLIES

Glial cells are a diverse group composed of both central and peripheral nervous system (CNS and PNS) constituents. As defined for vertebrates, the major classes of glia include astrocytes, oligodendrocytes (and Schwann cells in the PNS), and microglia. Considering that *Drosophila melanogaster* has been a popular model for investigating glial influences on sleep and circadian rhythms, we also review the fly glial classes as a representative of invertebrates.

Astrocytes are the most abundant and well-characterized glial cell type in the mammalian brain and are intimately tied to the metabolic, circulatory, and neuromodulatory control of neurons. Even still, the range of astrocyte morphological and functional specializations is only beginning to be understood (Ben Haim & Rowitch 2017). In general, astrocytes surround cell bodies, dendrites, and axons in a nonoverlapping manner within the adult nervous system, with individual cells capable of interacting with multiple neuronal soma, hundreds of dendrites, and thousands of synapses (Bushong et al. 2002, Halassa et al. 2007). This function is accomplished through the branched processes of astrocytes, which are highly refined and interact closely with axonal boutons and dendritic spines to the extent that they have been considered a third component of the synapse (Araque et al. 1999). *Drosophila* contain a neuropil class of glial cells, which are known as astrocyte-like glia (Awasaki et al. 2008). As in mammals, astrocyte-like glia extend projections that interdigitate with synapses.

In *D. melanogaster*, there are two additional glial classes that nominally lack a mammalian analog, but based on morphology and function they can be compared to astrocytes and perhaps viewed as specialized subtypes of the class (Freeman 2015). The first of these are cortex glia, which are expansive cells that surround the cell bodies of many neurons. In this manner, cortex glia are similar to the protoplasmic type of astrocyte (Freeman 2015). The functions of cortex glia are not well established, but since at their superficial boundary these cells contact the fly equivalent of

the blood–brain barrier (discussed below), a parallel can again be drawn to mammalian astrocytes, which are at the interface of nutrient exchange between the vasculature and neurons and have remote influence on barrier permeability and function (Abbott et al. 2006). The second class are the ensheathing glia, which can also be seen as functionally similar to astrocytes due to their role in engulfing degenerating neurons (Hilu-Dadia et al. 2018, MacDonald et al. 2006). Due to the characteristic of isolating projections, ensheathing glia can also be compared to another mammalian glial class, the oligodendrocytes.

Oligodendrocytes are an essential contributor to neuronal function. Through repetitive wrapping of their myelin-rich membranes around axons, oligodendrocytes provide the insulation necessary for rapid and energy-efficient salutatory conduction. Additionally, emerging work suggests that these cells independently provide metabolic support to neurons (Lee et al. 2012). Mammals also have oligodendrocyte precursor cells (OPCs) or NG2 cells, a progenitor population that continues to give rise to oligodendrocytes in the adult brain (Richardson et al. 2011) and has interesting properties such as electrical communication with neurons (Bergles et al. 2000). While there are isolated examples of myelination of axons in invertebrates, the general principle of glia insulating projections is evident. *Drosophila* ensheathing glia compartmentalize neuropil and surround fiber tracts as well as individual projections. Similarly, in the *Drosophila* periphery, wrapping glia insulate projections, as do Schwann cells in the mammalian PNS.

Microglia are the dedicated immune and macrophage cells of the brain, which surveil the CNS through elaborately branched and labile projections (Nimmerjahn et al. 2005, Wake et al. 2009). Activated microglia scavenge the brain for apoptotic cells and other injuries, but microglial phagocytosis is also important in pruning synapses during development (Paolicelli et al. 2011). *Drosophila* contain immune cells within the body (hemocytes), but no directly comparable cell exists for the CNS. As stated above, engulfment of apoptotic or cellular debris appears to be accomplished by ensheathing glia in flies (Hilu-Dadia et al. 2018, MacDonald et al. 2006).

An important distinction between the blood (or hemolymph)–brain barriers of most vertebrates and those of flies and other relevant invertebrates rests in whether the stringent barrier-forming cell type is endothelial (for mammals) or glial [for invertebrates as well as elasmobranch fish (Bundgaard & Abbott 2008)]. Therefore, flies also contain two prominent populations of barrier-forming or surface glial cells, neither of which have mammalian glial counterparts but are analogous in function to endothelial blood–brain barrier cells and the supporting pericytes and astrocytic end feet. The fly hemolymph–brain barrier is a continuous enveloping bilayer, up to several microns in thickness, which is formed by the perineurial glia (PG) and the subperineurial glia (SPG). The PG are the apical layer, representing a couple thousand cells that overlap to form a loose physical barrier. The role of the PG layer is not well established, but it appears to have metabolic significance, as the main sugar energy source, trehalose, is taken up and processed through glycolysis by PG cells (Volkenhoff et al. 2015). Through sensing nutrition status, this population also plays a developmental signaling role for neural stem cells (Chell & Brand 2010, Speder & Brand 2014). The basal SPG layer comprises large, polyploid (Unhavaithaya & Orr-Weaver 2012) cells numbering only in the hundreds (Kremer et al. 2017), which are bound together by septate junctions, thereby forming the tight diffusion barrier (Stork et al. 2008). The barrier populations are linked by gap junctions (Speder & Brand 2014). The barrier populations work in concert to control solute transport between the brain and periphery, whether ions, nutrients, metabolites, signaling molecules, or xenobiotics (Hindle & Bainton 2014). The two layers are known to a certain extent to have nonoverlapping transporter and receptor expression (DeSalvo et al. 2014), although they are unlikely to be completely mutually exclusive, and the degree to which functions are segregated between the populations is an open question.

This review examines the evidence for how these glial classes, in mammals and flies, are involved with two phenomena—sleep and circadian rhythms—that profoundly shape behavior but are also reflected in various cellular, molecular, and genetic correlates. The early studies in both fields have found glia to influence circadian and sleep behavior, while also showing state- and time-dependent aspects of glial physiology. We begin by discussing the extant research on glial roles in sleep.

CURRENT EVIDENCE FOR GLIAL INVOLVEMENT IN SLEEP REGULATION AND FUNCTION

Sleep is a fundamental behavioral state found in essentially all animals. Most simply, it is defined as reversible behavioral inactivity occurring at a species-appropriate time of day and under a homeostatic influence. The homeostatic component is often described as sleep pressure, which builds as a function of time spent awake and can be experimentally manipulated by depriving sleep and examining subsequent recovery sleep. The two-process model (Borbély 1982) postulates that typically homeostatic forces and circadian influences are aligned to promote sleep at the end of a day of wake, but under conditions of sleep loss, the homeostat can override the clock to force sleep at different times of day.

While sleep has always been of interest, the state still remains enigmatic, largely because the manifestation of sleep in the brain (cellular/molecular/genetic levels) and the functions it serves are still being described and contested. There is not yet a unifying theory for the function(s) of sleep, nor have proposed functions been connected to the circuitry that controls state transitions.

