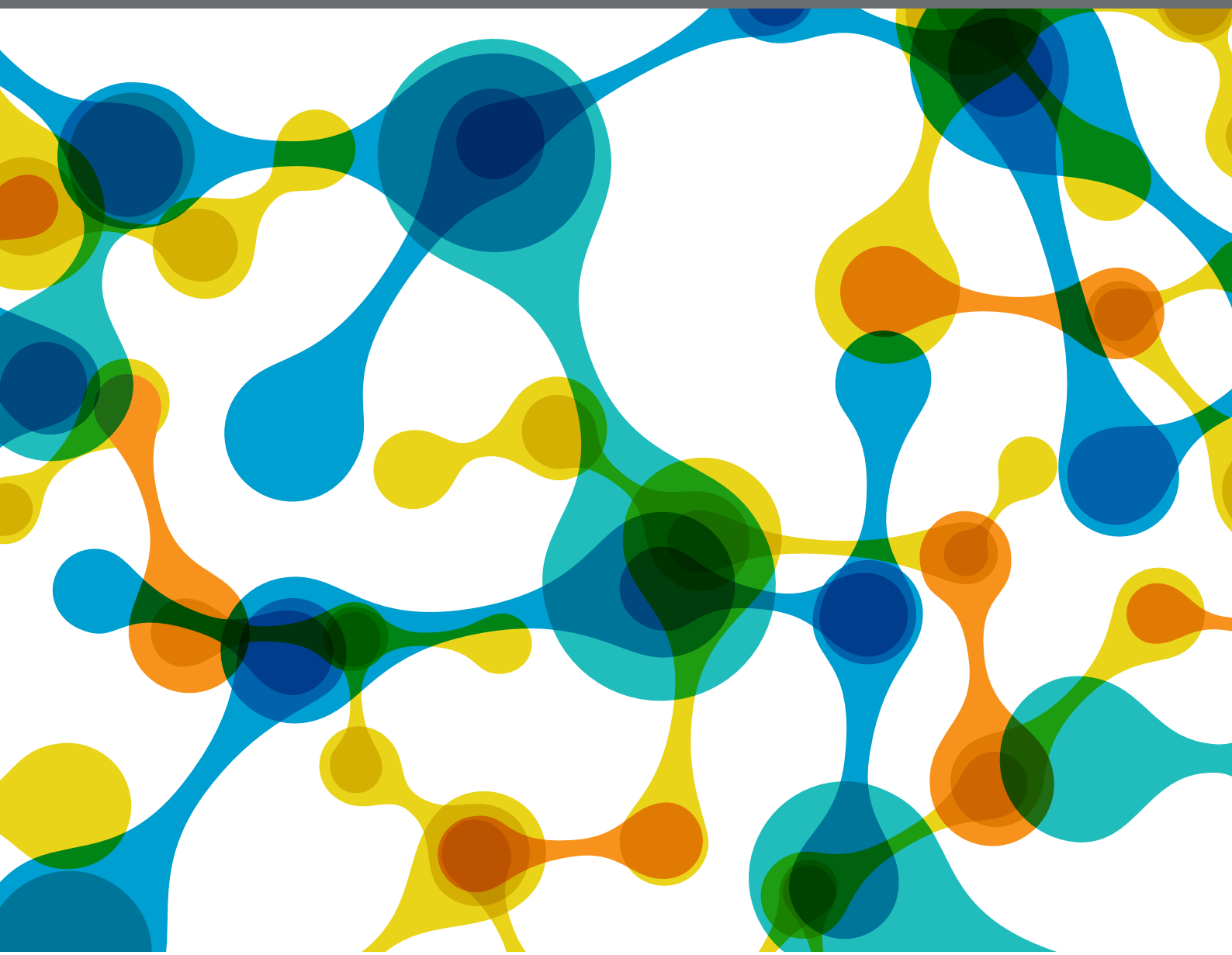
CIRCADIAN PLASTICITY—A COLLABORATION BETWEEN NEURONAL AND GLIAL OSCILLATORS

EDITED BY: Jolanta Górska-Andrzejak and Elżbieta Pyza
PUBLISHED IN: Frontiers in Physiology



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ISSN 1664-8714
ISBN 978-2-88963-141-4
DOI 10.3389/978-2-88963-141-4

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CIRCADIAN PLASTICITY—A COLLABORATION BETWEEN NEURONAL AND GLIAL OSCILLATORS

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Citation: Górska-Andrzejak, J., Pyza, E., eds. (2019). Circadian Plasticity—A Collaboration Between Neuronal and Glial Oscillators. Lausanne: Frontiers Media. doi: 10.3389/978-2-88963-141-4

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frontiers in Physiology

EDITORIAL
published: 30 July 2019
doi: 10.3389/fphys.2019.00951

Editorial: Circadian Plasticity—A Collaboration Between Neuronal and Glial Oscillators

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Keywords: circadian rhythms, biological clock, clock genes, oscillators, glial cells, astrocytes, plasticity, neuron-glia interactions

Editorial on the Research Topic

Circadian Plasticity—A Collaboration Between Neuronal and Glial Oscillators

The amounts of glia in the brains of *Drosophila* (25%), rodents (65%), and humans (90%) suggest that increasing complexity of the brain is accompanied by growing numbers of glial cells (Losada-Perez, 2018). In view of this observation it comes as no surprise that glial cells that were once perceived merely as the supportive constituent for neurons are currently regarded as neuronal partners and important contributors to neuronal circuits formation and functioning.

This Research Topic focuses on the team work of neurons and glial cells in the oscillatory systems. It mostly focuses on glial contribution to the circadian rhythms that adapt organisms to the solar cycle, but it also explores the role of glia in the brain oscillator that drives discharge of the electric organ of the weakly electric fish, *Apteronotus leptorhynchus* (Zupanc). The latter has recently emerged as an excellent model of the oscillatory network (Sîrbulescu et al., 2014; Zupanc et al., 2014). This collection of articles gives new information on the still elusive role played by glial cells in such networks and reviews our current understanding of glial functioning in oscillatory systems.

A review paper by Chi-Castañeda and Ortega recapitulates the current information on the role of glial cells in the circadian regulation of synaptic plasticity, exploiting its genetic, molecular, and physiological aspects. The authors provide an overview of literature that not only links circadian pacemakers with glial function but also gives the clinical implication of circadian clock and glial dysfunctions in diverse brain pathologies.

Another in-depth review, given by Lindberg et al. links the purinergic signaling to both circadian clock function and alcohol use disorder (AUD), as the normal diurnal oscillation of the key neurotransmitters like glutamate, GABA, dopamine and ATP adenosine, which underlie the circadian homeostasis, can be disrupted by excessive alcohol consumption. Considering the regulation of purinergic signaling and circadian oscillations by both neurons and astrocytes, as well as their interactions, they review the diverse mechanisms by which purinergic malfunction may contribute to circadian disruption or alcohol abuse.

Studies by Duhart et al. show effects of human glioma cells (the primary brain tumor with the highest incidence and mortality) in the hypothalamic region on the circadian behavioral output in mice. Their report might be of relevance for glioma diagnosis as it provides the foundation work for future research aimed to understand the pathological consequences of astrocytic dysfunction in the circadian time-keeping, which have recently come to light as a major player in SCN (Brancaccio et al., 2017; Tso et al., 2017).

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: *12 June 2019*
Accepted: *09 July 2019*
Published: *30 July 2019*

Citation:
Górska-Andrzejak J and Pyza E (2019) Editorial: Circadian Plasticity—A Collaboration Between Neuronal and Glial Oscillators. Front. Physiol. 10:951. doi: 10.3389/fphys.2019.00951

The research by Guissoni Campos et al., on the other hand, contributes to current knowledge of rhythmic characteristics of the cerebellar cortex (the major center of motor activity), including the neuron-glia interactions that may impact the processing in the cerebellum. The authors show possible relation between neurons expressing the clock protein PERIOD (which is a hallmark of a functioning molecular oscillator; PER) and Bergmann glia, which are demonstrated to express melatonin receptors.

Functioning of both neuronal and glial oscillators is the foundation of the circadian plasticity of the visual system of *Drosophila melanogaster* (Górska-Andrzejak, 2013). The studies on *Drosophila* included in this Research Topic reveal the heterogeneity of glial oscillators in the *Drosophila* optic lobe. This line of research has opened new perspectives in studying the glial circadian clock with regard to different subtypes of glial cells (Górska-Andrzejak et al.; Krzeptowski et al.). Górska-Andrzejak et al. bring attention to the glial cells located in the output of the circadian pacemaker in the neuropil of optic medulla and infer the possibility of interactions between Pigment Dispersing Factor (PDF) releasing clock neurons and the medulla glia expressing PDF receptors. Further characterization of the distal medulla glia by Krzeptowski et al. reveals two populations of cells that differ with respect to expression level of PER and the glial marker REPO. Interestingly, the authors have observed that the elevated levels of PER are characteristic for the ensheathing glia, but not the astrocyte-like ones (no staining of PER in the astrocyte-like glia was also reported by Long and Giebultowicz), even though the latter are well-known to influence the circadian rhythms of *Drosophila* locomotor activity (Suh and Jackson, 2007; Ng et al., 2011). It has to be taken into account, therefore, that the glial cells with low level of PER may also play a role in *Drosophila* circadian network (Krzeptowski et al.). Long and Giebultowicz additionally demonstrate that the rhythmic expression of PER dampens with advanced age in most of the glial subtypes. Thus, their study bring into focus the glia-related circadian changes that may significantly contribute to the loss of homeostasis in the aging brain.

Another study on *Drosophila* (Herrero et al.) emphasizes the important role of neuron-glial interactions in the structural plasticity of the circadian network. The article by Ceriani group (Herrero et al.) reveals the involvement of glial cells in the structural remodeling of neuronal pacemakers (Fernández et al., 2008), which changes the degree of the circadian network connectivity during the day. In this research, they have found that the molecular clocks in both clock neurons and glia are required for sustaining the circadian structural plasticity of the clock neuron projections. Yet another model of the pacemaker nucleus in *A. leptorhynchus* also reveals glial involvement in the plasticity of neuronal oscillators (Zupanc). It explains how the dynamic morphological changes of dense astrocytic meshwork might modulate the output activity of the neuronal oscillators to produce dimorphic behavior of females and males.

Eventually, the last paper on *Drosophila* (Cusumano et al.) corroborates the hypothesis that the circadian clock may also adopt post-transcriptional mechanisms regulating transposable elements (TEs) in order to ensure proper rhythmicity. Its authors argue that BELLE, a conserved DEAD-box RNA helicase that acts as an important piRNA-mediated regulator of TEs, is a putative clock component present in both the clock neurons and the glial cells of *Drosophila* brain (Cusumano et al.) and influences circadian rhythmicity of *Drosophila* locomotor activity.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was funded by grants from Institute of Zoology and Biomedical Research (N18/DBS/000015) and National Science Centre in Poland (14/15/B/NZ3/04754).

REFERENCES

Brancaccio, M., Patton, A. P., Chesham, J. E., Maywood, E. S., and Hastings, M. H. (2017). Astrocytes control circadian timekeeping in the suprachiasmatic nucleus via glutamatergic article astrocytes control circadian timekeeping in the suprachiasmatic nucleus via glutamatergic signaling. *Neuron* 93, 1420–1435.e5. doi: 10.1016/j.neuron.2017.02.030

Fernández, M. P., Jimena, B., and Ceriani, M. F. (2008). Circadian remodeling of neuronal circuits involved in rhythmic behavior. *PLoS Biol.* 6:e69. doi: 10.1371/journal.pbio.0060069

Górska-Andrzejak, J. (2013). Glia-related circadian plasticity in the visual system of diptera. *Front. Physiol.* 4:36. doi: 10.3389/fphys.2013.00036

Losada-Perez, M. (2018). Glia: from "just glue" to essential players in complex nervous systems: a comparative view from flies to mammals. *J. Neurogenet.* 32, 78–91. doi: 10.1080/01677063.2018.1464568

Ng, F. S., Tangredi, M. M., and Jackson, F. R. (2011). Glial cells physiologically modulate clock neurons and circadian behavior in a calcium-dependent manner. *Curr. Biol.* 21, 625–634. doi: 10.1016/j.cub.2011.03.027

Sîrbulescu, R. F., Ilieş, I., and Zupanc, G. K. (2014). Quantitative analysis reveals dominance of gliogenesis over neurogenesis in an adult brainstem oscillator. *Dev. Neurobiol.* 74, 934–952. doi: 10.1002/dneu.22176

Suh, J., and Jackson, F. R. (2007). Drosophila ebony activity is required in glia for the circadian regulation of locomotor activity. *Neuron* 55, 435–447. doi: 10.1016/j.neuron.2007.06.038

Tso, C. F., Simon, T., Greenlaw, A. C., Puri, T., Mieda, M., and Herzog, E. D. (2017). Astrocytes regulate daily rhythms in the suprachiasmatic nucleus and behavior. *Curr. Biol.* 27, 1055–1061. doi: 10.1016/j.cub.2017.02.037

Zupanc, G. K. H., Ilieş, I., Sîrbulescu, R. F., and Zupanc, M. M. (2014). Large-scale identification of proteins involved in the development of a sexually dimorphic behavior. *J. Neurophysiol.* 111, 1646–1654. doi: 10.1152/jn.00750.2013

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 12 February 2018
doi: 10.3389/fphys.2018.00088

Glial Cells in the Genesis and Regulation of Circadian Rhythms

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Circadian rhythms are biological oscillations with a period of ∼24 h. These rhythms are orchestrated by a circadian timekeeper in the suprachiasmatic nucleus of the hypothalamus, the circadian "*master clock,*" which exactly adjusts clock outputs to solar time via photic synchronization. At the molecular level, circadian rhythms are generated by the interaction of positive and negative feedback loops of transcriptional and translational processes of the so-called "*clock genes.*" A large number of clock genes encode numerous proteins that regulate their own transcription and that of other genes, collectively known as "*clock-controlled genes.*" In addition to the sleep/wake cycle, many cellular processes are regulated by circadian rhythms, including synaptic plasticity in which an exquisite interplay between neurons and glial cells takes place. In particular, there is compelling evidence suggesting that glial cells participate in and regulate synaptic plasticity in a circadian fashion, possibly representing the missing cellular and physiological link between circadian rhythms with learning and cognition processes. Here we review recent studies in support of this hypothesis, focusing on the interplay between glial cells, synaptic plasticity, and circadian rhythmogenesis.