For all of the major proposed functions of sleep, there are immediate, if not central, implications for how glial cells would contribute. In metabolic or energetic hypotheses, glia would be pivotal as intermediaries between circulation and neurons, containing the enzymatic machinery necessary to provide energy substrates; whether it is glucose/glycogen or lactate shuttling that is proposed, astrocytes are the relevant glial type (Benington & Heller 1995, Petit & Magistretti 2016). For the glymphatic hypothesis, which proposes enhanced flow of interstitial fluid during sleep, glia are the essential cell type because the flow is thought to depend on aquaporin-4 in astrocytes (Mestre et al. 2018, Xie et al. 2013). In consideration of immune functions and sleep, glial cells are both the targets and sources of sleep-promoting cytokine signals, as astrocytes release and respond to multiple cytokines (Sofroniew 2014) and as microglia, which are the CNS immune cells, release interleukin (IL)-1 and tumor necrosis factor alpha (TNFα) in vitro (Bianco et al. 2005, Hide et al. 2000). Finally, in regard to the synaptic hypothesis of sleep that postulates downscaling of synaptic strength during the state, while TNFα can itself regulate plasticity (Stellwagen & Malenka 2006), astrocytes have the potential to shape neuronal synaptic strength through gliotransmitters and to balance local neurotransmitter environments (De Pitta et al. 2016). Glial manipulations that affect sleep also modulate memory (Halassa et al. 2009, Seugnet et al. 2011).

Given this potential overlap between sleep and glial function, it is surprising that few studies have investigated glial impact on sleep or characteristics of glia across states.

Astrocytes

Astrocytes are among the best-studied glial subpopulations, and they are implicated in sleep by the early studies in both mammalian and invertebrate model organisms. Genetic manipulation of astrocytes has shown that these glia contribute to homeostatic sleep response following sleep deprivation (SD) (Halassa et al. 2009, Seugnet et al. 2011) and also to baseline sleep amount, which is also used as a measure of homeostatic sleep drive. These effects appear to be attributable to the astrocytic functions of signaling and neurotransmitter recycling, with both potentially being

consequences of metabolic/energetic sensing. Still other phenomena regarding astrocytes have been observed to depend on sleep state and suggest novel functions of sleep (Bellesi et al. 2015, Xie et al. 2013), although at present it is unclear if the direct manipulation of this biology would alter sleep amounts.

One of the earliest and most compelling studies to implicate astrocytes as regulators of sleep focused on inhibiting exocytotic signaling from glia, a process known as gliotransmission, through the conditional, adult-specific expression of a dominant-negative domain of synaptobrevin (dnSNARE) (Halassa et al. 2009). Mice with blocked gliotransmission had normal baseline sleep, apart from diminished light-phase slow-wave activity (SWA), but they did not display the typically elevated sleep time and delta power that follow during recovery sleep after SD. What is more, SD did not affect memory performance on a novel object recognition task in the dnSNARE mice, suggesting that astrocytes release some substance(s) that impairs cognitive function and promotes homeostatic sleep responses. Optogenetic stimulation of hypothalamic astrocytes also increased non-rapid eye movement (NREM) and rapid eye movement (REM) sleep time during the treatment, although by unknown mechanisms (Pelluru et al. 2016). Among other neurotransmitters, metabolites, and cytokines, astrocytes are known to release ATP, which is converted to adenosine within the synaptic cleft (Blutstein & Haydon 2013). Adenosine was proposed to mediate astrocytic effects on homeostatic sleep, since application of an A1 receptor antagonist would recapitulate the phenotype of the dnSNARE mice (Halassa et al. 2009).

As a neurotransmitter, adenosine is supported by numerous studies to be a somnogen, as concentrations rise with time awake and SD (Porkka-Heiskanen et al. 1997, 2000). Furthermore, caffeine, which is perhaps the most widely used stimulant, is an antagonist to the adenosine receptors (Huang et al. 2005). Conditional astrocytic knockdown of adenosine kinase, which breaks down adenosine to AMP and ADP, produced mice with greater SWA during baseline and recovery sleep, although sleep time was not affected in either condition (Bjorness et al. 2016). This study proposed that astrocytic uptake and processing of adenosine, in response to metabolic factors, modulates sleep through its role in SWA (Bjorness et al. 2016).

Using the fly model, the first studies to assess sleep upon glial manipulation also found astrocytes to be important for homeostatic sleep (Seugnet et al. 2011). Expression of the intracellular domain of Notch in astrocytes, or its receptor delta in the mushroom body neurons, eliminated homeostatic recovery sleep in sleep-deprived flies. Mirroring the experiments of Halassa et al. (2009) in the mouse, expression of Notch also prevented memory impairments following SD, as evaluated by the aversive phototaxic suppression task (Seugnet et al. 2011). These findings substantiate that astrocytic signaling is a conserved and hence vital contributor to sleep regulation and, interestingly, advance the idea that adenosine is not the sole mediator. While the effect of the classical neurotransmitters on sleep is remarkably consistent between mammals and flies (Nall & Sehgal 2014), knockout of the only known adenosine receptor in the fly does not alter sleep (Wu et al. 2009).

In line with a role for sleep in immune function, astrocytes are known to secrete cytokines and respond to immune signals (Sofroniew 2014). Astrocytic knockdown of the *Drosophila* homolog of mammalian TNFα, *eiger*, reduces baseline sleep (Vanderheyden et al. 2018). Knockdown of the TNF receptor homolog, *wengen*, in neurons did not reduce baseline sleep but negated the homeostatic recovery sleep after SD. Injection of human recombinant TNFα elevated sleep in the fly but was abrogated by loss of *wengen* in neurons. In sum, these findings establish yet another mechanism for an astrocyte–neuron axis of sleep regulation. Interestingly, knockdown of *eiger* in astrocytes also blocked the sleep-promoting effects of a socially rich environment (Ganguly-Fitzgerald et al. 2006, Vanderheyden et al. 2018), representing the first example of a glial contribution to this experience-dependent input into sleep-wake regulation.

Ubiquitous overexpression of fabp7, a member of the fatty acid–binding proteins that help transport lipids, increases sleep in the fly (Gerstner et al. 2011), and astrocytic expression of a human mutant form (T61M) diminishes daytime sleep to produce shorter and more numerous bouts when compared to astrocytic expression of a wild-type human fabp7 (Gerstner et al. 2017). Fabp7 expression in mammals appears to be enriched in astrocytes, and impressively, both fabp7 knockout mice and humans with the fabp7.T61M mutation display shorter and more numerous sleep bouts (Gerstner et al. 2017).

While astrocytes may potentiate cytokine and adenosine signaling, a different mode of astrocytic influence on sleep occurs through the well-recognized functions of recycling and buffering classical neurotransmitters, particularly glutamate and γ aminobutyric acid (GABA) (Schousboe et al. 2013). Glial uptake and catabolism of GABA may significantly contribute to sleep amount in the fly. The short-sleeping *sleepless* mutants have decreased GABA levels and increased expression of γ aminobutyric acid transaminase (GABAT), an enzyme that breaks down GABA (Chen et al. 2015). While *sleepless* is necessary in neurons, the mutant appears to disrupt GABAT non-cell-autonomously, since a partial rescue of sleep is accomplished in *sssP1*;*gabatF* double mutants by glial expression of GABAT (Chen et al. 2015).

In mammals, EAAT1 and especially EAAT2 (known as GLT-1) are recognized as glutamate transporters and are enriched in astrocytes (Rothstein et al. 1994). Knocking down the glutamate transporter, *Eaat1*, in the cortex glia/astrocytes of flies decreases sleep (Luna et al. 2017). Manipulation of the fly homolog of amyloid precursor protein, *Appl*, decreases sleep with overexpression and increases sleep while decreasing glutamine synthase protein levels upon glial knockdown (Luna et al. 2017). Knockdown of *Eaat2* in ensheathing glia increases daytime sleep amount in adult flies (Stahl et al. 2018), potentially acting on the transport of taurine. Feeding of taurine increased sleep in wild-type but not in *Eaat2* mutant flies, while expression of *Eaat2* in ensheathing glia in the mutant background rescued the taurine effect on sleep (Stahl et al. 2018). These studies defined a contribution of glial amino acid transporters to sleep, but it remains unclear if these effects are through altered global levels or if they act specifically on sleep-wake circuitry. A noteworthy study examined GLT-1 expression in glia surrounding sleep- and wake-promoting neurons in rats. After six hours of SD, the extent of GLT-1 apposition to neuronal soma was affected in opposite ways, with an ∼10% decrease at orexinergic neurons and an ∼16% increase at melanin-concentrating hormone cells, which was reversible with recovery sleep (Briggs et al. 2018). These changes were associated with divergent electrophysiological effects in these populations.

Astrocytes can also signal between each other through extensive gap junction networks, which are thought to underlie the ability of these populations to sample metabolic conditions from wide swaths of the nervous system. Global astrocytic knockout of Cx43, an essential gap junction component, produces mice with greater NREM and REM sleep during the active phase and also frequent transitions between states (Clasadonte et al. 2017). Orexinergic neurons, which promote wake and are thought to stabilize the switch sleep circuitry of mammals (Saper et al. 2010), are lost in narcoleptics, who similarly display state instability. Viral knockout of gap junctions amid orexin cells in the lateral hypothalamus also produced mice with greater transitions and increased NREM during the active period. Orexinergic neurons from knockout animals showed decreased spontaneous and induced firing, which could be rescued by lactate dialysis into astrocytes within the area or extracellular lactate. Likewise, infusion of lactate to virally treated animals also restored sleep characteristics (Clasadonte et al. 2017). Presumably other components of sleep-wake circuitry would also be sensitive to changing energy demands, so it remains unknown whether astrocytic lactate availability in other parts of the brain would also influence state transitions.

Astrocytes phagocytize neuronal material and prune synaptic connections during development (Chung et al. 2015). Furthermore, astrocytic projections are quite dynamic in developed animals,

being capable of protracting and retracting in response to synaptic activity (Bernardinelli et al. 2014, Hirrlinger et al. 2004). Recently, the use of serial block-face electron microscopy (EM) across the brains of mice in sleep, wake, and sleep-deprived conditions has led to several insights concerning cellular changes with state (Bellesi et al. 2015, 2017; de Vivo et al. 2017). Astrocytic projections were found engulfing axonal and dendritic components to a greater extent in acutely sleep-deprived or chronically (multiday) sleep-restricted conditions as compared to naturally sleeping or awake time points (Bellesi et al. 2017). There was no difference between sleep and wake conditions, which might suggest that sleep loss such as from enforced wake, which exceeds daily amounts, is an additional pathological burden that is potentially due to enhanced oxidative stress (Bellesi et al. 2017).

The venerated neuroanatomist Santiago Ramón y Cajal seemingly recognized the plasticity of astrocytic projections even by static histological techniques, as he presciently offered glia the commanding role in state control, suggesting that sleep is produced when astrocytes physically abrogate synaptic connections by intrusion of their projections (Frank 2013, Tso & Herzog 2015). This question was also examined by EM, and the amount of astrocytic apposition of neuronal spines in layer II of the prefrontal cortex was significantly higher in mice exposed to four days of chronic sleep restriction (CSR) than those who had been sleeping or were sleep deprived for about six hours (Bellesi et al. 2015). The degree to which the synaptic cleft was apposed by the astrocytic perimeter was also increased in CSR-condition mice over sleep and SD, while mice taken from wake had greater synaptic coverage than ones that had been sleeping (Bellesi et al. 2015). Perisynaptic astrocytic processes were also found to have more glycogen granules in all wake and deprivation conditions, as compared to sleep (Bellesi et al. 2018a). This singular evidence suggests a state dependence to astrocytic proximity, if not as dramatic as Ramón y Cajal's prediction. Interestingly, a specific demonstration of glial regulation of a sleep circuit was discovered recently in *Caenorhabditis elegans*. Ablation of the CEPsh glia, which surround the synapse between the sleep-promoting ALA and the AVE neurons, produces worms that sleep longer and show other movement differences (Katz et al. 2018).

Finally, the newly emerged glymphatic hypothesis states that the movement of cerebrospinal fluid along periarterial space to eventually mix and result in exchange with interstitial fluid in the brain is dependent on aquaporin-4 expression in the vascular end feet of astrocytes (Iliff et al. 2014). Mice were found to have substantially greater volumes of interstitial space during sleep than during wake, which allowed for greater clearance of injected Aβ during the state (Xie et al. 2013), while conversely, SD inhibited the spread of injected apoE3 beyond the arteries (Achariyar et al. 2016). The importance of astrocytic aquaporin channels was specifically challenged on the grounds that knockout animals did not have a detectable difference in tracer movement in the extracellular space (Smith et al. 2017), but a subsequent rebuttal substantiated aquaporin-4 dependence in multiple knockout lines, including the one used in the dissenting study (Mestre et al. 2018). Nevertheless, whether interstitial space or rates of convective flow are altered by sleep and sleep loss has yet to be disputed or confirmed.

Microglia

Several studies have examined a link between sleep loss and microglial activation, which typically occurs in the event of injury/stress in order to facilitate functions like pruning. Protracted sleep restriction by the disc-over-water method suggested greater microglial activation in the hippocampus of rats after five days of restriction (Hsu et al. 2003), but this was measured by OX-42 staining, which is not entirely specific to microglia (Jeong et al. 2013). In another work comparing sleep, SD, and CSR conditions, microglial number was not different and neither was the number

of processes, although the length of these processes was diminished only in the CSR condition (Bellesi et al. 2017). This group also examined microglial phagocytosis of neuronal material across these conditions by counterstaining for VGLUT1-positive terminals to find that the number and volume of phagocytosed puncta are higher in CSR than in sleep (Bellesi et al. 2017). Under these conditions, TNFα was highest in sleep, although SD is generally considered to raise TNFα levels (Rockstrom et al. 2018). In contrast to CSR, a different study used quite moderate SD, revealing a lowered expression of microglial Cd11b, which could lead to greater cytokine signaling from these cells (Wisor et al. 2011). A converse strategy was to prevent microglial activation by the drug minocycline (Yrjanheikki et al. 1998) and examine the effect on subsequent sleep (Wisor et al. 2011). NREM sleep amount was not altered, but animals on the drug had diminished delta power during NREM, a marker of sleep depth. Overt microglial activation may only be a consequence of pathological extended periods of sleep loss, but these studies were far from exhaustive, as microglia also have considerable signaling ability, which remains to be investigated in the context of sleep. Just as one preliminary example, cathepsin S is a proteolytic protein, which, in the brain, is only expressed by microglia. Global cathepsin S knockout mice showed lower delta activity during the light phase than wild-type mice and also exhibited increased locomotor activity in both light and dark periods (Hayashi et al. 2013a).