Keywords: circadian rhythms, clock genes, glial oscillators, learning, memory, plasticity

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: *29 September 2017*
Accepted: *26 January 2018*
Published: *12 February 2018*

Citation:
Chi-Castañeda D and Ortega A (2018) Glial Cells in the Genesis and Regulation of Circadian Rhythms. Front. Physiol. 9:88. doi: 10.3389/fphys.2018.00088

INTRODUCTION

Most light-sensitive organisms have an internal timekeeping mechanism to anticipate daily changes associated with the transition of day to night that is commonly known as "circadian clock". In 1959, Halberg denominated "circadian rhythms" the biological rhythms that have a period of ∼24 h (Halberg, 1959). These rhythms regulate a large number of physiological and behavioral functions in vertebrates, such as hormone secretion, body temperature, metabolism, and even memory processes. The sleep-wake cycle is one the most studied rhythms (Schibler and Sassone-Corsi, 2002; Stratmann and Schibler, 2006; Walker and Stickgold, 2006).

Sleep is a highly conserved process (Hartse, 2011), and several hypotheses support the notion that sleep supersedes learning and memory, possibly through the control of synaptic plasticity (Benington and Frank, 2003; Frank and Benington, 2006; Frank, 2011; Fellin et al., 2012; Frank and Cantera, 2014; De Pittà et al., 2016). Synaptic plasticity refers to the biochemical processes by which synaptic strength changes in an activity-dependent fashion. These cellular cascades are a combination of post-translational modifications that change neural activity, and also result in the reshaping of synaptic terminals (Lohmann and Kessels, 2014). Depending on its temporal course, synaptic plasticity is distinguished into three classes: (1) short-term plasticity, that occurs in the milliseconds to minutes range, and includes the modulation of neurotransmitter release, and depends

on post-translational modifications via phosphorylation, ubiquitination, and several other molecular processes (Bliss and Collingridge, 1993; Martin et al., 2000); (2) long-term plasticity, such as long-term potentiation (LTP) and depression (LTD), which may last from hours to months and is represented by cellular changes involving modification of the cellular protein repertoire that may require changes in transcriptional activity and are strictly dependent on protein synthesis (Martin et al., 2000); (3) homeostatic plasticity, which is the result of a variety of molecular and cellular events that shape neuronal circuits, continuously occurs in parallel with other plasticity phenomena and is thought to prevent runaway of neural activity by excessive excitation (Turrigiano, 2011).

Various studies have shown that disruptions of circadian rhythms alter synaptic plasticity and thus, learning and memory, including spatial and place learning and trace fear memory (Winocur and Hasher, 2004; Van der Zee et al., 2008; Wang et al., 2009; Jilg et al., 2010; Kondratova et al., 2010). Based on these studies, it has been suggested that a functional circadian clock is required for optimal learning and memory formation and consolidation (Becker-Weimann et al., 2004; Eckel-Mahan and Storm, 2009). The neural correlates and the mechanisms underpinning these clocks remain largely unknown. In the past two decades, the notion that brain function exclusively relies on neuronal signaling has been challenged by evidence that glial cells work in coordination with neurons, to regulate neurotransmission (Araque et al., 1999). These regulatory events occur through a set of molecular mechanisms that control neurotransmitter recycling (Danbolt et al., 2016), energy requirements (Newman et al., 2011; Suzuki et al., 2011), and eventually sleep homeostasis (Halassa et al., 2009b). This often involves, but is not limited to, the secretion of neuroactive molecules (or "gliotransmitters") in an activity-dependent manner which target synaptic terminals modulating synaptic transmission (Bergles et al., 1997; Haydon, 2001; Lin and Bergles, 2004; Fellin et al., 2006; Perea et al., 2009).

Interestingly, astrocytes which are the most common glial cells in the cortex, have also been implicated in the circadian control of synaptic plasticity (Lavialle and Servière, 1993; Du et al., 2005; Lavialle et al., 2011; Hayashi et al., 2013a,b), suggesting a possible non-neuronal, glial candidate for the regulation of circadian rhythms that control learning and memory processes. This review focuses on this hypothesis, further elaborating on the possible clinical implications associated with disruptions of glial-mediated pathways on circadian rhythms related to high brain function.

CIRCADIAN CLOCKS AND THEIR MOLECULAR/GENETIC BASES

Mammalian circadian clocks are hierarchically organized by a "*master clock*" in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. This clock coordinates independent peripheral clocks (Reppert and Weaver, 2002; Lowrey and Takahashi, 2004). At the molecular level, all of these clocks are the result of a translation-based, interconnected feedback loops in which the transcription factors Brain and Muscle ARNT-Like Protein 1 (BMAL1) and Circadian Locomotor Output Cycles Kaput (CLOCK) form heterodimers that regulate the circadian expression of *Cryptochrome* (*Cry*) and *Period* (*Per;* Dunlap, 1999; Reppert and Weaver, 2001), whose products lead to the inhibition of their own transcription. Additionally, an accessory regulatory loop involves the regulation of *Bmal1* transcription by the coordinated action of the orphan nuclear receptors *Reverse erb* α (*Rev-erbα*, repressor) and *Retinoid-related orphan receptor-*α (*Rorα*, activator) through the binding to the evolutionarily conserved nucleotide sequence [A/T]A[A/T]NT[A/G]GGTCA present in the promoter region of *Bmal1* (Dunlap, 1999; Harmer et al., 2001; Reppert and Weaver, 2001; Preitner et al., 2002).

Significantly, a large number of circadian transcription factors not only regulate their own transcription, but also the expression of numerous other "*clock-controlled genes*" (CCGs) (Dunlap, 1999; Reppert and Weaver, 2001) whose protein products are not essential for the core clock mechanism itself. Among the genes that are part of the CCGs, various enzymes are included, like *phosphoenolpyruvate carboxykinase*, *glycogen phosphorylase*, and *glucose-6-phosphatase* (Panda et al., 2002); ion channels, like cGMP-gated cation channels, various voltage-gated calcium and potassium channels, the Na^+/K^+*-ATPase*, and a long-opening cation channel (Ko et al., 2009); peptides, such as *arginine-vasopressin* (*Avp*; Jin et al., 1999) and *D element-binding protein* (*DBP*; Le Martelot et al., 2009). In fact, cells rhythmically synthesize about 10% of their transcripts, including those involved in neuronal signaling and synaptic plasticity (Panda et al., 2002; Lowrey and Takahashi, 2004).

CIRCADIAN CLOCKS, SLEEP, AND THEIR INVOLVEMENT IN SYNAPTIC PLASTICITY

In recent years, numerous reports of *in vitro* and *in vivo* studies, have suggested an essential functional role of sleep in synaptic plasticity (Frank, 2011; Fellin et al., 2012). Accordingly, sleep has been proposed to strengthen, stabilize, or weaken synapses (Benington and Frank, 2003; Frank and Benington, 2006; Frank and Cantera, 2014). The molecular basis of these synaptic changes and whether sleep is necessary for their occurrence remain largely unknown. While sleep is the result of a combination of circadian rhythms and homeostatic mechanisms (Frank and Cantera, 2014), a clear causal connection between circadian clocks, sleep homeostasis, and synaptic plasticity has not been demonstrated.

In this context, it is noteworthy that the recycling of glutamate (Glu) is regulated by clock components, strongly suggesting a functional interplay between circadian rhythms and excitatory synaptic transmission (Beaulé et al., 2009). In fact, glial glutamate transporters are regulated by clock genes having a significant impact in the dynamic, activity-dependent metabolic coupling of glial cells with glutamatergic neurons. This glia/neuron interplay is mediated by the glutamate/glutamine cycle and the astrocyte/neuron/lactate shuttle (Martínez-Lozada and Ortega, 2015). In the same vein, taking into consideration the major role of Glu as the most abundant excitatory transmitter and its

role in the molecular models of synaptic plasticity, like LTP and LTD, it is tempting to speculate that another molecular loop between clock genes' expression and glia/neuron coupling via the glutamatergic tripartite synapses control synaptic plasticity at the immediate, mediate and long-term ranges (Flores-Méndez et al., 2016).

GLIAL REGULATION OF SYNAPTIC PLASTICITY

Astrocytes

Beyond their recognized role in synapse development and neurodegeneration, astrocytes provide a delicate ensheathment of synapses in the mature brain (Chao et al., 2002; Theodosis et al., 2008). It is well-established that the degree of astrocytic ensheathing greatly changes with the brain area, hinting local specialization. In the hippocampus for example, a single astrocyte is in close proximity to few hundreds dendrites of different neurons, but can ensheathe up to several hundred thousands synapses (Bushong et al., 2002; Halassa et al., 2007; Agulhon et al., 2008). Such morphological arrangement provides the structural substrate for tight functional interactions between astrocytes and neurons (Saab et al., 2012; Bernardinelli et al., 2014).

Astrocytes are also recognized for their role in clearance of neurotransmitters, such as Glu and gamma-aminobutyric acid (GABA), from the synaptic cleft. Perisynaptic astrocytes processes are indeed enriched in transporters that guarantee rapid and efficient removal of the released neurotransmitters (Anderson and Swanson, 2000; Conti et al., 2004). Interestingly, the regulation of the kinetics and the extent of neurotransmitter clearance by astrocytes have been related to synaptic plasticity insofar as they both affect the degree of postsynaptic activation and desensitization (Tzingounis and Wadiche, 2007). Moreover, through the release of a variety of neuroactive molecules, such as Glu, D-serine, adenosine triphosphate (ATP), adenosine, GABA, tumor necrosis factor-α (TNFα), prostaglandins, proteins and peptides, astrocytes are capable of regulating synaptic transmission and plasticity (Halassa and Haydon, 2010; Araque et al., 2014). These neuroactive molecules activate extrasynaptic metabotropic and ionotropic receptors, modifying neurotransmitter release and regulating short-term plasticity and synaptic efficacy (Parpura et al., 1994; Araque et al., 1998a,b, 2014; Halassa et al., 2009a; Halassa and Haydon, 2010).

Increasing evidence indicates that astrocytes could be involved in the synchronization of cortical firing. Cortical circuits for sensory integration are known to display transient synchrony of neuronal ensembles (Harris et al., 2003; Haider and McCormick, 2009). The hallmark of this synchronized activity is the alternation of UP states—i.e., episodes of persistent neuronal firing lasting few milliseconds—and DOWN states—i.e., episodes of neuronal hyperpolarization (Steriade et al., 2001; Brecht and Sakmann, 2002; Cossart et al., 2003; Kenet et al., 2003). UP and DOWN states are common in a wide range of conditions, including quiescent wakefulness (Gentet et al., 2010), anesthesia (Steriade et al., 1993; Ramaswamy and Muller, 2015), and sleep itself (Massimini and Amzica, 2001). Moreover, astrocytes have been implicated in UP state genesis through the release of D-serine, adenosine and ATP (Fellin et al., 2009, 2012; Halassa et al., 2009b; Poskanzer and Yuste, 2011, 2016). Interestingly, gliotransmission has been proposed to operate on different time scales (Fellin et al., 2012). According to the Hill and Tononi's model of sleep and in agreement with the modulation cortical UP and DOWN states, Fellin and colleagues have demonstrated that the depolarizing effect of NMDA receptors currents preserves the UP state (Hill and Tononi, 2004; Fellin et al., 2012). Such a role for NMDA receptors is thought to be dependent on the availability of glia-released D-serine (Fellin et al., 2012), again demonstrating a prominent role of glia/neuron coupling.

Oligodendrocytes

Oligodendrocytes projections wrap neuronal axons forming the myelin sheaths in the central nervous system (CNS). These myelin sheaths insulate the fibers, and help them to carry the nerve impulses. Interestingly, myelin can influence conduction velocity of the electrical impulse by regulating the axon diameter, thickness of the myelin sheath, the number and spacing of nodes of Ranvier, and nodal structure and molecular composition of ion channels in the node and paranodal region (Berthold et al., 1983; Wurtz and Ellisman, 1986; Baker and Stryker, 1990; Carr and Konishi, 1990; Dupree et al., 2004). Taking this into a consideration, it has been shown that myelin specific proteins, including Nogo-A (Chen et al., 2000; GrandPré et al., 2000), myelin-associated glycoprotein (MAG; McKerracher et al., 1994) and oligodendrocyte-myelin glycoprotein (OMgp; Wang et al., 2002; Huang et al., 2005), inhibit directly axon sprouting and synaptogenesis and constrain nervous system plasticity. This finding indicates the participation of myelin in learning, memory, and cognition.

Microglia

These glial cells are part of the brain's immune system and are mainly involved in the phagocytosis of foreign matter and cellular wastes of the CNS (Aloisi, 2001). Moreover, during postnatal development and adaptation to novel environments, microglia has a critical role in synaptic remodeling through the elimination of synapses and axon terminals. Additionally, increasing evidence points out that microglia could regulate synaptic plasticity and neurotransmission through the release of gliotransmitters (Batchelor et al., 1999, 2002; Zhong et al., 2010; Harry and Kraft, 2012; Sierra et al., 2013), as well as an increase hippocampal LTP and NMDA receptor-mediated responses via the secretion of glycine (Thomson et al., 1989; Abe et al., 1990; Hayashi et al., 2006). During neuroinflammation, microglia is capable to regulate excitatory neurotransmission by the rapid production of small amounts of ATP, that in turn, recruit astrocytes to augment ATP formation and Glu exocytosis enhancing synaptic transmission via metabotropic Glu receptors (Pascual et al., 2012). In fact, several reports reveal that some of the established astrocytic functions are regulated by the upstream activation of microglia (Ben Achour and Pascual, 2010; Pascual et al., 2012).

CIRCADIAN MODULATION OF THE SYNAPTIC PLASTICITY IN GLIAL CELLS

Since 1978, it has been demonstrated that diverse cognitive processes are regulated by the circadian clocks in a phase-specific manner (Monk and Folkard, 1978). Particularly, in long-term memories generated in diverse learning paradigms, a role for the endogenous circadian clock has been reported both in vertebrates and invertebrates (Rudy and Pugh, 1998; Valentinuzzi et al., 2004; Rawashdeh et al., 2007). However, the circadian modulation of short-term memory formation has been almost impossible to prove. In this section, we summarize the evidence that involves different glial cells in processes of synaptic plasticity regulated by circadian generators.

Astrocytes

Gliotransmission is the process by which astrocytes interact with nearby neurons via the release of transmitters, like ATP and Glu (Haydon, 2001; Perea et al., 2009; Parpura and Zorec, 2010). Remarkably, ATP has been linked to modulation of LTP but also of synaptic depression (Gordon et al., 2005; Pascual et al., 2005; Bains and Oliet, 2007). *In vivo*, an astrocyte-dependent rhythmic ATP release is present in the SCN. Although the mechanisms responsible for these ATP oscillations are unknown, calcium-dependent signaling seems to be involved (Womac et al., 2009). Subsequently, it was shown that astrocytes display daily extracellular ATP oscillations that depend on the clock genes (*Clock*, *Per,* and *Bmal1*) and in inositol triphosphate (IP_3) signaling, indicating that extracellular ATP levels increase at a specific time of day and suggest a clock-induced increase in energy metabolism and glial activity, which participate in sleep-wake changes in the brain and in control synaptic transmission (Marpegan et al., 2011).