Oligodendrocytes

To date, oligodendrocytes have not been intensively studied in relationship to sleep. To assess the status of OPCs across states, one group injected BrdU to mark replicating cells in mice prior to 8-hour periods of sleep, wake, or forced wake through SD and revealed a greater colocalization with an OPC marker during sleep over both forms of wake, meaning that OPC proliferation rate is higher during the sleep state (Bellesi et al. 2013). Specifically, the amount of new OPCs was correlated with time in REM sleep. It is unclear what the physiological significance of this enhanced proliferation would be, as OPC numbers are thought to be stable in the brain (Psachoulia et al. 2009), and a commensurate increase in differentiation of OPCs was not reported, save for a modest increase occurring inexplicably during SD (Bellesi et al. 2013).

If sleep/wake state is reflected in oligodendrocyte function, it would be important to examine characteristics of mature oligodendrocytes in response to sleep loss. A study using EM determined that myelin thickness was diminished in mice that had been chronically sleep restricted as compared to those who engaged in spontaneous sleep (Bellesi et al. 2018b). Nevertheless, there was no appreciable difference between unperturbed animals and those that had more limited, acute periods of SD; neither were other characteristics such as myelin density or distance between nodes of Ranvier significantly altered between conditions.

Ultimately, this is only one study, so many more questions can be imagined. The emerging metabolic roles of oligodendrocytes (Lee et al. 2012), which may change as a function of sleep-wake state but not be evident in anatomy, could be of particular interest. It has been proposed that myelin might impact sleep (Morelli et al. 2011) through delivery of ATP, although the idea that myelin itself can generate ATP is debated (Harris & Attwell 2013, Ravera et al. 2009).

Barrier Glia

The fly hemolymph–brain barrier is composed of two glial populations, which as a unit show considerable similarity to functions and expression profiles of the mammalian blood–brain barrier (Weiler et al. 2017), a cohesive unit of tight endothelial cells, adjoining pericytes, and regulatory astrocytic end feet. In *Drosophila*, barrier glia have been found to affect sleep, as disruption of membrane trafficking in these populations by expression of the dominant-negative dynamin *Shibire*, or

a constitutively active *Rab11*, increased total sleep amounts (Artiushin et al. 2018). Conversely, endocytosis at the barrier is elevated during daily sleep as well as during recovery sleep following deprivation relative to times of wake; in short, this finding advances the idea that some trafficking at the barrier is influenced by sleep state, which is important for regulating the behavior (Artiushin et al. 2018).

The glial intersection with sleep and blood–brain barrier function is worth considering in mammals, given that astrocytes impact barrier endothelial cell properties and may do so in response to sleep and sleep loss via cytokine (Hurtado-Alvarado et al. 2016, Wang et al. 2014) or adenosinergic signaling (Carman et al. 2011). Furthermore, blood–brain barrier (BBB) integrity is susceptible to sleep loss. Restriction of REM sleep in rats increased permeability of the endothelial layer of the BBB to dye, which was correlated with increased caveolae (Gomez-Gonzalez et al. 2013). Interestingly, this was quickly reversed after recovery sleep, suggesting that altered transport at the BBB may be quite dynamic and not necessarily a reflection of pathology (Gomez-Gonzalez et al. 2013). Multiple days of sleep restriction in mice increased permeability of the barrier in several brain regions and also decreased tight junction expression and glucose uptake (He et al. 2014). Again, this effect was reversible with a day of recovery sleep (He et al. 2014).

Future Directions for Glial Involvement in Sleep Regulation and Function

Studies to date in both mammalian and fly models have demonstrated a potent ability of astrocytes to impact homeostatic and, more recently, baseline sleep by mechanisms such as gliotransmission (of ATP), cytokine signaling, and neurotransmitter recycling, among others (**Figure 1**).

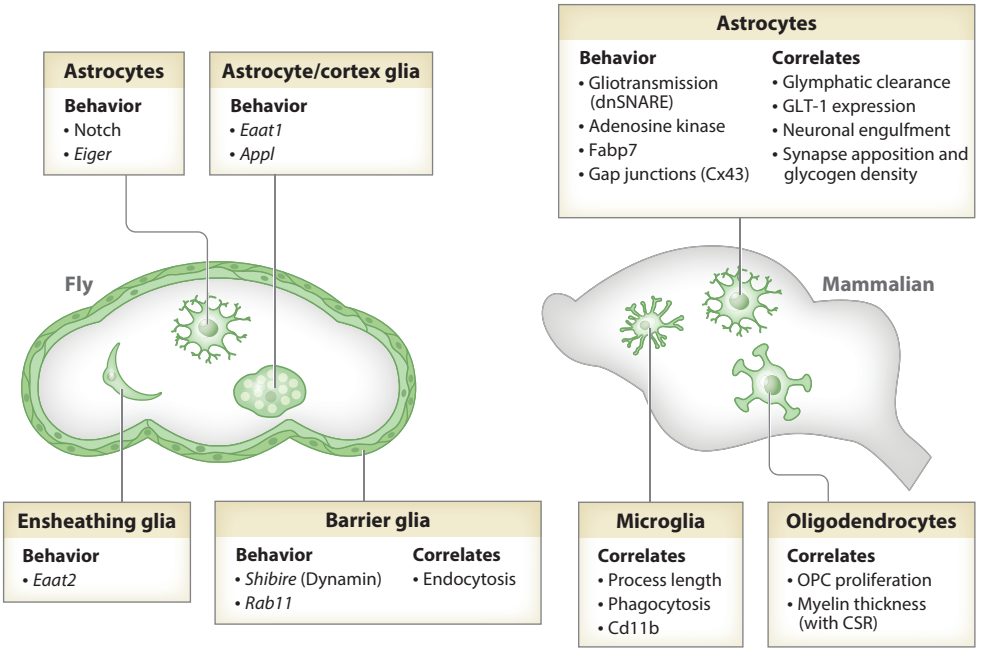

Figure 1

Fly and mammalian glial class influences on sleep behavior and correlates of the state. Behavioral points refer to genes and processes whose manipulation in glia produces changes in sleep amount or quality. Correlates refer to cellular and molecular aspects of glial cells, which have been observed to change as a function of sleep-wake state.