To date, there is no report demonstrating that the circadian clock regulates Glu release. In contrast, accumulating evidence indicates that the glutamate/aspartate transporter (*Glast*) gene expression and protein levels exhibit a diurnal rhythm in a light/dark 12/12 h cycle (Spanagel et al., 2005). These findings are consistent with the absence of rhythmicity of GLAST in the *Per2* mutant mice, pointing out the presence of a circadian control (Spanagel et al., 2005). Later on, using cultured cortical astrocytes from *Clock* mutant animals, it was observed a marked decrease in *Glast* mRNA and protein levels, proposing that glial Glu uptake activity is a function of the clock genes: *Clock*, *Npas2,* and *Per2* (Beaulé et al., 2009). Specifically, the dependence related to CLOCK and NPAS2 may be due to their involvement in *Glast* transcription, or in GLAST stability and/or localization (Danbolt, 2001). It is important to mention that no conclusive evidence has been shown for a circadian-dependent change in Glu uptake, suggesting a non-circadian role for clock proteins in *Glast* transcription or *Glast* mRNA translation and/or stability (Beaulé et al., 2009). However, it is clear that the regulation and precise function of this transporter is very important to guarantee an efficient glutamatergic neurotransmission. A failure in synaptic Glu clearance is neurotoxic due to a hyperactivation of postsynaptic Glu receptors resulting in the phenomena known as excitotoxicity, which is implicated in most of neurodegenerative diseases (McEntee and Crook, 1993; Domingues et al., 2010; Gegelashvili and Bjerrum, 2014).

In the adult brain, the distribution of the specific astrocyte marker, glial fibrillary acidic protein (GFAP), has been reported to peak during daily rhythms in the SCN (Lavialle and Servière, 1993). Furthermore, it has been demonstrated that this peak also prevails in constant darkness (Lavialle and Servière, 1993; Moriya et al., 2000), strongly suggesting that these rhythms are essential and independent of environmental light. Although the function of circadian fluctuations of GFAP immunoreactivity is unknown, it has been observed that mice lacking the *gfap* gene show reduced eyeblink training and impaired LTD in the cerebellum (Shibuki et al., 1996), suggesting that this protein plays a role in the regulation of neuronal functions.

CNS excitatory synapses are extremely dynamic structures that show stabilization in response to learning and memory process. These synapses are surrounded by intricate astrocytic processes denominated perisynaptic astrocytic processes (PAPs; Iino et al., 2001; Hirrlinger et al., 2004; Nishida and Okabe, 2007). It has been described in primary cultured astrocytes that ezrin (an actin-binding protein) is required for filopodia formation and motility of PAPs, such motility can be induced by Glu via activation of metabotropic Glu receptors 3 and 5 (Lavialle et al., 2011). Moreover, changes in glutamatergic circadian activity in the hamster SCN are in synchrony with changes in ezrin immunoreactivity which is consistent with Glu-induced perisynaptic glial motility (Lavialle et al., 2011). These results suggest that ezrin is essential for Glu-induced PAPs plasticity that could be regulated by circadian system.

On the other hand, the brain fatty-acid binding protein (FABP7) localizes in astrocytes and neuronal cell precursors in the mature brain, and presents a high binding affinity to long chain fatty acids whose effects on brain function include development, emotion, learning, and memory (Yamamoto et al., 1987; Jensen et al., 1996; Wainwright et al., 1997; Moriguchi et al., 2000; Takeuchi et al., 2003). Gerstner and colleagues demonstrated in adult murine brain that levels of *Fabp7* mRNA oscillate over a 24 h period in brain areas that participate in daily activity and sleep like the hypothalamus, the tuberomammilary nucleus, the pons and the *locus coeruleus*. In these areas, *Fabp7* diminish in the dark phase and increase instead in the light phase (Gerstner et al., 2006, 2008). In contrast with its mRNA levels, FABP7 levels are higher during the dark phase (Gerstner et al., 2008), indicating that expression of this protein is delayed by 12 h with regards to its mRNA. More recently, the same group demonstrated that FABP7 is specifically augmented in the perisynaptic compartment of fine astrocytic processes that surround synapses. Furthermore, CPEB-mediated cytoplasmic polyadenylation controls the diurnally regulated *Fabp7* mRNA levels (Gerstner et al., 2012). Accordingly, in plasticity terms, targeting of *Fabp7* and CPEB-mediated polyadenylation could participate in controlling astrocytic process extension, although it is unknown if variations in synaptic plasticity and/or neuronal activity modify polyadenylation and trafficking of *Fabp7* mRNA resulting in morphological modifications of the astrocytic processes (Gerstner et al., 2012). In addition, variations in cycle-dependent memory formation and synaptic plasticity could

regulate the circadian variations in subcellular trafficking and localization of *Fabp7* mRNA in hippocampal tripartite synapses (Gerstner et al., 2009, 2012).

Oligodendrocytes

To date, there is no evidence that oligodendrocytes have an internal circadian clock; however, it has been reported that oligodendrocytes precursor cells (OPCs) proliferation in the hippocampus could be regulated by clock genes (Matsumoto et al., 2011). It should be noted that OPCs proliferation itself could modulate the synaptic plasticity of the hippocampus in response to neuronal activity, thus circadian proliferation of these cells could regulate hippocampal function. Particularly, the OPCs give rise to mature oligodendrocytes, and are thought to be a constitutive reservoir of oligodendrocytes that replace damaged myelin (Levine et al., 1993) or add *de novo* myelination (McCarthy and Leblond, 1988). Interestingly, *myelin proteolipid protein* (*plp*), a myelin-specific gene, is regulated by *Clock* (Du et al., 2005), suggesting that the circadian clock controls myelin formation.

Microglia

Microglial cells constantly retract and extend their processes to sense their local environment contributing to the maintenance of healthy neuronal circuits (Kirchhoff, 2013). There is evidence that an intrinsic molecular clock exists in cortical microglia which controls diurnal morphological changes of its processes, and whereby these cells regulate the sleep-wake cycle-dependent changes in synaptic strength (Hayashi et al., 2013a,b). In line with these findings, it has been reported that the microglia-specific lysosomal cysteine protease Cathepsin S (CatS) exhibits a circadian expression in cortical microglia. The expression of CatS is involved in diurnal variations of synaptic strength in cortical neurons via the proteolytic modification of the perineuronal environment. However, disruptions in CatS lead to hyperlocomotor activity and to the deletion of the diurnal variations in spine density and synaptic activity of these cortical neurons as a consequence of the failure to downscale synaptic strength during sleep (Hayashi et al., 2013a). Since downscaling of synaptic strength is required for the acquisition and consolidation of novel information upon awakening, it is evident that dysfunction of the microglial intrinsic circadian clock is involved in neuropsychiatric disorders based on sleep disturbance, including depression and cognitive impairment (Bhattacharjee, 2007; Hayashi et al., 2014).

On the other hand, microglial cells express ATP receptors of the P2X (P2XR, ligand-gated ion-channel receptor) and P2Y subtypes (P2YR, G protein-coupled receptor). ATP released by glial cells during neuronal activity is then, capable to influence synaptic transmission. In fact in microglial cells, ATP increases the number their branch points, extension of their processes and morphological complexity (Fontainhas et al., 2011). Specifically, it has been demonstrated that the degree of microglial process extension is controlled by microglial $P2Y_{12}$Rs (Haynes et al., 2006). Moreover, Hayashi and colleagues reported that microglial $P2Y_{12}$Rs present circadian oscillations regardless that microglia would be isolated under constant darkness conditions (Hayashi et al., 2013b). Interestingly, inhibition of these purinergic receptors disrupts the rhythmic patterns of synaptic strength or spine density, while upregulated $P2Y_{12}$Rs during the dark phase results in extension of the microglial processes that are partially retracted during the light phase resulting in a decrease of synaptic strength or spine density (Hayashi et al., 2013b). In the same fashion of CatS disruptions, dysfunctions in microglia-synapse interactions participate in neuropsychiatric disorders (Bhattacharjee, 2007; Hayashi et al., 2014).

Concerning P2X purinergic receptors, Nakazato and coworkers demonstrated that ATP selectively promotes the expression of the protein and mRNA of *Per1* through the activation of P2X7R in microglial cells (Nakazato et al., 2011). While the outcome of this upregulation is not completely clear, it has been reported that *Per* is not only crucial for long-term memory formation (LTM), but overexpression of this gene also enhances memory formation (Sakai et al., 2004). Taken together these evidences suggest that *Per* has an important function in the regulation of circadian synaptic plasticity in microglia.

Additionally, several reports indicate that ATP promotes microglial cells to secrete several signaling molecules, like interleukin-1 beta (IL-1β), TNFα, and plasminogen (Inoue et al., 1998; Hide et al., 2000; Sanz and Di Virgilio, 2000; Suzuki et al., 2004); which are involved in the modulation of synaptic transmission and plasticity (Ikegaya et al., 2003; Becker et al., 2013; Liu et al., 2014). Finally, it should be noted that microglia display rhythmic fluctuations in the gene expression of these mediators (Fonken et al., 2015).

CLINICAL IMPLICATIONS

Although synaptic dysfunction is the cellular basis of most mental illnesses, disturbance of the circadian clock system and dysfunctions of glial cells are likely to be involved in diverse brain pathologies. Up to now, only few studies, summarized in **Table 1**, provide a link between brain disorders, circadian rhythm dysfunction and glial physiology. For example, a study using $Per2^{Brdm1}$ mutant mice demonstrated that a nonfunctional *Per2* results in a hyperglutamatergic state due to a reduced GLAST expression and as a consequence, Glu uptake by astrocyte is diminished (Spanagel et al., 2005). Accordingly, one could expect that a reduction of astrocytic Glu uptake would be related to severe pathophysiological implications as shown in several disease models, including multiple sclerosis, Alzheimer's, and Huntington diseases (Domingues et al., 2010). Additionally, several studies related to proteins that are involved in the regulation of the astrocytic processes extension that surround synapses, like ezrin and FABP7, have shown that dysfunctions in these proteins lead to impairment in processes, like development, learning, memory, and emotion (Lavialle et al., 2011; Gerstner et al., 2012).

The fact that microglia exhibits circadian rhythmicity, such as oscillating expression patterns of clock genes that regulate the expression of $P2Y_{12}$R and of the CatS protease suggests that alteration of these two factors disrupts the rhythmic patterns of synaptic strength and spine density (Hayashi et al., 2013a,b).

TABLE 1 | Clock-controlled genes (CCG) and their implications in brain pathologies.

CCG	Preparation	Pathological implications	References
ASTROCYTES			
ATP	Cortical astrocyte cultures	Disruptions in sleep-wake changes in the brain and in control synaptic transmission.	Marpegan et al., 2011
GLAST	*Per2* mutant mice	Dysregulation in the Glu uptake process.	Spanagel et al., 2005
GFAP	GFAP mutant mice	Impaired LTD in the cerebellum, as well as reduced eyeblink conditioning.	Shibuki et al., 1996
Ezrin	Primary astrocytes cultures	Alterations in the Glu-induced PAPs plasticity.	Lavialle et al., 2011
FABP7	Primary mouse astrocyte culture	Dysregulation of astrocytic processes extension.	Gerstner et al., 2012
OLIGODENDROCYTES			
OPCs	Mouse hippocampus slices	Alterations in synaptic plasticity for the hippocampal function.	Matsumoto et al., 2011
MICROGLIA			
CatS	CatS$^{-/-}$ mice	Neurological disorders by disruption of the circadian oscillation patterns of synaptic strength and spine density in cortical neurons.	Hayashi et al., 2013a
P2Y$_{12}$R	Cortical microglia cultures	Neurological disorders by disruption of the rhythmic patterns of synaptic strength or spine density.	Hayashi et al., 2013b
P2X7R	Cultured murine microglia and BV-2 cells	Downregulates *Per1* mRNA expression.	Nakazato et al., 2011
		Reduces the number of processes in microglial cells as a result of cellular activation.	

ATP, Adenosine triphosphate; CatS, cathepsin S; FABP7, brain-type fatty acid binding protein; GFAP, glial fibrillary acidic protein; GLAST, Glu/aspartate transporter; LTD, long-term depression; OPCs, oligodendrocytes precursor cells; P2X7R, P2X7 receptor; P2Y$_{12}$R, P2Y$_{12}$ receptor; PAPs, perisynaptic astrocytic processes.

In this context, healthy brain synaptic homeostasis depends on microglia-synapse interactions controlled by the intrinsic microglial clock, so the dysfunction of this clock most probably leads to neuropsychiatric disorders, like depression and cognitive deficits (Bhattacharjee, 2007; Hayashi et al., 2014).

Finally, it has been demonstrated that sleep disturbances are involved with multiple negative effects on human physiology, including neuronal dysfunction (Joo et al., 2013), mood disturbances (Dinges et al., 1997), cognitive impairments (Lo et al., 2012), and disruption to circadian rhythmicity (Möller-Levet et al., 2013).

CONCLUSIONS

Glial cells have long been regarded as simple supportive cells of neuronal function. However, in recent years, several reports have demonstrated the involvement glial cells in diverse processes required for proper brain function, including contribution to the regulation of the synaptic plasticity. Taking into consideration that clock genes modify glial Glu transporters and, by these means, control the strength and continuity of the major excitatory system, their role in higher brain functions is likely of a high relevance. Accordingly, specific alterations of the circadian system are related to various diseases in which glutamatergic transmission is impaired. Additionally, dysfunction of astrocyte-neuron signaling plays a critical role in the pathology of most of the neurodegenerative diseases, such as Alzheimer, Parkinson, and Huntington. Altogether, these findings make it clear that glial cells are an important tool to understand the circadian regulation of synaptic plasticity, both in the short and in the long terms. Certainly, characterization of the activity-dependent and clock-dependent changes in glial proteins repertoire will provide a major input to our understanding of the pivotal role of glial cells in higher brain functions.

AUTHOR CONTRIBUTIONS

DC-C gathered the relevant information, wrote the manuscript, as well as elaborated the table. AO reviewed and edited the final version of the manuscript.

FUNDING

The work in the lab is supported by grants from Conacyt-México (255087) and "Soluciones para un México Verde S.A. de C.V." to AO; DC-C is supported by SNI-Conacyt.

REFERENCES

Abe, K., Xie, F. J., Watanabe, Y., and Saito, H. (1990). Glycine facilitates induction of long-term potentiation of evoked potential in rat hippocampus. *Neurosci. Lett.* 117, 87–92. doi: 10.1016/0304-3940(90)90124-R

Agulhon, C., Petravicz, J., McMullen, A. B., Sweger, E. J., Minton, S. K., Taves, S. R., et al. (2008). What is the role of astrocyte calcium in neurophysiology? *Neuron* 59, 932–946. doi: 10.1016/j.neuron.2008.09.004

Aloisi, F. (2001). Immune function of microglia. *Glia* 36, 165–179. doi: 10.1002/glia.1106

Anderson, C. M., and Swanson, R. A. (2000). Astrocyte glutamate transport: review of properties, regulation, and physiological functions. *Glia* 32, 1–14. doi: 10.1002/1098-1136(200010)32:1<1::AID-GLIA10>3.0.CO;2-W

Araque, A., Carmignoto, G., Haydon, P. G., Oliet, S. H., Robitaille, R., and Volterra, A. (2014). Gliotransmitters travel in time and space. *Neuron* 81, 728–739. doi: 10.1016/j.neuron.2014.02.007

Araque, A., Parpura, V., Sanzgiri, R. P., and Haydon, P. G. (1998a). Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. *Eur. J. Neurosci.* 10, 2129–2142.

Araque, A., Parpura, V., Sanzgiri, R. P., and Haydon, P. G. (1999). Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci.* 22, 208–215. doi: 10.1016/S0166-2236(98)01349-6

Araque, A., Sanzgiri, R. P., Parpura, V., and Haydon, P. G. (1998b). Calcium elevation in astrocytes causes an NMDA receptor-dependent increase in the frequency of miniature synaptic currents in cultured hippocampal neurons. *J. Neurosci.* 18, 6822–6829.

Bains, J. S., and Oliet, S. H. R. (2007). Glia: they make your memories stick! *Trends Neurosci.* 30, 417–424. doi: 10.1016/j.tins.2007.06.007

Baker, G. E., and Stryker, M. P. (1990). Retinofugal fibres change conduction velocity and diameter between the optic nerve and tract in ferrets. *Nature* 344, 342–345. doi: 10.1038/344342a0

Batchelor, P. E., Liberatore, G. T., Wong, J. Y., Porritt, M. J., Frerichs, F., Donnan, G. A., et al. (1999). Activated macrophages and microglia induce dopaminergic sprouting in the injured striatum and express brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor. *J. Neurosci.* 19, 1708–1716.

Batchelor, P. E., Porritt, M. J., Martinello, P., Parish, C. L., Liberatore, G. T., Donnan, G. A., et al. (2002). Macrophages and microglia produce local trophic gradients that stimulate axonal sprouting toward but not beyond the wound edge. *Mol. Cell. Neurosci.* 21, 436–453. doi: 10.1006/mcne.2002.1185

Beaulé, C., Swanstrom, A., Leone, M. J., and Herzog, E. D. (2009). Circadian modulation of gene expression, but not glutamate uptake, in mouse and rat cortical astrocytes. *PLoS ONE* 4:e7476. doi: 10.1371/journal.pone.0007476

Becker, D., Zahn, N., Deller, T., and Vlachos, A. (2013). Tumor necrosis factor alpha maintains denervation-induced homeostatic synaptic plasticity of mouse dentate granule cells. *Front. Cell. Neurosci.* 7:257. doi: 10.3389/fncel.2013.00257

Becker-Weimann, S., Wolf, J., Herzel, H., and Kramer, A. (2004). Modeling feedback loops of the mammalian circadian oscillator. *Biophys. J.* 87, 3023–3034. doi: 10.1529/biophysj.104.040824

Ben Achour, S., and Pascual, O. (2010). Glia: the many ways to modulate synaptic plasticity. *Neurochem. Int.* 57, 440–445. doi: 10.1016/j.neuint.2010.02.013

Benington, J. H., and Frank, M. G. (2003). Cellular and molecular connections between sleep and synaptic plasticity. *Prog. Neurobiol.* 69, 71–101. doi: 10.1016/S0301-0082(03)00018-2

Bergles, D. E., Dzubay, J. A., and Jahr, C. E. (1997). Glutamate transporter currents in Bergmann glial cells follow the time course of extrasynaptic glutamate. *Proc. Natl. Acad. Sci. U.S.A.* 94, 14821–14825. doi: 10.1073/pnas.94.26.14821

Bernardinelli, Y., Muller, D., and Nikonenko, I. (2014). Astrocyte-synapse structural plasticity. *Neural Plast.* 2014, 1–13. doi: 10.1155/2014/232105

Berthold, C. H., Nilsson, I., and Rydmark, M. (1983). Axon diameter and myelin sheath thickness in nerve fibres of the ventral spinal root of the seventh lumbar nerve of the adult and developing cat. *J. Anat.* 136, 483–508.

Bhattacharjee, Y. (2007). Is internal timing key to mental health? *Science* 317, 1488–1490. doi: 10.1126/science.317.5844.1488

Bliss, T. V., and Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361, 31–39. doi: 10.1038/361031a0

Brecht, M., and Sakmann, B. (2002). Dynamic representation of whisker deflection by synaptic potentials in spiny stellate and pyramidal cells in the barrels and septa of layer 4 rat somatosensory cortex. *J. Physiol.* 543, 49–70. doi: 10.1113/jphysiol.2002.018465

Bushong, E. A., Martone, M. E., Jones, Y. Z., and Ellisman, M. H. (2002). Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. *J. Neurosci.* 22, 183–192.

Carr, C. E., and Konishi, M. (1990). A circuit for detection of interaural time differences in the brain stem of the barn owl. *J. Neurosci.* 10, 3227–3246.

Chao, T. I., Rickmann, J. R., and Wolff, J. R. (2002). "The synapse-astrocyte boundary: An anatomical basis for an integrative role of glia in synaptic transmission," in *The Tripartite Synapse: Glia in Synaptic Transmission*, eds A. Volterra, P. J. Magistretti, and P. G. Haydon (Oxford: Oxford University Press), 3–23.

Chen, M. S., Huber, A. B., van der Haar, M. E., Frank, M., Schnell, L., Spillmann, A. A., et al. (2000). Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. *Nature* 403, 434–439. doi: 10.1038/35000219

Conti, F., Minelli, A., and Melone, M. (2004). GABA transporters in the mammalian cerebral cortex: localization, development and pathological implications. *Brain Res. Rev.* 45, 196–212. doi: 10.1016/j.brainresrev.2004.03.003

Cossart, R., Aronov, D., and Yuste, R. (2003). Attractor dynamics of network UP states in the neocortex. *Nature* 423, 283–288. doi: 10.1038/nature01614

Danbolt, N. C. (2001). Glutamate uptake. *Prog Neurobiol.* 65, 1–105. doi: 10.1016/S0301-0082(00)00067-8

Danbolt, N. C., Furness, D. N., and Zhou, Y. (2016). Neuronal vs glial glutamate uptake: resolving the conundrum. *Neurochem. Int.* 98, 29–45. doi: 10.1016/j.neuint.2016.05.009

De Pittà, M., Brunel, N., and Volterra, A. (2016). Astrocytes: Orchestrating synaptic plasticity? *Neuroscience* 323, 43–61. doi: 10.1016/j.neuroscience.2015.04.001

Dinges, D. F., Pack, F., Williams, K., Gillen, K. A., Powell, J. W., Ott, G. E., et al. (1997). Cumulative sleepiness, mood disturbance, and psychomotor vigilance performance decrements during a week of sleep restricted to 4-5 hours per night. *Sleep* 20, 267–277.

Domingues, A. M., Taylor, M., and Fern, R. (2010). Glia as transmitter sources and sensors in health and disease. *Neurochem. Int.* 57, 359–366. doi: 10.1016/j.neuint.2010.03.024

Du, Y. Z., Fan, S. J., Meng, Q. H., Wang, G. Q., and Tong, J. (2005). Circadian expression of clock and screening of clock-controlled genes in peripheral lymphocytes of rat. *Biochem. Biophys. Res. Commun.* 336, 1069–1073. doi: 10.1016/j.bbrc.2005.08.228

Dunlap, J. C. (1999). Molecular bases for circadian clocks. *Cell* 96, 271–290. doi: 10.1016/S0092-8674(00)80566-8

Dupree, J. L., Mason, J. L., Marcus, J. R., Stull, M., Levinson, R., Matsushima, G. K., et al. (2004). Oligodendrocytes assist in the maintenance of sodium channel clusters independent of the myelin sheath. *Neuron Glia Biol.* 1, 179–192. doi: 10.1017/S1740925X04000304

Eckel-Mahan, K. L., and Storm, D. R. (2009). Circadian rhythms and memory: not so simple as cogs and gears. *EMBO Rep.* 10, 584–591. doi: 10.1038/embor.2009.123

Fellin, T., Ellenbogen, J. M., De Pittà, M., Ben-Jacob, E., and Halassa, M. M. (2012). Astrocyte regulation of sleep circuits: experimental and modeling perspectives. *Front. Comput. Neurosci.* 6:65. doi: 10.3389/fncom.2012.00065

Fellin, T., Halassa, M. M., Terunuma, M., Succol, F., Takano, H., Frank, M., et al. (2009). Endogenous nonneuronal modulators of synaptic transmission control cortical slow oscillations *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 15037–15042. doi: 10.1073/pnas.0906419106

Fellin, T., Pascual, O., and Haydon, P. G. (2006). Astrocytes coordinate synaptic networks: balanced excitation and inhibition. *Physiology* 21, 208–215. doi: 10.1152/physiol.00161.2005

Flores-Méndez, M., Mendez-Flores, O. G., and Ortega, A. (2016). Glia plasma membrane transporters: key players in glutamatergic neurotransmission. *Neurochem. Int.* 98, 46–55. doi: 10.1016/j.neuint.2016.04.004

Fonken, L. K., Frank, M. G., Kitt, M. M., Barrientos, R. M., Watkins, L. R., and Maier, S. F. (2015). Microglia inflammatory responses are controlled by an intrinsic circadian clock. *Brain Behav. Immun.* 45, 171–179. doi: 10.1016/j.bbi.2014.11.009

Fontainhas, A. M., Wang, M., Liang, K. J., Chen, S., Mettu, P., Damani, M., et al. (2011). Microglial morphology and dynamic behavior is regulated by ionotropic glutamatergic and GABAergic neurotransmission. *PLoS ONE* 6:e15973. doi: 10.1371/journal.pone.0015973

Frank, M. G. (2011). Sleep and developmental plasticity not just for kids. *Prog. Brain Res.* 193, 221–232. doi: 10.1016/B978-0-444-53839-0.00014-4

Frank, M. G., and Benington, J. H. (2006). The role of sleep in memory consolidation and brain plasticity: dream or reality? *Neuroscience* 12, 477–488. doi: 10.1177/1073858406293552

Frank, M. G., and Cantera, R. (2014). Sleep, clocks, and synaptic plasticity. *Trends Neurosci.* 37, 491–501. doi: 10.1016/j.tins.2014.06.005

Gegelashvili, G., and Bjerrum, O. J. (2014). High-affinity glutamate transporters in chronic pain: an emerging therapeutic target. *J. Neurochem.* 131, 712–730. doi: 10.1111/jnc.12957

Gentet, L. J., Avermann, M., Matyas, F., Staiger, J. F., and Petersen, C. C. H. (2010). Membrane potential dynamics of GABAergic neurons in the barrel cortex of behaving mice. *Neuron* 65, 422–435. doi: 10.1016/j.neuron.2010.01.006

Gerstner, J. R., Bremer, Q. Z., Vander Heyden, W. M., LaVaute, T. M., Yin, J. C., and Landry, C. F. (2008). Brain fatty acid binding protein (Fabp7) is diurnally regulated in astrocytes and hippocampal granule cell precursors in adult rodent brain. *PLoS ONE* 3:e1631. doi: 10.1371/journal.pone.0001631

Gerstner, J. R., Lyons, L. C., Wright, K. P., Loh, D. H., Rawashdeh, O., Eckel-Mahan, K. L., et al. (2009). Cycling behavior and memory formation. *J. Neurosci.* 29, 12824–12830. doi: 10.1523/JNEUROSCI.3353-09.2009

Gerstner, J. R., Vander Heyden, W. M., LaVaute, T. M., and Landry, C. F. (2006). Profiles of novel diurnally regulated genes in mouse hypothalamus: expression analysis of the cysteine and histidine-rich domain-containing, zinc-binding protein 1, the fatty acid-binding protein 7 and the GTPase, ras-like family member 11b. *Neuroscience* 139, 1435–1448. doi: 10.1016/j.neuroscience.2006.01.020

Gerstner, J. R., Vanderheyden, W. M., LaVaute, T., Westmark, C. J., Rouhana, L., Pack, A. I., et al. (2012). Time of day regulates subcellular trafficking, tripartite synaptic localization, and polyadenylation of the astrocytic Fabp7 mRNA. *J. Neurosci.* 32, 1383–1394. doi: 10.1523/JNEUROSCI.3228-11.2012

Gordon, G. R., Baimoukhametova, D. V., Hewitt, S. A., Rajapaksha, W. R., Fisher, T. E., and Bains, J. S. (2005). Norepinephrine triggers release of glial ATP to increase postsynaptic efficacy. *Nat. Neurosci.* 8, 1078–1086. doi: 10.1038/nn1498

GrandPré, T., Nakamura, F., Vartanian, T., and Strittmatter, S. M. (2000). Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. *Nature* 403, 439–444. doi: 10.1038/35000226

Haider, B., and McCormick, D. A. (2009). Rapid neocortical dynamics: cellular and network mechanisms. *Neuron* 62, 171–189. doi: 10.1016/j.neuron.2009.04.008

Halassa, M. M., Fellin, T., and Haydon, P. G. (2009a). Tripartite synapses: roles for astrocytic purines in the control of synaptic physiology and behavior. *Neuropharmacology* 57, 343–346. doi: 10.1016/j.neuropharm.2009.06.031

Halassa, M. M., Fellin, T., Takano, H., Dong, J.-H., and Haydon, P. G. (2007). Synaptic islands defined by the territory of a single astrocyte. *J. Neurosci.* 27, 6473–6477. doi: 10.1523/JNEUROSCI.1419-07.2007

Halassa, M. M., Florian, C., Fellin, T., Munoz, J. R., Lee, S.-Y., Abel, T., et al. (2009b). Astrocytic modulation of sleep homeostasis and cognitive consequences of sleep loss. *Neuron* 61, 213–219. doi: 10.1016/j.neuron.2008.11.024

Halassa, M. M., and Haydon, P. G. (2010). Integrated brain circuits: astrocytic networks modulate neuronal activity and behavior. *Annu. Rev. Physiol.* 72, 335–355. doi: 10.1146/annurev-physiol-021909-135843

Halberg, F. (1959). Physiologic 24-hour periodicity; general and procedural considerations with reference to the adrenal cycle. *Int. Z. Vitaminforsch. Beih.* 10, 225–296.