Future work should build on these mechanisms by understanding the astrocytic conditions that give rise to them, as this could bridge the execution of sleep behavior with its underlying purposes. In astrocytes, the most appealing avenue would be a metabolic underpinning (Bjorness et al. 2016, Clasadonte et al. 2017), which can incorporate the resultant gliotransmission and potentially even differences in neurotransmitter uptake. But can glia reveal new circuit elements of sleep regulation? As of yet, most studies in flies and mammals do not localize effects of manipulations to particular astrocytes interacting with defined neuronal circuits. Given the importance of gap junctions, astrocytes may also be acting broadly to sense conditions and relay between neuronal populations.

Work on oligodendrocyte and microglia functions with sleep has been quite preliminary. Thus far, the intensive EM analyses have not revealed obvious differences between sleep and wake states, short of prolonged restriction conditions. This may indicate that potential changes with state are not morphological but are reflected in intercellular conditions, and further study of signaling from these populations could be informative. The findings of barrier trafficking with sleep in flies could point to a renewed appreciation of brain-periphery exchange as an influence on sleep. Clearly, the reciprocal relationship between blood–brain barrier function and sleep requires further study. Such findings could complement glymphatic functions, as Aβ, a model solute in these studies (Xie et al. 2013), also crosses the BBB layers (Deane et al. 2009) through a mechanism potentiated by astrocytic adenosine (Carman et al. 2011).

CIRCADIAN RHYTHMS AND GLIA

Perhaps even more so than the state of sleep, circadian rhythms are a nearly ubiquitous phenomenon in nature, which can be recognized in a wide breadth of organizational levels, from the high-order behavioral patterns of complex animals to even simple metabolic processes in unicellular organisms. The triumph of the circadian field has been the discovery of the genetic elements of the circadian clock, based in transcription-translation feedback loops (TTFLs), which assemble in varied but conceptually similar ways in quite evolutionarily distant forms of life.

This review assumes a basic familiarity with the homologous elements of the molecular circadian clock in mammals and *Drosophila* and the general anatomic locations of these oscillators in the master neuronal populations [suprachiasmatic nucleus (SCN) in mammals and clock cell network in flies] essential for rhythmic behavioral output. These subjects continue to be extensively reviewed elsewhere (Dubowy & Sehgal 2017, Herzog et al. 2017, Mohawk et al. 2012).

Particularly in mammalian tissues, circadian clocks are widely distributed, including in glial populations. As with sleep, one can ask how glial cells affect circadian behavior and outputs, and conversely, what glial components and functions (e.g., transcripts and proteins, secreted factors, morphology) can be shown to adhere to a circadian cycle? If glia exhibit a functional TTFL clock, are the circadian qualities of glia dependent on it? These questions and others are beginning to be unraveled in mammalian and fly model organisms.

ASTROCYTES

Given that astrocytes are the most intensively studied of the glial subclasses, it is natural that most experiments examining circadian rhythms in glia also uncover effects in these populations. Early investigations into the location of circadian clock cells in the fruit fly described the presence of the period clock protein (PER) in disparate neurons, as well as in what appeared to be astrocytic glial cells, based on colabeling with a neuronal marker (Ewer et al. 1992). It is important to note

that circadian clock proteins are present in various populations, but this alone is not evidence of a circadian function, as these proteins may serve purposes other than supporting a TTFL clock.

Beyond the mere presence of clock proteins, an essential step was to establish that these genes cycle in glia as well. Cortical cultures of astrocytes from rats exhibited Per2 and Per1::Luc rhythms, which could be sustained for days and entrained by various stimuli such as doses of calcimycin and forskolin or simply a media change, demonstrating that glia can display circadian rhythms in clock gene expression (Prolo et al. 2005). Coculture with SCN neurons provided a marginally more stable rhythm in astrocytes, thus presaging recently emerging evidence of a mutually supportive role of astrocytes and neurons on SCN rhythmicity (Brancaccio et al. 2017, Tso et al. 2017). Likewise, PER was found to cycle in glia of the fly brain (Ng et al. 2011).

Evidence from *Drosophila melanogaster*

The first studies to demonstrate that genetic manipulations in glia produced tangible alterations in behavioral rhythms were another accomplishment of the *Drosophila* model. Ebony is a β-alanyl-biogenic amine synthase, whose mRNA and protein levels cycle in the fly brain, where its expression is exclusively glial (Suh & Jackson 2007). This cycling is dependent on clock function, as it is lost in clock mutants, but it is not clear whether glial ebony rhythms result from functional clocks within glia or are driven by signals from neuronal clock cells. At the behavioral level, ebony mutants have dampened locomotor rhythmicity, which can be rescued by glial overexpression of the enzyme, suggesting that a constitutively high level of the enzyme is permissive for robust rhythms. Loss of ebony does not substantially alter clock cycling in neurons, so it is likely that glial ebony alters circadian locomotor behavior downstream of clock neurons, perhaps by acting on biogenic amines.

A key set of experiments that demonstrated the influence of astrocytes on circadian behavior in the fly focused on the necessity of vesicular trafficking. By blocking trafficking in glia for several days with conditional expression of the temperature-sensitive dominant-negative dynamin (*Shibire*), it was shown that flies would become arrhythmic and that this was reversible with a shift back to permissive temperature for *Shibire* (Ng et al. 2011). Likewise, conditional expression of a Na^+ channel, NaChBac, or knockdown of the endoplasmic reticulum (ER) Ca^{2+}-ATPase pump (SERCA) also eliminated rhythms. Targeting of the major glial subclasses revealed that blockade of vesicular trafficking in astrocytes was sufficient to cause arrhythmicity.

A natural hypothesis follows that astrocytic manipulation disrupts timekeeping in the neuronal clock cells to produce behavioral differences. While *Shibire* expression in astrocytes did not alter neuronal cycling of PER or another clock protein (PDP1ε), it reduced expression of a circadian neuropeptide, PDF, in clock neurons (Ng et al. 2011). Interestingly, knockdown of PER in all glia did not affect the period length or degree of rhythmicity for locomotor activity in flies (Ng et al. 2011), suggesting that effects of glia on circadian behavior do not require glial clocks and are likely downstream of neuronal timekeeping mechanisms.

Given that the disruption of dynamin (with *Shibire*) or Ca^{2+} signaling is a broad manipulation that could implicate various endocytic and exocytic processes, further experiments along this line of inquiry have attempted to refine which genes and trafficking pathways are relevant in glia for the maintenance of robust behavioral rhythms (Ng & Jackson 2015, Ng et al. 2016). Some genes identified by genetic knockdown in adult glia include other components of trafficking machinery. *ROP*, a Sec1-homolog, is involved in vesicle fusion and release and diminishes rhythmicity when conditionally knocked down in astrocytes (Ng & Jackson 2015). This loss of behavioral rhythmicity occurred without degradation of PER rhythms or PDF expression patterns in clock

neurons, although an elevated level of PDP1ε was observed. The loss of clathrin-mediated endocytosis factor *AP-2σ*, SNARE components *Syx5* and *6*, and solute carrier (SLC) transporters such as *Ncc69* and *CG9657* produced less robust rhythms (Ng & Jackson 2015, Ng et al. 2016).