Harmer, S. L., Panda, S., and Kay, S. A. (2001). Molecular bases of circadian rhythms. *Annu. Rev. Cell Dev. Biol.* 17, 215–253. doi: 10.1146/annurev.cellbio.17.1.215

Harris, K. D., Csicsvari, J., Hirase, H., Dragoi, G., and Buzsáki, G. (2003). Organization of cell assemblies in the hippocampus. *Nature* 424, 552–556. doi: 10.1038/nature01834

Harry, G. J., and Kraft, A. D. (2012). Microglia in the developing brain: a potential target with lifetime effects. *Neurotoxicology* 33, 191–206. doi: 10.1016/j.neuro.2012.01.012

Hartse, K. M. (2011). "The phylogeny of sleep," in *Handbook of Clinical Neurology, Vol. 98*, eds P. Montagna and S. Chokroverty (Amsterdam: Elsevier), 97–109.

Hayashi, Y., Ishibashi, H., Hashimoto, K., and Nakanishi, H. (2006). Potentiation of the NMDA receptor-mediated responses through the activation of the glycine site by microglia secreting soluble factors. *Glia* 53, 660–668. doi: 10.1002/glia.20322

Hayashi, Y., Koyanagi, S., Kusunose, N., Okada, R., Wu, Z., Tozaki-Saitoh, H., et al. (2013a). The intrinsic microglial molecular clock controls synaptic strength via the circadian expression of cathepsin S. *Sci. Rep.* 3:2744. doi:10.1038/srep02744

Hayashi, Y., Koyanagi, S., Kusunose, N., Takayama, F., Okada, R., Wu, Z., et al. (2013b). Diurnal spatial rearrangement of microglial processes through the rhythmic expression of P2Y12 receptors. *J. Neurol. Disord.* 1:120. doi: 10.4172/2329-6895.1000120

Hayashi, Y., Wu, Z., and Nakanishi, H. (2014). A possible link between microglial process dysfunction and neuropsychiatric disorders. *J. Neurol. Disord. Stroke* 2:1060.

Haydon, P. G. (2001). GLIA: listening and talking to the synapse. *Nat. Rev. Neurosci.* 2, 185–193. doi: 10.1038/35058528

Haynes, S. E., Hollopeter, G., Yang, G., Kurpius, D., Dailey, M. E., Gan, W.-B., et al. (2006). The P2Y12 receptor regulates microglial activation by extracellular nucleotides. *Nat. Neurosci.* 9, 1512–1519. doi: 10.1038/nn1805

Hide, I., Tanaka, M., Inoue, A., Nakajima, K., Kohsaka, S., Inoue, K., et al. (2000). Extracellular ATP triggers tumor necrosis factor-alpha release from rat microglia. *J. Neurochem.* 75, 965–972. doi: 10.1046/j.1471-4159.2000.0750965.x

Hill, S., and Tononi, G. (2004). Modeling sleep and wakefulness in the thalamocortical system. *J. Neurophysiol.* 93, 1671–1698. doi: 10.1152/jn.00915.2004

Hirrlinger, J., Hülsmann, S., and Kirchhoff, F. (2004). Astroglial processes show spontaneous motility at active synaptic terminals *in situ*. *Eur. J. Neurosci.* 20, 2235–2239. doi: 10.1111/j.1460-9568.2004.03689.x

Huang, J. K., Phillips, G. R., Roth, A. D., Pedraza, L., Shan, W., Belkaid, W., et al. (2005). Glial membranes at the node of Ranvier prevent neurite outgrowth. *Science* 310, 1813–1817. doi: 10.1126/science.1118313

Iino, M., Goto, K., Kakegawa, W., Okado, H., Sudo, M., Ishiuchi, S., et al. (2001). Glia-synapse interaction through Ca2+-permeable AMPA receptors in Bergmann glia. *Science* 292, 926–929. doi: 10.1126/science.1058827

Ikegaya, Y., Delcroix, I., Iwakura, Y., Matsuki, N., and Nishiyama, N. (2003). Interleukin-1beta abrogates long-term depression of hippocampal CA1 synaptic transmission. *Synapse* 47, 54–57. doi: 10.1002/syn.10154

Inoue, K., Nakajima, K., Morimoto, T., Kikuchi, Y., Koizumi, S., Illes, P., et al. (1998). ATP stimulation of Ca2+-dependent plasminogen release from cultured microglia. *Br. J. Pharmacol.* 123, 1304–1310. doi: 10.1038/sj.bjp.0701732

Jensen, C. L., Chen, H., Fraley, J. K., Anderson, R. E., and Heird, W. C. (1996). Biochemical effects of dietary linoleic/alpha-linolenic acid ratio in term infants. *Lipids* 31, 107–113. doi: 10.1007/BF02522419

Jilg, A., Lesny, S., Peruzki, N., Schwegler, H., Selbach, O., Dehghani, F., et al. (2010). Temporal dynamics of mouse hippocampal clock gene expression support memory processing. *Hippocampus* 20, 377–388. doi: 10.1002/hipo.20637

Jin, X., Shearman, L. P., Weaver, D. R., Zylka, M. J., de Vries, G. J., and Reppert, S. M. (1999). A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. *Cell* 96, 57–68. doi: 10.1016/S0092-8674(00)80959-9

Joo, E. Y., Noh, H. J., Kim, J.-S., Koo, D. L., Kim, D., Hwang, K. J., et al. (2013). Brain gray matter deficits in patients with chronic primary insomnia. *Sleep* 36, 999–1007. doi: 10.5665/sleep.2796

Kenet, T., Bibitchkov, D., Tsodyks, M., Grinvald, A., and Arieli, A. (2003). Spontaneously emerging cortical representations of visual attributes. *Nature* 425, 954–956. doi: 10.1038/nature02078

Kirchhoff, F. (2013). "Role of microglia in the normal brain," in *Neuroglia*, eds H. Kettenmann, and B. R. Ransom (New York, NY: Oxford University Press), 605–613.

Ko, G. Y., Shi, L., and Ko, M. L. (2009). Circadian regulation of ion channels and their functions. *J. Neurochem.* 110, 1150–1169. doi: 10.1111/j.1471-4159.2009.06223.x

Kondratova, A. A., Dubrovsky, Y. V., Antoch, M. P., and Kondratov, R. V. (2010). Circadian clock proteins control adaptation to novel environment and memory formation. *Aging* 2, 285–297. doi: 10.18632/aging.100142

Lavialle, M., Aumann, G., Anlauf, E., Pröls, F., Arpin, M., and Derouiche, A. (2011). Structural plasticity of perisynaptic astrocyte processes involves ezrin and metabotropic glutamate receptors. *Proc. Natl. Acad. Sci. U.S.A.* 108, 12915–12919. doi: 10.1073/pnas.1100957108

Lavialle, M., and Servière, J. (1993). Circadian fluctuations in GFAP distribution in the Syrian hamster suprachiasmatic nucleus. *Neuroreport* 4, 1243–1246. doi: 10.1097/00001756-199309000-00008

Le Martelot, G., Claudel, T., Gatfield, D., Schaad, O., Kornmann, B., Lo Sasso, G., et al. (2009). REV-ERBalpha participates in circadian SREBP signaling and bile acid homeostasis. *PLoS Biol* 7:e1000181. doi: 10.1371/journal.pbio.1000181

Levine, J. M., Stincone, F., and Lee, Y.-S. (1993). Development and differentiation of glial precursor cells in the rat cerebellum. *Glia* 7, 307–321. doi: 10.1002/glia.440070406

Lin, S.-C., and Bergles, D. E. (2004). Synaptic signaling between neurons and glia. *Glia* 47, 290–298. doi: 10.1002/glia.20060

Liu, Z., Li, Y., Qian, J., Cui, Y., and Chopp, M. (2014). Plasminogen deficiency causes reduced corticospinal axonal plasticity and functional recovery after stroke in mice. *PLoS ONE* 9:e94505. doi: 10.1371/journal.pone.0094505

Lo, J. C., Groeger, J. A., Santhi, N., Arbon, E. L., Lazar, A. S., Hasan, S., et al. (2012). Effects of partial and acute total sleep deprivation on performance

across cognitive domains, individuals and circadian phase. *PLoS ONE* 7:e45987. doi: 10.1371/journal.pone.0045987

Lohmann, C., and Kessels, H. W. (2014). The developmental stages of synaptic plasticity. *J. Physiol.* 592, 13–31. doi: 10.1113/jphysiol.2012.235119

Lowrey, P. L., and Takahashi, J. S. (2004). Mammalian circadian biology: elucidating genome-wide levels of temporal organization. *Annu. Rev. Genomics Hum. Genet.* 5, 407–441. doi: 10.1146/annurev.genom.5.061903.175925

Marpegan, L., Swanstrom, A. E., Chung, K., Simon, T., Haydon, P. G., Khan, S. K., et al. (2011). Circadian regulation of ATP release in astrocytes. *J. Neurosci.* 31, 8342–8350. doi: 10.1523/JNEUROSCI.6537-10.2011

Martínez-Lozada, Z., and Ortega, A. (2015). Glutamatergic transmission: a matter of three. *Neural Plast.* 2015, 1–11. doi: 10.1155/2015/787396

Martin, S. J., Grimwood, P. D., and Morris, R. G. (2000). Synaptic plasticity and memory: an evaluation of the hypothesis. *Annu. Rev. Neurosci.* 23, 649–711. doi: 10.1146/annurev.neuro.23.1.649

Massimini, M., and Amzica, F. (2001). Extracellular calcium fluctuations and intracellular potentials in the cortex during the slow sleep oscillation. *J. Neurophysiol.* 85, 1346–1350. doi: 10.1152/jn.2001.85.3.1346

Matsumoto, Y., Tsunekawa, Y., Nomura, T., Suto, F., Matsumata, M., Tsuchiya, S., et al. (2011). Differential proliferation rhythm of neural progenitor and oligodendrocyte precursor cells in the young adult hippocampus. *PLoS ONE* 6:e27628. doi: 10.1371/journal.pone.0027628

McCarthy, G. F., and Leblond, C. P. (1988). Radioautographic evidence for slow astrocyte turnover and modest oligodendrocyte production in the corpus callosum of adult mice infused with 3H-thymidine. *J. Comp. Neurol.* 271, 589–603. doi: 10.1002/cne.902710409

McEntee, W. J., and Crook, T. H. (1993). Glutamate: its role in learning, memory, and the aging brain. *Psychopharmacology* 111, 391–401. doi: 10.1007/BF02253527

McKerracher, L., David, S., Jackson, D. L., Kottis, V., Dunn, R. J., and Braun, P. E. (1994). Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. *Neuron* 13, 805–811. doi: 10.1016/0896-6273(94)90247-X

Möller-Levet, C. S., Archer, S. N., Bucca, G., Laing, E. E., Slak, A., Kabiljo, R., et al. (2013). Effects of insufficient sleep on circadian rhythmicity and expression amplitude of the human blood transcriptome. *Proc. Natl. Acad. Sci. U.S.A.* 110, E1132–E1141. doi: 10.1073/pnas.1217154110

Monk, T. H., and Folkard, S. (1978). Concealed inefficiency of late-night study. *Nature* 273, 296–297. doi: 10.1038/273296a0

Moriguchi, T., Greiner, R. S., and Salem, N. (2000). Behavioral deficits associated with dietary induction of decreased brain docosahexaenoic acid concentration. *J. Neurochem.* 75, 2563–2573. doi: 10.1046/j.1471-4159.2000.0752563.x

Moriya, T., Yoshinobu, Y., Kouzu, Y., Katoh, A., Gomi, H., Ikeda, M., et al. (2000). Involvement of glial fibrillary acidic protein (GFAP) expressed in astroglial cells in circadian rhythm under constant lighting conditions in mice. *J. Neurosci. Res.* 60, 212–218. doi: 10.1002/(SICI)1097-4547(20000415)60:2<212::AID-JNR10>3.0.CO;2-P

Nakazato, R., Takarada, T., Yamamoto, T., Hotta, S., Hinoi, E., and Yoneda, Y. (2011). Selective upregulation of Per1 mRNA expression by ATP through activation of P2X7 purinergic receptors expressed in microglial cells. *J. Pharmacol. Sci.* 116, 350–361. doi: 10.1254/jphs.11069FP

Newman, L. A., Korol, D. L., and Gold, P. E. (2011). Lactate produced by glycogenolysis in astrocytes regulates memory processing. *PLoS ONE* 6:e28427. doi: 10.1371/journal.pone.0028427

Nishida, H., and Okabe, S. (2007). Direct astrocytic contacts regulate local maturation of dendritic spines. *J. Neurosci.* 27, 331–340. doi: 10.1523/JNEUROSCI.4466-06.2007

Panda, S., Antoch, M. P., Miller, B. H., Su, A. I., Schook, A. B., Straume, M., et al. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* 109, 307–320. doi: 10.1016/S0092-8674(02)00722-5

Parpura, V., Basarsky, T. A., Liu, F., Jeftinija, K., Jeftinija, S., and Haydon, P. G. (1994). Glutamate-mediated astrocyte–neuron signalling. *Nature* 369, 744–747. doi: 10.1038/369744a0

Parpura, V., and Zorec, R. (2010). Gliotransmission: exocytotic release from astrocytes. *Brain Res. Rev.* 63, 83–92. doi: 10.1016/j.brainresrev.2009.11.008

Pascual, O., Ben Achour, S., Rostaing, P., Triller, A., and Bessis, A. (2012). Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission. *Proc. Natl. Acad. Sci. U.S.A.* 109, E197–E205. doi: 10.1073/pnas.1111098109

Pascual, O., Casper, K. B., Kubera, C., Zhang, J., Revilla-Sanchez, R., Sul, J.-Y., et al. (2005). Astrocytic purinergic signaling coordinates synaptic networks. *Science* 310, 113–116. doi: 10.1126/science.1116916

Perea, G., Navarrete, M., and Araque, A. (2009). Tripartite synapses: astrocytes process and control synaptic information. *Trends Neurosci.* 32, 421–431. doi: 10.1016/j.tins.2009.05.001

Poskanzer, K. E., and Yuste, R. (2011). Astrocytic regulation of cortical UP states. *Proc. Natl. Acad. Sci. U.S.A.* 108, 18453–18458. doi: 10.1073/pnas.1112378108

Poskanzer, K. E., and Yuste, R. (2016). Astrocytes regulate cortical state switching *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 113, E2675–E2684. doi: 10.1073/pnas.1520759113

Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., et al. (2002). The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* 110, 251–260. doi: 10.1016/S0092-8674(02)00825-5

Ramaswamy, S., and Muller, E. B. (2015). Cell-type specific modulation of neocortical UP and DOWN states. *Front. Cell. Neurosci.* 9:370. doi: 10.3389/fncel.2015.00370

Rawashdeh, O., de Borsetti, N. H., Roman, G., and Cahill, G. M. (2007). Melatonin suppresses nighttime memory formation in zebrafish. *Science* 318, 1144–1146. doi: 10.1126/science.1148564

Reppert, S. M., and Weaver, D. R. (2001). Molecular analysis of mammalian circadian rhythms. *Annu. Rev. Physiol.* 63, 647–676. doi: 10.1146/annurev.physiol.63.1.647

Reppert, S. M., and Weaver, D. R. (2002). Coordination of circadian timing in mammals. *Nature* 418, 935–941. doi: 10.1038/nature00965

Rudy, J. W., and Pugh, C. R. (1998). Time of conditioning selectively influences contextual fear conditioning: further support for a multiple-memory systems view of fear conditioning. *J. Exp. Psychol. Anim. Behav. Process.* 24, 316–324. doi: 10.1037/0097-7403.24.3.316

Saab, A. S., Neumeyer, A., Jahn, H. M., Cupido, A., Šimek, A. A. M., Boele, H.-J., et al. (2012). Bergmann glial AMPA receptors are required for fine motor coordination. *Science* 337, 749–753. doi: 10.1126/science.1221140

Sakai, T., Tamura, T., Kitamoto, T., and Kidokoro, Y. (2004). A clock gene, period, plays a key role in long-term memory formation in Drosophila. *Proc. Natl. Acad. Sci. U.S.A.* 101, 16058–16063. doi: 10.1073/pnas.0401472101

Sanz, J. M., and Di Virgilio, F. (2000). Kinetics and mechanism of ATP-dependent IL-1 beta release from microglial cells. *J. Immunol.* 164, 4893–4898. doi: 10.4049/jimmunol.164.9.4893

Schibler, U., and Sassone-Corsi, P. (2002). A web of circadian pacemakers. *Cell* 111, 919–922. doi: 10.1016/S0092-8674(02)01225-4

Shibuki, K., Gomi, H., Chen, L., Bao, S., Kim, J. J., Wakatsuki, H., et al. (1996). Deficient cerebellar long-term depression, impaired eyeblink conditioning, and normal motor coordination in GFAP mutant mice. *Neuron* 16, 587–599. doi: 10.1016/S0896-6273(00)80078-1

Sierra, A., Abiega, O., Shahraz, A., and Neumann, H. (2013). Janus-faced microglia: beneficial and detrimental consequences of microglial phagocytosis. *Front. Cell. Neurosci.* 7:6. doi: 10.3389/fncel.2013.00006

Spanagel, R., Pendyala, G., Abarca, C., Zghoul, T., Sanchis-Segura, C., Magnone, M. C., et al. (2005). The clock gene Per2 influences the glutamatergic system and modulates alcohol consumption. *Nat. Med.* 11, 35–42. doi: 10.1038/nm1163

Steriade, M., Nuñez, A., and Amzica, F. (1993). Intracellular analysis of relations between the slow (< 1 Hz) neocortical oscillation and other sleep rhythms of the electroencephalogram. *J. Neurosci.* 13, 3266–3283.

Steriade, M., Timofeev, I., and Grenier, F. (2001). Natural waking and sleep states: a view from inside neocortical neurons. *J. Neurophysiol.* 85, 1969–1985. doi: 10.1152/jn.2001.85.5.1969

Stratmann, M., and Schibler, U. (2006). Properties, entrainment, and physiological functions of mammalian peripheral oscillators. *J. Biol. Rhythms* 21, 494–506. doi: 10.1177/0748730406293889

Suzuki, A., Stern, S. A., Bozdagi, O., Huntley, G. W., Walker, R. H., Magistretti, P. J., et al. (2011). Astrocyte-neuron lactate transport is required for long-term memory formation. *Cell* 144, 810–823. doi: 10.1016/j.cell.2011.02.018

Suzuki, T., Hide, I., Ido, K., Kohsaka, S., Inoue, K., and Nakata, Y. (2004). Production and release of neuroprotective tumor necrosis

factor by P2X7 receptor-activated microglia. *J. Neurosci.* 24, 1–7. doi: 10.1523/JNEUROSCI.3792-03.2004

Takeuchi, T., Iwanaga, M., and Harada, E. (2003). Possible regulatory mechanism of DHA-induced anti-stress reaction in rats. *Brain Res.* 964, 136–143. doi: 10.1016/S0006-8993(02)04113-6

Theodosis, D. T., Poulain, D. A., and Olliet, S. H. (2008). Activity-dependent structural and functional plasticity of astrocyte-neuron interactions. *Physiol. Rev.* 88, 983–1008. doi: 10.1152/physrev.00036.2007

Thomson, A. M., Walker, V. E., and Flynn, D. M. (1989). Glycine enhances NMDA-receptor mediated synaptic potentials in neocortical slices. *Nature* 338, 422–424. doi: 10.1038/338422a0

Turrigiano, G. (2011). Too many cooks? Intrinsic and synaptic homeostatic mechanisms in cortical circuit refinement. *Annu. Rev. Neurosci.* 34, 89–103. doi: 10.1146/annurev-neuro-060909-153238

Tzingounis, A. V., and Wadiche, J. I. (2007). Glutamate transporters: confining runaway excitation by shaping synaptic transmission. *Nat. Rev. Neurosci.* 8, 935–947. doi: 10.1038/nrn2274

Valentinuzzi, V. S., Menna-Barreto, L., and Xavier, G. F. (2004). Effect of circadian phase on performance of rats in the Morris water maze task. *J. Biol. Rhythms* 19, 312–324. doi: 10.1177/0748730404265688

Van der Zee, E. A., Havekes, R., Barf, R. P., Hut, R. A., Nijholt, I. M., Jacobs, E. H., et al. (2008). Circadian time-place learning in mice depends on Cry genes. *Curr. Biol.* 18, 844–848. doi: 10.1016/j.cub.2008.04.077

Wainwright, P. E., Xing, H. C., Mutsaers, L., McCutcheon, D., and Kyle, D. (1997). Arachidonic acid offsets the effects on mouse brain and behavior of a diet with a low (n-6):(n-3) ratio and very high levels of docosahexaenoic acid. *J. Nutr.* 127, 184–193.

Walker, M. P., and Stickgold, R. (2006). Sleep, memory, and plasticity. *Annu. Rev. Psychol.* 57, 139–166. doi: 10.1146/annurev.psych.56.091103.070307

Wang, K. C., Koprivica, V., Kim, J. A., Sivasankaran, R., Guo, Y., Neve, R. L., et al. (2002). Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. *Nature* 417, 941–944. doi: 10.1038/nature 00867

Wang, L. M.-C., Dragich, J. M., Kudo, T., Odom, I. H., Welsh, D. K., O'Dell, T. J., et al. (2009). Expression of the circadian clock gene Period2 in the hippocampus: possible implications for synaptic plasticity and learned behaviour. *ASN Neuro* 1:e00012. doi: 10.1042/AN20090020

Winocur, G., and Hasher, L. (2004). Age and time-of-day effects on learning and memory in a non-matching-to-sample test. *Neurobiol. Aging* 25, 1107–1115. doi: 10.1016/j.neurobiolaging.2003.10.005

Womac, A. D., Burkeen, J. F., Neuendorff, N., Earnest, D. J., and Zoran, M. J. (2009). Circadian rhythms of extracellular ATP accumulation in suprachiasmatic nucleus cells and cultured astrocytes. *Eur. J. Neurosci.* 30, 869–876. doi: 10.1111/j.1460-9568.2009.06874.x

Wurtz, C. C., and Ellisman, M. H. (1986). Alterations in the ultrastructure of peripheral nodes of Ranvier associated with repetitive action potential propagation. *J. Neurosci.* 6, 3133–3143.

Yamamoto, N., Saitoh, M., Moriuchi, A., Nomura, M., and Okuyama, H. (1987). Effect of dietary alpha-linolenate/linoleate balance on brain lipid compositions and learning ability of rats. *J. Lipid Res.* 28, 144–151.

Zhong, Y., Zhou, L.-J., Ren, W.-J., Xin, W.-J., Li, Y.-Y., Zhang, T., et al. (2010). The direction of synaptic plasticity mediated by C-fibers in spinal dorsal horn is decided by Src-family kinases in microglia: the role of tumor necrosis factor-α. *Brain Behav. Immun.* 24, 874–880. doi: 10.1016/j.bbi.2010.01.007

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 06 February 2018
doi: 10.3389/fphys.2018.00009

Purinergic Signaling in Neuron-Astrocyte Interactions, Circadian Rhythms, and Alcohol Use Disorder

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Alcohol use disorder (AUD) is a debilitating condition marked by cyclic patterns of craving, use, and withdrawal. These pathological behaviors are mediated by multiple neurotransmitter systems utilizing glutamate, GABA, dopamine, ATP, and adenosine. In particular, purines such as ATP and adenosine have been demonstrated to alter the phase and function of the circadian clock and are reciprocally regulated by the clock itself. Importantly, chronic ethanol intake has been demonstrated to disrupt the molecular circadian clock and is associated with altered circadian patterns of activity and sleep. Moreover, ethanol has been demonstrated to disrupt purinergic signaling, while dysfunction of the purinergic system has been implicated in conditions of drug abuse such as AUD. In this review, we summarize our current knowledge regarding circadian disruption by ethanol, focusing on the reciprocal relationship that exists between oscillatory neurotransmission and the molecular circadian clock. In particular, we offer detailed explanations and hypotheses regarding the concerted regulation of purinergic signaling and circadian oscillations by neurons and astrocytes, and review the diverse mechanisms by which purinergic dysfuction may contribute to circadian disruption or alcohol abuse. Finally, we describe the mechanisms by which ethanol may disrupt or hijack endogenous circadian rhythms to induce the maladaptive behavioral patterns associated with AUD.

Keywords: circadian, adenosine, glutamate, AUD, ethanol, astrocyte

OPEN ACCESS

Edited by:
Jolanta Górska-Andrzejak, Jagiellonian University, Poland

Reviewed by:
Alan Rosenwasser, University of Maine, France
Ali Keshavarzian, Rush University, United States

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Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: *20 November 2017*
Accepted: *05 January 2018*
Published: *06 February 2018*

Citation:
Lindberg D, Andres-Beck L, Jia Y-F, Kang S and Choi D-S (2018) Purinergic Signaling in Neuron-Astrocyte Interactions, Circadian Rhythms, and Alcohol Use Disorder. Front. Physiol. 9:9. doi: 10.3389/fphys.2018.00009

DISRUPTION OF CIRCADIAN RHYTHMS IN ADDICTIVE BEHAVIORS

Time is an important factor governing human behavior. Each day we engage in repeated patterns of activity regarding work, sleep, and eating. Although artificial and cultural traditions may inform our daily schedules and activities, robust, intrinsic physiological rhythms heavily influence our thoughts and behaviors. These circadian rhythms are entrained by external lighting cues and are reciprocally modified by our behaviors and environment, which may alter the phase and amplitude of biological rhythms controlling sleep, consumption, and other behaviors (Partch et al., 2014).

As such, disruption of circadian rhythms often results in maladaptive or pathologic behavior. Conversely, our behaviors and environment can adversely affect our circadian rhythms, producing maladaptive behavioral patterns that can contribute to substance abuse and alcohol use disorder (AUD) (Parekh et al., 2015).

At the molecular level, circadian rhythms are governed by auto-inhibitory transcriptional feedback loops mediated by the transcriptional activators *Clock* and *Bmal* and their inhibitory targets *Period 1 (Per1), Period 2 (Per2), Cryptochrome 1 (Cry1),* and *Cryptochrome 2 (Cry2)* (Partch et al., 2014; Videnovic et al., 2014). More specifically, *Clock* and *Bmal* are transcriptional activators that induce the expression of *Period* and *Cryptochrome* proteins, which progressively accumulate throughout the circadian night and inhibit the activity of *Clock* and *Bmal* (Partch et al., 2014; Takahashi, 2015). Although this cycle is ubiquitous, and occurs in multiple cell types throughout the body, the mammalian suprachiasmatic nucleus (SCN) is the epicenter and central pacemaker of the mammalian circadian clock. As illustrated in **Figure 1**, the SCN receives photic input from the retina, which entrains molecular rhythms to environmental stimuli via the light-dependent degradation of *Cryptochrome* proteins, and subsequently conveys this information to distant regions of the CNS and periphery in order to synchronize independently functioning circadian clocks throughout the body (Partch et al., 2014; Takahashi, 2015). Interestingly, other environmental stimuli such as food, sex, and drugs can also influence the phase and amplitude of the circadian clock, both within the SCN as well as other regions of the brain more directly involved in behavior and reward (Rosenwasser, 2010; Albrecht, 2013; Damaggio and Gorman, 2014; Parekh et al., 2015).

Many humans live irregular lives that are incompatible with the natural cycles of the outside world. We work unnatural shifts, sleep at irregular hours for truncated or protracted periods of time, and eat with profound temporal indiscretion. These types of circadian disruptions can have tremendous impact on our intrinsic circadian clocks and adversely affect our health and behavior (**Figure 1**). Multiple studies indicate that circadian disruption caused by irregular shift work or repeated jetlag increases the risk of developing AUD (Trinkoff and Storr, 1998; Hasler et al., 2014). Similarly, work in animal models has demonstrated that circadian disruption via exposure to constant light or continuously variable light-dark cycles can increase ethanol drinking, promote relapse, and contribute to the development of AUD and mood disorders such as anxiety and depression (Rosenwasser and Fixaris, 2013; Rosenwasser et al., 2015). Genetic models disrupting the molecular circadian clock have revealed similar results. For example, Per2 mutant mice exhibit increased ethanol preference, and Per1 knockout animals fail to sensitize to cocaine and display increased conditioned place preference in response to drugs (Abarca et al., 2002; Spanagel et al., 2005a; Perreau-Lenz et al., 2009). In *Drosophila,* genetic disruption of the molecular circadian clock abolishes the development of ethanol tolerance and inhibits sensitization to the behavioral effects of cocaine (Andretic et al., 1999; Pohl et al., 2013). Alternatively, other studies have demonstrated that circadian disruption has little effect on ethanol intake. For example, Summa et al. showed that environmental or genetic disruption of circadian rhythms via 12 weeks of light/dark reversal or clock mutation, respectively, had little effect on ethanol intake in mice administered a liquid diet (Summa et al., 2013, 2015). This may be due to the methods utilized to measure ethanol intake or the lack of liquid choice imposed by such a restricted liquid diet. However, these studies also open the possibility that other factors related to circadian disruption as opposed to disruption of rhythms itself contribute to increased ethanol consumption.