A different approach to understanding glial genes involved in circadian behavior has been to inhibit microRNAs (miRs), which are noncoding RNAs involved in widespread translation control of multiple gene targets per miR. Through the targeted and adult-specific expression of miR sponges, which adhere to miRs through a complementary sequence, it was found that the conditional inhibition of miR-263b and miR-274 in astrocytes diminished the robustness of locomotor rhythms (You et al. 2018). Interestingly, overexpression of these miRs also produced a similar effect. This was not the case when these constructs were expressed by a pan-neuronal driver, although undoubtedly other miRs are involved in circadian control in neuronal populations (Xue & Zhang 2018). Again, as has generally been the case for manipulations in flies, glial expression of miRs did not obviously impact PER cycling in clock neurons, nor did it alter PDF staining (You et al. 2018). Inquiry into the putative targets of these miRs added two other genes to the list of those that degrade rhythmicity when knocked down in glia: *CG4328*, a transcription factor related to LMX1A and B in mammals, and *MESK2*, a gene involved in Ras signaling (You et al. 2018).

Evidence from Mammalian Models

More recently, several studies have demonstrated that the clocks of mammalian astrocytes are capable of influencing neuronal oscillators and subsequent behavioral rhythms, suggesting a model in which SCN neurons and glia operate harmoniously to shape circadian rhythms.

Several groups chose to inhibit *Bmal1* expression in astrocytes by slightly different methodologies. Knockdown of *Bmal1* in astrocytes of SCN slices produced animals with a longer locomotor activity period, which was mirrored by a long period in whole-SCN Per2::Luc activity (Tso et al. 2017). To further demonstrate the in vivo influence of astrocytic clocks, two independent studies used mice with a floxed Casein kinase 1 epsilon (CK1ε) tau mutation. While global tau mutant mice have short periods, it was found that excision of the mutation in neurons (Brancaccio et al. 2017) or just in astrocytes (whether by Aldh1l1-Cre line or injection of GFAP-Cre virus) was sufficient to rescue the period in locomotor activity rhythms and, according to one study, also in Per2::Luc in the SCN (Brancaccio et al. 2017, Tso et al. 2017). A separate study also knocked down *Bmal1* in astrocytes but did not find overt changes in period length for locomotor activity (Barca-Mayo et al. 2017). However, this group employed a tamoxifen-inducible Cre line, which was much less effective at knocking down *Bmal1* expression in astrocytes and also caused an almost equivalent reduction in neurons of the SCN.

One of the most impressive demonstrations of the potency of astrocytes in dictating SCN rhythms came from yet another approach, that of viral reinstatement of *Cry1* in the SCN of animals that were *Cry1/2* null (Brancaccio et al. 2019). Although occurring more slowly than if expressed in neurons, the rescue by *Cry1* in astrocytes alone was capable of inducing Per2::Luc and Ca^{2+} rhythms in SCN neurons and also driving behavioral rhythms whose period was slightly longer than 24 hours (Brancaccio et al. 2019).

What mechanisms might govern the mutual influence of SCN neuronal and astrocytic clocks on each other's oscillators as well as on shaping behavioral output? ATP and adenosinergic signaling has been at the forefront of gliotransmission as a mechanism for astrocyte-neuron communication (Hines & Haydon 2014) and hence would be an attractive hypothesis for SCN synchronization. Microdialysis measurements of extracellular ATP from rat SCN showed a circadian rhythm,

with ATP peaking during the dark phase (Womac et al. 2009). ATP levels in the medium were also found to be circadian, using immortalized SCN cell lines or cortical astrocyte cultures. In this preparation, rhythms of cytosolic Ca^{2+} in astrocytes were antiphasic to extracellular ATP as well as to rhythms of Ca^{2+} in mitochondria (Burkeen et al. 2011). ATP rhythms could be inhibited by chelation of intracellular Ca^{2+} with BAPTA, although inhibition of SERCA with THAPS did not substantially impact the ATP rhythm (Burkeen et al. 2011). Murine astrocytic cultures also showed rhythmic ATP release, which was affected by the glial clock, as Clock and Per1/2 mutants tended to show diminished ATP release and a greater propensity to be arrhythmic in their extracellular accumulation of ATP (Marpegan et al. 2011). ATP release was dependent on IP_3 signaling but not on synaptobrevin-2-dependent vesicular release (Marpegan et al. 2011). The extent to which these ATP rhythms in the SCN affect neuronal and behavioral oscillations in vivo largely remains to be determined.

Other work in culture has suggested that GABAergic signaling is important for glial communication in the SCN. Employing a method of coculturing astrocytes and neurons that are partitioned but bathed in a common culture media (Barca-Mayo et al. 2019), researchers established that astrocytes could, via a factor in the media, induce rhythms of the canonical clock genes in the neurons, and this was not possible with *Bmal1* knocked down in the astrocytes (Barca-Mayo et al. 2017). In such a setup, a pulse of GABA is sufficient to induce clock gene rhythms in neurons, and if dexamethasone-synchronized astrocytes are placed adjacent to asynchronous neurons, their ability to induce rhythms in the neurons is abrogated by the presence of the GABA-A receptor blocker bicuculline. Animals with loss of *Bmal1* in astrocytes showed some differences in the expression of astrocytic GABA transporters (GAT1 and 3), although this was complicated and varied by the time of day and brain region in question. Nevertheless, clearance of GABA was inhibited by *Bmal1* knockout (Barca-Mayo et al. 2017).

In vivo experiments have instead forwarded a model in which glutamate is the essential neurotransmitter governing astrocyte-neuronal circadian regulation, underpinned by Ca^{2+} rhythms in each population. In the dorsal SCN, GCaMP recordings from both neurons and astrocytes revealed circadian rhythms in Ca^{2+} levels. At least for neurons, Ca^{2+} coincides with synaptic activity, as confirmed by coexpression of genetic voltage indicators. Notably, neuronal and astrocytic Ca^{2+} rhythms are almost exactly antiphasic, peaking about 12 hours apart (Brancaccio et al. 2017). A genetic sensor of glutamate targeted to either population revealed a rhythm of extracellular Glu, which coincided with the astrocytic rise in Ca^{2+}. A previous study had also reported that glutamate uptake and glutamine synthetase activity changed with time of day in the SCN, although not during constant dark conditions (Leone et al. 2015). Using several methods, researchers found that the extracellular glutamate arose from astrocytes. Selective astrocytic ablation in slices decreased extracellular glutamate, as did pharmacological blockade of glutamate metabolism, which occurs in astrocytes. Inhibition of glutamate transporters (Eaat1–3) desynchronized Per2::Luc rhythms in the SCN, and curiously, this effect was strongest when a drug that also blocked neuronal Eaat3 glutamate transporters was used. Further experiments showed that the desynchronization of clocks in the SCN was dependent on *N*-methyl-D-aspartate (NMDA) receptors, specifically those containing the NMDA 2C subunit-containing receptor (NR2C) (Brancaccio et al. 2017). Therefore, because neuronal Ca^{2+} and Per2::Luc rhythms drop off with protracted NR2C antagonism, the model holds that extracellular glutamate is high when astrocytic Ca^{2+} is high (during the night), which serves to inhibit neuronal activity via glutamatergic NR2C-containing NMDA receptors. Inhibition is relieved during the day, thereby establishing circadian rhythms in the SCN (Brancaccio et al. 2017). Supporting this model, the astrocytic rescue of *Cry1* in a null background, which eventually drives Per2::Luc and Ca^{2+} rhythms in SCN neurons, can be suppressed by DQP-1105, an antagonist to NR2C (Brancaccio et al. 2019). There appears to also be some role for gap

junctions, as inhibition of Cx43 in this experiment dampened amplitude of Per2::Luc rhythms in the neurons (Brancaccio et al. 2019).

MICROGLIA

Global and constitutive clock gene knockout animals often exhibit increased inflammation and microglial activation (Griffin et al. 2019, Musiek et al. 2013), but these phenotypes are not necessarily due to the clock roles of these genes. At the same time, it should be recognized that nongenetic alterations, such as extended exposure to light, which disrupts circadian clocks, also lead to enhanced susceptibility to immune challenges (Fonken et al. 2013). While these alterations are important due to the health impacts of behavioral circadian misalignment and comorbidities of circadian dysfunction with various diseases, from the vantage of understanding the purely glial contribution, studies that limit manipulations to microglia are necessary.

Initial work in microglial cultures from mice demonstrated *Per1* transcripts in these cells and found expression levels to be sensitive to ATP administration (Nakazato et al. 2011). Furthermore, pulling down murine microglia by fluorescence-activated cell sorting across the circadian day demonstrated mRNA cycling of *Per1*, *Per2*, *Rev-erbα*, and *Bmal1* (Hayashi et al. 2013a), which was confirmed by a different isolation technique in rats (Fonken et al. 2015). In culture, various clock gene cycling in microglia is disrupted by cannabidiol application (Lafaye et al. 2019).

Microglia are dynamic and become activated in response to challenges through movement and expansion of their processes. In the hippocampus, imaging of microglia by Iba1 staining or intracellular dye injection revealed a greater volume and branch complexity of microglia during the dark phase, as compared to a time point 12 hours prior, in the somatosensory cortex (Hayashi et al. 2013b, Takayama et al. 2016) and the hippocampus (Griffin et al. 2019). In vivo imaging of microglial dynamics in response to focal injections in mice revealed that microglia extended processes more readily in response to ATP injection at ZT14, while bacterial injection elicited a greater response at ZT14 (Takayama et al. 2016). A study employing EM imaging of microglial processes did not report morphological differences as a function of time of day, but the focus there was on sleep and sleep restriction (Bellesi et al. 2017). It is not yet clear whether diurnal changes in microglia are a function of the microglial clock.

Another essential characteristic of microglia is their secretion of numerous cytokines and other factors. Microglial transcript levels of IL-1β, IL-6, and TNFα were found to cycle and peak during the light phase (inactive period), with IL-1R1 also being rhythmic but peaking near lights off (ZT12) (Fonken et al. 2015). Likewise, cytokine responses in the hippocampus of rats injected with lipopolysaccharide differed with the time of day, with protein levels of IL-1, IL-6, and TNFα elevated when animals were injected during the light phase but not during the dark phase (when they are active) (Fonken et al. 2015). Beyond cytokines, cathepsin S transcripts oscillate in microglia of wild-type animals, closely matched to the peak of *Per1* and *Per2* expression, but their cycling is blunted in *Clock* mutant animals (Hayashi et al. 2013a). Several cortical synaptic properties such as mEPSC amplitude, frequency, and spine density are increased during the early light phase as compared to the early dark phase in mice, and the differences persist in constant darkness, but are absent in *Clock* mutants as well as in cathepsin S knockouts (Hayashi et al. 2013a, Takayama et al. 2017). This suggests that rhythmic cathepsin S secretion from microglia, perhaps governed by their autonomous clock, may impact patterns of cortical synaptic transmission.

Drosophila do not have microglia, although, with respect to debris engulfment, a similar function is accomplished by ensheathing glia (Doherty et al. 2009). Ensheathing glia show some PER protein expression (Long & Giebultowicz 2017), which is particularly strong in the medulla glia (Krzeptowski et al. 2018), but the extent to which this generates a functional clock and what relationship it holds to the potentially rhythmic properties of this population are unknown.

OLIGODENDROCYTES

As has been the case for sleep, there is not a strong literature on molecular clocks or circadian correlates in the function of oligodendrocytes and their precursor cells. There is evidence of a circadian pattern of OPC proliferation in the hippocampus, although this is variable even within different areas of this structure (Matsumoto et al. 2011). Isolated studies suggest that some gene expression may be rhythmic in these glia. One group found that roughly 2% of oligodendrocyte genes (357 in their data set) fluctuate as a function of circadian time (Bellesi et al. 2013). Additionally, in the corpus callosum, time of day differences in *Sgk1* (a protein kinase) mRNA expression were reported and found to be dependent on corticosterone levels (Hinds et al. 2017). While clock genes are expressed widely in mammalian cells, there is no definitive evidence of clocks in oligodendrocytes (Colwell & Ghiani 2020). As discussed above, oligodendrocytes do not have a perfect analog in flies, although superficially, the ensheathing glia might again be comparable due to their encasement of axonal projections.

BARRIER GLIA

Penetration of the blood–brain barrier is a major obstacle in drug delivery to the CNS. A growing literature points to circadian differences in efficacies of medication (Kreuter 2015), and therefore, apart from the basic biological question, clock control of barrier transport and permeability represents a considerable clinical interest.

Importantly, the principal restrictive layer in mammalian blood–brain barriers is an endothelial layer, and therefore not developmentally glial, as in invertebrates. Nevertheless, as discussed above, there are many functional similarities, suggesting that invertebrate models may provide fundamental lessons that are translational. Furthermore, the mammalian BBB is also composed of pericytes, as well as astrocytes with specialized end feet, whose signaling is known to alter properties of the other layers. Since the emerging research on circadian and sleep influences at the blood–brain barrier has recently been reviewed (Cuddapah et al. 2019), we focus the current references to those that directly interrogate circadian clocks in the glial components of barriers.

The barrier glia of *Drosophila* contain clocks, as PER expression has been found to cycle in the PG (Zhang et al. 2018), and a separate study also reported that this was the case in SPG (Long & Giebultowicz 2017). Flies were observed to show circadian rhythms in xenobiotic permeability of the barrier, with greater presence of the markers in the brain at night (Zhang et al. 2018). The barrier glial clocks were consequential to this, as expression of a dominant-negative *Cyc* to disrupt the clock in PG eliminated the nighttime difference, while doing so in SPG did not alter permeability. The time of day difference in drug presence was explained by greater efflux during the day, attributable in part to the activity of the P-glycoprotein (Pgp) transporter Mdr65 in the subperineurial layer. Rhythmic activity of efflux transporters is driven by a synchronous oscillation of Mg^{2+} within the SPG, which promotes activity of Pgp transporters. These rhythms are abolished if the gap junctions between the two populations are disrupted, yielding a model in which the molecular clock within the PG controls rhythms of gap junction expression and thereby creates a cycle in intracellular Mg^{2+} that acts to vary the activity of xenobiotic transporters in the SPG across the day (Zhang et al. 2018).