Conversely, chronic alcohol abuse has been demonstrated to disrupt circadian rhythms (Rajakrishnan et al., 1999; Rosenwasser et al., 2005; Spanagel et al., 2005b; Kosobud et al., 2007; Ruby et al., 2009). Human patients suffering from AUD display diverse but markedly abnormal patterns of circadian activity, which may persist during abstinence and increase the risk of relapse (Brower, 2001). Furthermore, AUD has been associated with mutations and single-nucleotide polymorphisms (SNPs) in circadian genes including *Clock, Per1, Per2, and Per3* (Spanagel et al., 2005a; Kovanen et al., 2010; Sjöholm et al., 2010; Dong et al., 2011; Gamble et al., 2011; Blomeyer et al., 2013; Baranger et al., 2016). Although relatively few human studies have examined the effects of ethanol on the circadian clock, *Clock* mRNA expression was significantly lower in peripheral mononuclear cells isolated from AUD patients (Huang et al., 2010), and fibroblasts isolated from AUD patients exhibited Per2 cycles whose duration was inversely proportional to disease severity (Huang et al., 2010; McCarthy et al., 2013). In animal models, chronic ethanol administration disrupts photic entrainment of the SCN molecular clock and alters circadian patterns of locomotion (Ruby et al., 2009). Other studies have demonstrated that ethanol shortens the free-running period of mice in constant darkness, supporting the findings of truncated Per2 cycles in human patients with severe AUD (Seggio et al., 2009). These behavioral changes may be associated with ethanol-mediated modulation of the molecular circadian clock. Gene expression analysis of the nucleus accumbens revealed that chronic ethanol administration increases the expression of multiple components of the circadian clock, including *Bmal,* and *Per3* (Melendez et al., 2012). Others have shown that chronic ethanol administration via a two-bottle choice paradigm, decreases *Clock* expression in both the nucleus accumbens and the VTA (Ozburn et al., 2013). In the SCN, ethanol has been demonstrated to alter the phase of *Per2* and *Per3* rhythms, perhaps providing some explanation for ethanol-mediated disruption of circadian patterns of sleep and activity (Chen et al., 2004). Thus, ethanol has been demonstrated to alter circadian rhythms of gene expression and behavior, exerting combinatorial affects on both the central pacemaker of the SCN as well as peripheral sites involved in behavior and reward. Although these effects have been widely reported, it is possible that the circadian disrupting effects of ethanol are dependent upon the dose or time of ethanol exposure. For example, in human patients, administration of a single dose of alcohol does not significantly alter the circadian phase or

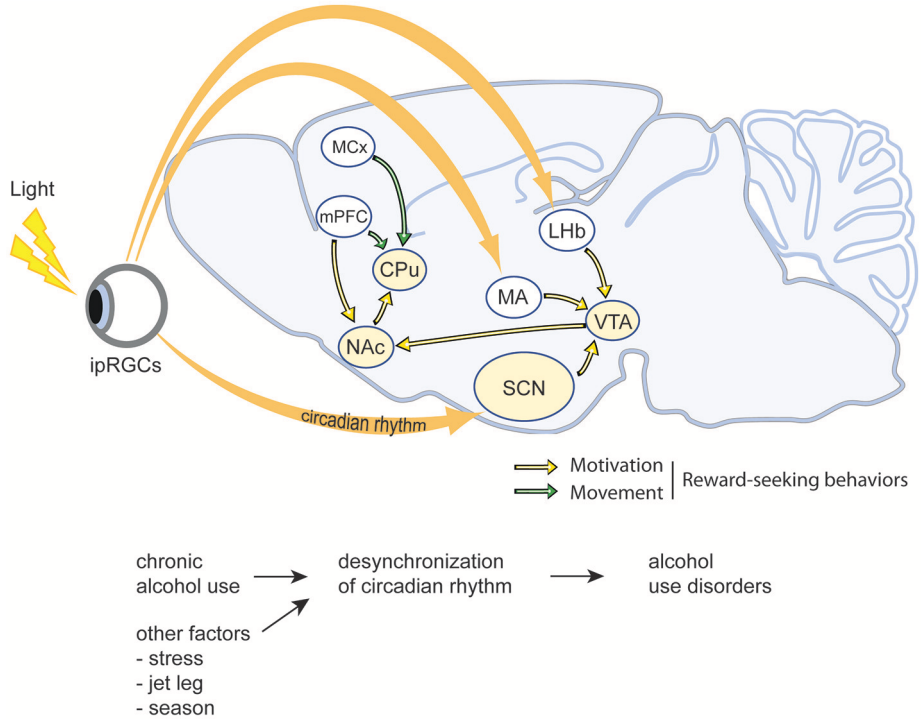

FIGURE 1 | Schematic illustration of the interactions between circadian rhythms and reward-related brain regions. As the central pacemaker, the suprachiasmatic nucleus (SCN) receives photic input from the retina and thereby synchronizes internal rhythmicity with the 24 h light-dark cycle to regulate the circadian rhythms. The ventral tegmental area (VTA), nucleus accumbens (NAc), caudate putamen (CPu), as well as the medial prefrontal cortex (mPFC) and motor cortex (MCx) are the major regions involving in motivation and movement, thus regulate reward-seeking behaviors. ipRGCs, intrinsically photosensitive retinal ganglion cells; LHb, lateral habenula; SCN, suprachiasmatic nucleus; MA, medial amygdala; VTA, ventral tegmental area; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; MCx, motor cortex; CPu, caudate-putamen.

amplitude (Burgess et al., 2016). Alternatively, chronic ethanol administration greatly affects circadian rhythms (Rajakrishnan et al., 1999; Rosenwasser et al., 2005; Spanagel et al., 2005b; Kosobud et al., 2007; Ruby et al., 2009). Furthermore, ethanol has been demonstrated to exert increased sedative effects when consumed during the circadian night, perhaps exacerbating the circadian disrupting effects of even small doses of alcohol (Walsh et al., 1991).

Circadian clock genes are expressed in regions of the brain associated with reward, including the nucleus accumbens, caudate putamen, medial prefrontal cortex, and ventral tegmental area (Logan et al., 2014; Parekh et al., 2015). Although these regions are dependent on the SCN to synchronize the circadian oscillations of individual cells, these molecular cycles may be out of phase with the SCN, owing to their differential sensitivity to light-independent external stimuli, including drugs of abuse (Logan et al., 2014). For example, ethanol, cocaine, methamphetamine, and nicotine have been shown to induce anticipatory behavior and entrain Per1 expression rhythms in the nucleus accumbens, prefrontal cortex, and amygdala (Kosobud et al., 1998, 2007; White et al., 2000; Gillman et al., 2008). Other studies have shown that chronic ethanol intake via a restrictive liquid diet alters the phase of *Per1* rhythms in the arcuate nucleus, which sends projections to the nucleus accumbens and is heavily involved in consummatory behavior (Chen et al., 2004). Importantly, chronic ethanol intake has been demonstrated to reduce the expression of *Clock* within the ventral tegmental area (Ozburn et al., 2013; Parekh et al., 2015). Conversely, RNAi-mediated knockdown of *Clock* expression within the VTA increases ethanol intake (Roybal et al., 2007; Mukherjee et al., 2010). Furthermore, *Clock* mutant mice exhibit increased locomotor activity, reduced anxiety-like and depression-like behaviors, and more frequent intracranial self-stimulation at a lower threshold (McClung et al., 2005; Roybal et al., 2007). These behaviors are associated with an increase in dopaminergic activity in the VTA and a general increase in glutamatergic tone, suggesting that circadian disruption may induce both region-specific and global changes in synaptic signaling which may further desynchronize circadian rhythms and contribute to the behavioral manifestations of AUD (McClung et al., 2005; Beaule et al., 2009).

Together, these results suggest that circadian rhythms and reward-regulated behaviors exist in a reciprocal relationship wherein molecular circadian oscillations dictate rhythms of anticipation and reward seeking, which may be re-entrained by ethanol and drugs of abuse. Oscillations of the circadian clock may help to establish behavioral rhythms of reward by maintaining a fluctuating baseline of anticipation against which the hedonic value of rewarding stimuli may be judged. Drugs of abuse may coopt this system by altering the phase, frequency,

or amplitude of endogenous circadian rhythms, prompting maladaptive manifestations such as craving and drug seeking behaviors. These drug-induced alterations of circadian cycles may occur not only at the level of the molecular circadian clock but also affect the rhythmicity of multiple neurotransmitter systems utilizing glutamate, dopamine, and adenosine.

PURINERGIC SIGNALING AND CIRCADIAN RHYTHMS

Each night, we abate the bustling activities of daytime and retire to our beds, where we sleep until awakening the following morning. Cyclic, circadian behaviors such as these are necessarily driven by periodic physiological processes, most often governed by autonomous, regularly-occurring negative feedback loops. At the molecular level, circadian timing is regulated by oscillatory transcription and translation of auto-inhibitory clock genes (Partch et al., 2014; Takahashi, 2015). As shown in **Figure 2**, the molecular periodicity regulates innumerable neurological processes including neuronal excitability, glutamatergic transmission, dopamine signaling, neuronal and glial energy homeostasis, and ATP and adenosine handling (Bass and Takahashi, 2010; Ruby et al., 2014a; Parekh et al., 2015). In turn, functional fluctuations of these cellular, bioenergetic, and signaling processes manifest as behavioral rhythms that permeate our everyday life.

Purinergic signaling, mediated by adenosine and ATP, is a powerful regulator of sleep that reciprocally regulates circadian gene expression (Ruby et al., 2014a; Urry and Landolt, 2015; Reichert et al., 2016). ATP is the primary biochemical energy carrier, and is also utilized as a neurotransmitter at glutamatergic, GABAergic, dopaminergic, and adrenergic synapses, where it is co-secreted into the synaptic cleft by the presynaptic neuron or released via calcium-dependent exocytosis by astrocytes (Burnstock, 2007, 2008; Lindberg et al., 2015). Here, ATP modulates synaptic activity according to the subtypes of purinergic receptors expressed by neighboring neurons and astrocytes. ATP and its phosphate-bound hydrolysis products ADP and AMP activate P2 receptors, which are predominantly localized to the post-synaptic membrane as well as on astrocytes, microglia, and oligodendrocytes (Burnstock, 2007). P2 receptors are subdivided into P2X and P2Y subtypes; P2X receptors are ligand-gated ion channels with variable permeability to Na^+, K^+, and Ca^{2+}, while P2Y receptors are GPCRs coupled to G_s, $G_{i/o}$, or $G_{q/11}$ (Burnstock, 2007; Lindberg et al., 2015).

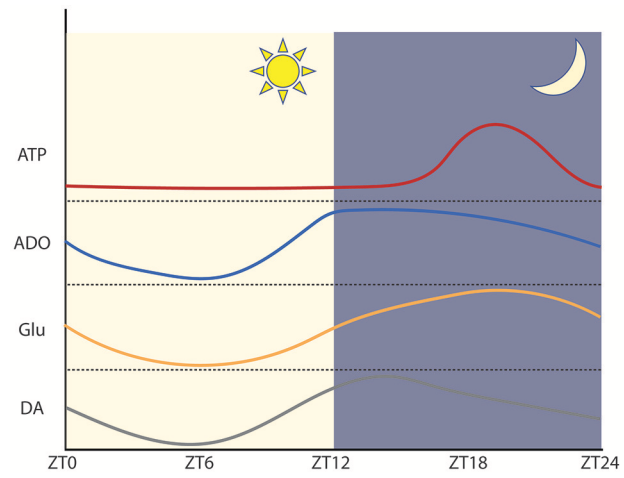

FIGURE 2 | Approximation of the phase and amplitude of circadian neurotransmitter oscillations within regions of abundance in mice. Within the SCN ATP and glutamate peak during the circadian night and reach a trough during the circadian day. In the SCN, adenosine peaks early during the circadian night. Within the dorsal striatum, dopamine peaks early during the circadian night. ATP: adapted from Womac et al. (2009) and Marpegan et al. (2011). Adenosine: adapted from Chagoya de Sánchez (1995). Glutamate: adapted from Brancaccio et al. (2017). Dopamine: adapted from Ferris et al. (2014).

Interestingly, concentrations of extracellular ATP exhibit marked circadian rhythmicity within the SCN, peaking during the latter half of the dark period (subjective night) and reaching a trough during the subjective day (Womac et al., 2009; Marpegan et al., 2011). Numerous metabolic processes including glycolysis, gluconeogenesis, and fatty acid oxidation are transcriptionally regulated by molecular components of the circadian clock, which bind to multiple regulatory elements effecting the expression of enzymes that carry out the rate-limiting steps of metabolic processes (Mazzoccoli et al., 2012; Eckel-Mahan and Sassone-Corsi, 2013). For example, the mammalian SCN displays circadian oscillations in the expression of hexose kinase 1 (HK1), which regulates the concentration of useable glucose, as well as several mitochondrial mediators of oxidative phosphorylation (Mazzoccoli et al., 2012). This temporal control of energy homeostasis may impose circadian limitations on the availability of ancillary ATP utilizable for vesicular transport and synaptic signaling, thus contributing to the circadian rhythmicity of synaptic ATP. This is supported by the observation that SCN astrocytes display circadian mitochondrial calcium rhythms which mirror the extracellular accumulation of ATP and are necessary for the nocturnal peak in synaptic ATP (Burkeen et al., 2011).

Alternatively, rhythmic circadian accumulation of synaptic ATP may be a consequence of intrinsic circadian rhythms governing neuronal and astrocytic excitability and activity. Both neurons and astrocytes within the SCN exhibit robust rhythmic circadian activity demonstrable by voltage indicators and calcium sensors (Brancaccio et al., 2017). These cycles are anti-phasic, with neuronal activity peaking during mid-circadian daytime and astrocytes displaying peak calcium concentrations during the circadian night (Brancaccio et al., 2017). Interestingly, extracellular concentrations of SCN glutamate display circadian oscillations that are synchronous with astrocytic calcium rhythms and anti-phasic to patterns of neuronal activity (Brancaccio et al., 2017). These extracellular glutamate rhythms appear to approximate the phase of synaptic ATP concentrations, suggesting that vesicular release of glutamate and ATP may be orchestrated by astrocytes and act to inhibit neuronal activity during the circadian night. Additionally, rhythmic cycles of extracellular ATP may help to set the gain or reinforce the phase of the molecular circadian clock.