Of note, although potentially a noncircadian role for *Bmal1*, loss of this clock gene in pericytes decreases expression of PDGFRβ, leading to disruption of barrier integrity and pericyte function (Nakazato et al. 2017). Apart from the blood–brain barrier, a short-lived rhythm in Per2::Luc expression was reported in slices for the ependymal cells lining the third ventricle (Guilding et al. 2009).

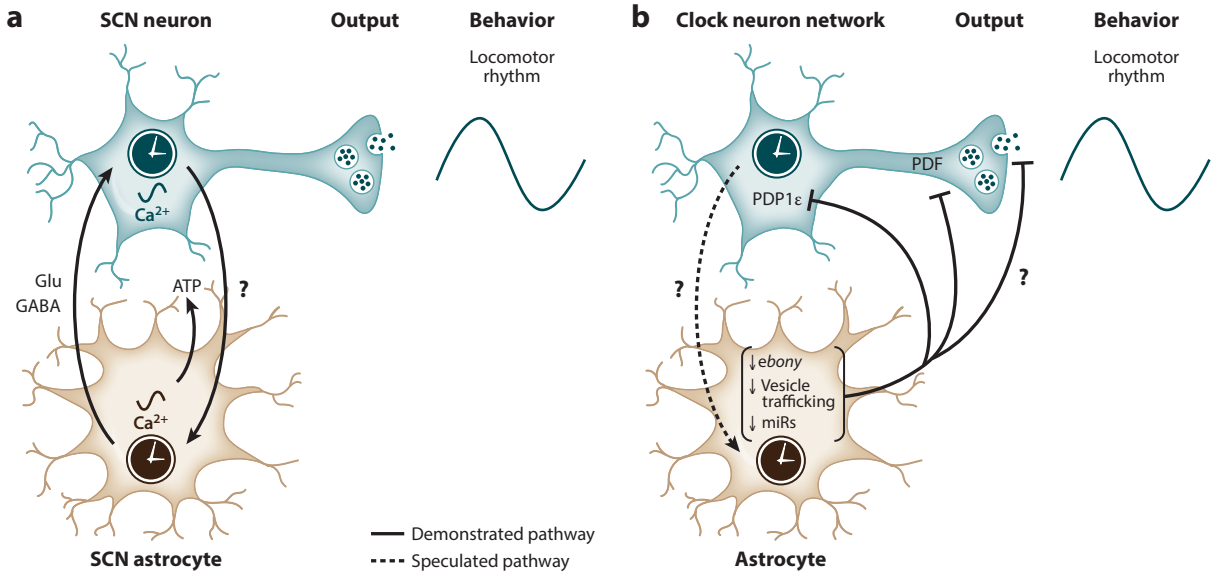

Figure 2

Astrocytic influence on neuronal clocks and behavioral output. (*a*) In mammals, astrocytic clocks of the suprachiasmatic nucleus (SCN) influence neuronal clocks via γ aminobutyric acid (GABA) in cell culture and glutamate in vivo. Both populations have rhythmic Ca^{2+} levels, which are antiphasic to each other throughout the day. Other signals such as extracellular ATP, which depends on astrocytic Ca^{2+}, might also act to influence neuronal clocks. Neuronal clocks can entrain astrocytic clocks by unknown mechanisms. (*b*) In flies, disruption of astrocytic vesicular trafficking, as well as enzymes such as *ebony* and certain transporters and receptors, produces deficits in behavioral output without affecting the core clock in neurons. Some manipulations eliminate rhythms of the pigment dispersing factor (PDF) or expression of the circadian gene *PDP1ε*, while others act independently. Knockout of the clock in glia has not been shown to affect neuronal clocks, and it is unknown whether neuronal clocks entrain glial ones.

SUMMARY AND FUTURE DIRECTIONS OF GLIAL INVOLVEMENT IN CIRCADIAN RHYTHMS

It is evident across model organisms that glial cells have circadian properties, and astrocytes in particular can impact neuronal clocks and/or their outputs to regulate rhythms of locomotor behavior. Nevertheless, findings from flies and mammals demonstrate different means by which this is accomplished (**Figure 2**).

The consistent result across the fly studies is that interruption of astrocytic function disrupts behavioral circadian rhythms, creating either arrhythmic or weakly rhythmic animals. Early work suggested that glial PER could be sufficient for weak rhythms, but due to methodology, it was uncertain whether expression was purely glial (Ewer et al. 1992). More recently, knockdown of glial PER was not sufficient to affect rhythms (Ng et al. 2011), thus perhaps warranting complete knockout of PER in glia and/or study of other clock proteins. Glial manipulations have also not readily affected the core neuronal clocks in flies. Therefore, determining the mechanism by which glial knockdown of trafficking and transporters affects output from clock neurons poses a challenge, since some manipulations act on PDF or PDP1ε (and still by unknown means), while others produce arrhythmic behavior independently. This may point to a greater unresolved complexity in the understanding of circadian output in the fly and glial participation in it. Another obstacle is the dispersed nature of fly clock neurons. Future studies might benefit by performing functional imaging of multiple populations concurrent with astrocytic manipulations to understand how and where output is disrupted.

Contrary to the fly, mammalian studies have demonstrated that a functional clock in astrocytes of the SCN is vital for contributing to the overall circadian output of the tissue. While astrocytic manipulation has not been demonstrated to completely eliminate rhythmicity in mammals, under certain experimental conditions (Brancaccio et al. 2019), a clock in astrocytes alone is sufficient to induce rhythms in neurons and drive behavior. This speaks to the flexibility and robustness of the SCN, which now must be understood as arising from an interplay between neuronal and astrocytic oscillators. How neurotransmitters such as GABA, glutamate, and perhaps ATP coordinate this communication remains to be fully worked out, and it will also be interesting to study how glial oscillators are entrained.

The other glial classes have thus far not been as intensively studied, particularly in vivo. There is good evidence that a functional microglial clock governs some cytokine signaling, although the full extent of this still needs to be examined.

CONCLUSION

To summarize, these pioneering studies have demonstrated that glial manipulation can affect adult behaviors, including sleep and circadian rhythms of locomotion, and the cellular correlates of sleep/wake and circadian oscillations are evident in glial populations. Astrocytes have thus far been the most strongly implicated glial population, with many major functions of this class being relevant to sleep, and they are the sole glial class recognized as affecting circadian oscillators and outputs in clock neurons. Nevertheless, the investigation of any one of these mechanisms has been limited to only a handful of studies, demonstrating that our understanding of the relationship of circadian rhythms and sleep to astrocytes, and especially to other glial classes, is only beginning.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

Work in the laboratory is supported in part by National Institutes of Health (NIH) grant R37NS048471. G.A. was supported by NIH grant T32 HL 7953-17.

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