Although clock-mediated control of metabolism, ATP synthesis, and purine and glutamate release may mediate the rhythmic oscillations of extracellular ATP, numerous investigations suggest that ATP may reciprocally regulate the activity of the molecular circadian clock. For example, exposure of murine microglia to ATP transiently induces Per1 expression in a manner sensitive to P2X7 antagonism (Nakazato et al., 2011). Furthermore, both the hypothalamus and somatosensory cortex exhibit circadian variation in the expression of P2X7 receptors, which is increased during the circadian day and decreased at night (Krueger et al., 2010). Interestingly, hypothalamic P2X7 expression is also induced by sleep deprivation (Krueger et al., 2010). Together, these results suggest that enhanced diurnal ATP signaling via P2X7 may partially contribute to the steady rise of Per1 expression during the circadian daytime. Perhaps more accurately, oscillatory P2X7 signaling may help control the gain or amplitude of the molecular clock, producing more dramatic variation of clock gene expression than would be possible in the absence of extracellular ATP.

Like ATP, adenosine has also been demonstrated to reciprocally regulate circadian rhythms (Ruby et al., 2014a; Urry and Landolt, 2015; Reichert et al., 2016). Although adenosine may be synthesized *de novo* from activated ribose (PRPP) in anabolic reactions requiring amino acids, it is more commonly derived from catabolism of high-energy ATP produced by oxidative phosphorylation (Lindberg et al., 2015). Synaptic ATP secreted via calcium-dependent exocytosis by neurons and astrocytes is quickly converted to adenosine by a family of exonucleotidases (Burnstock, 2007). Likewise, additional adenosine can be packaged into vesicles following intracellular ATP degradation or be transported across the membrane by nucleoside transporters such equilibrative nucleoside transporter 1 (ENT1) located on neurons and astrocytes (Burnstock, 2007). Extracellular adenosine acts upon P1 receptors, which are predominantly localized to the presynaptic membrane as well as on astrocytes, microglia, and oligodendrocytes (Burnstock, 2008). The four subtypes of P1 receptors (A1, A2A, A2B, and A3) activate varying subsets of G_s, $G_{i/o}$, and $G_{q/11}$ to exert diverse effects on cellular and CNS function (Burnstock, 2007, 2008). A1 receptors bind adenosine to activate $G_{i/o}$ and inhibit cAMP production, while A2A and A2B receptors (A2AR and A2BR) increase cAMP via G_s (Burnstock, 2007). A3 may activate either $G_{i/o}$ or $G_{q/11}$, resulting in decreased cellular cAMP and increased concentrations of intracellular inosine-1, 4, 5-triphosphate (IP3) (Burnstock, 2007). Importantly, the stepwise degradation of ATP to ADP, AMP, and finally adenosine produce a diverse and constantly varying array of ligands that may act upon both P1 and P2 receptors to exert a repertoire of cellular and signaling effects that may vary depending upon the relative concentration of each purine.

Similar to ATP, adenosine undergoes circadian oscillations of synaptic and extracellular concentrations (Chagoya de Sánchez, 1995; Porkka-Heiskanen et al., 1997, 2000). Interestingly, levels of extracellular adenosine peak early during circadian nighttime and progressively increase throughout the course of the circadian day (Porkka-Heiskanen et al., 1997). This circadian rhythm is synchronized with the cyclic expression of transporters responsible for regulating synaptic adenosine, including ENT1 (Ruby et al., 2014b). Interestingly, disruption of ENT1 function via genetic knockout reduces synaptic adenosine concentrations and lowers the peak expression of clock genes, including Per2 (Ruby et al., 2014b). Furthermore, ENT1 KO mice display altered circadian rhythms marked by early onset of an elongated and hyperactive active phase (Ruby et al., 2014b). Similar circadian disruptions are also induced by caffeine and A2AR antagonism, which lengthen the period of molecular clock oscillations and induce behavioral changes in the sleep-wake cycle (Antle et al., 2001; Burke et al., 2015). This suggests that adenosine signaling may not only regulate the amplitude of circadian oscillations but also modulate the phase of the circadian clock.

Perhaps the most well-established behavioral role of adenosine is the exertion of "sleep pressure." As previously described, adenosine accumulates in the extracellular environment during the daytime, peaking early during the circadian night and progressively declining during the circadian sleep phase. Sleep deprivation elevates extracellular levels of adenosine and produces a phase shift in the sleep-wake cycle, transiently lengthening the period of the sleep cycle and increasing the duration of slow wave sleep in a manner proportional to the duration of sleep deprivation (Porkka-Heiskanen et al., 1997; Wurts and Edgar, 2000). These results suggest that extracellular adenosine acts to promote the drive to sleep, continuously increasing in extracellular concentration until resolved by the induction of sleep.

Importantly, the somnogenic and circadian effects of adenosine are at least partially mediated by interactions with the dopaminergic and glutamatergic neurotransmitter systems. As previously described, astrocytes control circadian timekeeping in the SCN by regulating extracellular levels of glutamate (Brancaccio et al., 2017). This is associated with circadian fluctuations in glutamate uptake as well as diurnal cycles in the activity of glutamine synthetase (GS), which converts glutamate into glutamine (Leone et al., 2015). Interestingly, several regions of the brain including the cortex, nucleus accumbens, amygdala, and caudate putamen demonstrate cyclic expression of the metabotropic glutamate receptor 5 (mGluR5) (Elmenhorst et al., 2016). Furthermore, mGluR5 knockout animals exhibit altered sleep-wake architecture, marked by reduced REM sleep time during the light phase with shorter bouts of REM sleep and reduced state transitions in the non-REM sleep-REM cycle (Ahnaou et al., 2015). Moreover, these animals exhibited reduced slow wave activity and sleep drive after sleep deprivation, demonstrating that like adenosine, glutamate and mGluR5 are important for regulating the phase and amplitude of the sleep-wake cycle (Ahnaou et al., 2015). Importantly, mGluR5 has been demonstrated to form heterodimers with the A2AR within the striatum (**Figure 3**), where complex interactions between adenosine and glutamate signaling and cyclic circadian oscillations in purinergic and glutamatergic signaling modulate behaviors related to motivation and reward (Ferré et al., 2002; Brown et al., 2012). Other studies have demonstrated that both the type 2 and 3 metabotropic glutamate receptors (GluR2 and GluR3) are also important for regulating the circadian sleep cycle. For example, double knockout animals exhibit altered activity

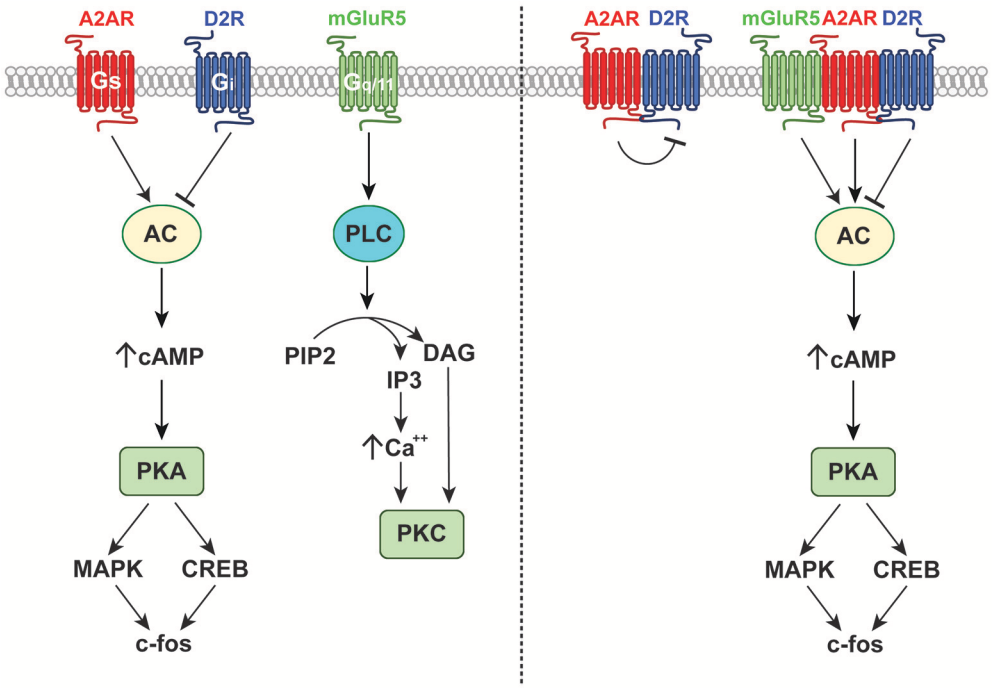

FIGURE 3 | Receptor and signaling interactions of the A2AR. The A2AR forms functional A2A-D2 receptor dimers as well as mGluR5-A2A-D2 receptor heteromers. These interactions affect binding with endogenous ligands and modulate downstream signaling events as shown.

cycles, marked by a decrease in immobility-determined sleep time, increased sleep fragmentation, and heightened sensitivity to the circadian effects of light (Pritchett et al., 2015). Given that adenosine has been demonstrated to gate glutamatergic photic input into the SCN and modulate non-photic circadian cycles of glutamatergic and non-glutamatergic transmission controlling sleep and activity, it is possible that adenosine acts to refine the relative sensitivity to numerous convergent neurotransmitter systems and that multiple different neurotransmitters act in concert to set the phase and amplitude of circadian rhythms.

A 2015 report by Morioka and colleagues partially demonstrated the complex interactions of multiple neurotransmitter systems in influencing the molecular clock. Simultaneous treatment of cultured astrocytes with glutamate, serotonin, and dopamine led to induction of *Per1* expression and a delayed increase of *Bmal1* expression and a decrease of *Cry1* expression (Morioka et al., 2015). In contrast, individual treatment with any one of these transmitters led only to a transient increase in Per1 expression, which was not maintained through further modulation of the molecular clock (Morioka et al., 2015). These results demonstrate the complex neurotransmitter interactions that govern circadian rhythms and demonstrate that dopamine may also play an important function in circadian control. Other studies have extended this finding, suggesting that the dopaminergic system is reciprocally regulated by the circadian clock and also mediates some cyclic circadian behaviors. This is exemplified by patients suffering from Parkinsonian dopamine deficiency, who often present with altered sleep-wake cycles and impaired entrainment to external lighting cues (Videnovic and Golombek, 2013). Like most neurotransmitters, dopamine undergoes circadian oscillations in extracellular abundance, peaking during the middle of the circadian night and reaching a trough during the circadian day (Ferris et al., 2014). Interestingly, these rhythms are in phase with circadian cycles of both the dopamine D2 receptor (D2R) and A2AR within the caudate putamen (**Figure 3**; Weber et al., 2004). Dopamine rhythms within regions of the brain related to reward and goal-directed behavior, including the nucleus accumbens and caudate putamen, mediate behavioral rhythms such as food anticipation and reward seeking behavior (Gallardo et al., 2014; Parekh et al., 2015). Moreover, rewarding environmental stimuli can entrain circadian dopamine rhythms and produce parallel phase changes in clock gene expression and extracellular adenosine (Kosobud et al., 2007; Angeles-Castellanos et al., 2008; Gillman et al., 2008). Together, these findings support the concept that dopamine, adenosine, and the circadian clock exist in a reciprocal relationship that coordinates reward-based consummatory behaviors, which may be altered by pathologic dysfunction such as substance use disorder (SUD) and AUD.

Numerous studies have demonstrated that adenosine and dopamine receptors form heterodimers that alter the activity and/or affinity of both receptors (Ferré et al., 2007). Within the striatum, dopamine D1 receptors (D1Rs) form heterodimers with adenosine A1 receptors (A1Rs), resulting in impaired D1R signaling (Ginés et al., 2000). This heterodimerization is promoted by A1R agonists, which impair D1R-mediated increases in cAMP, and is prevented by pretreatment with D1R agonists (Ginés et al., 2000). These results suggest that the

precise phase relationship of circadian adenosine and dopamine oscillations may dictate the precise response to either transmitter, and may permit changes in downstream signaling events without altering absolute neurotransmitter levels. Like A1R and D1R, dopamine D2R form heterodimers with adenosine A2AR, resulting in allosteric antagonism upon activation or inhibition of the purinergic receptor (Ferre et al., 1991; Dasgupta et al., 1996). Specifically, A2AR agonists and antagonists inhibit D2R activation, minimizing G_i signaling and promoting the activation of inhibitory GABAergic medium spiny neurons that affect action selection and reward-based decision making (Ferré et al., 1993; Strömberg et al., 2000). Conversely, D2R signaling via G_i directly counteracts A2AR-mediated increases in cAMP driven by G_s activation (Kull et al., 1999; Hillion et al., 2002). Therefore, given that A2AR and D2Rs undergo circadian oscillations, it is possible that shifting the phase of any one of these rhythms could drastically alter synaptic responses to multiple endogenous ligands or external stimuli in a manner dependent upon the current ratio of A2AR to D2R. Additional complexity arises from the fact that stimulation of GluR5 receptors potentiates the antagonistic effect of A2AR on D2R binding, suggesting the existence of mGluR5-A2A-D2 receptor heteromers (Popoli et al., 2001; Ferré et al., 2002). These complex receptors may mediate some synergistic actions of A2AR and mGluR5, including mGluR5R-mediated potentiation of A2AR signaling via MAPK and PKA with phosphorylation of dopamine- and cAMP-regulated neuronal phosphoprotein 32 (DARPP-32) (Popoli et al., 2001; Nishi et al., 2003). This may allow co-stimulation with glutamate and adenosine to successfully overcome tonic D2R-mediated inhibition of the indirect striatal pathway to induce activity-dependent changes in gene expression. In fact, central coadministration of selective A2AR and mGluR5 agonists induces an increase in the striatal expression of *c-fos*, which was unachievable by individual activation of either receptor type (Popoli et al., 2001; **Figure 3**).

Taken together, these findings demonstrate that multiple neural pathways and neurotransmitter systems converge to affect the molecular circadian clock and mediate rhythmic circadian behaviors. Under normal physiological conditions or mild external assault, this extraordinary convergence may underlie the tremendous stability and fidelity of the circadian system. However, this confluence of signaling pathways also provides an abundance of entry points by which external stimuli may modulate or adversely affect the circadian clock. Adenosine and ATP appear relatively unique in their nearly ubiquitous role of modulating other neurotransmitter systems that provide input into the molecular clock. As such, it is possible that purinergic signaling modulates the gain of individual clock inputs and minimizes the damage induced by disruption of any single neurotransmitter system. Conversely, ethanol and other drugs of abuse that adversely affect the purinergic system may induce dysfunction in disparate signaling pathways. Similarly, the circadian clock exerts divergent effects on dopaminergic, glutamatergic, and purinergic signaling pathways. Although this serves as an effective means to amplify and convey circadian signals, pathological disruption of the clock may be exaggerated and perpetuated by maladaptive downstream events. Likewise, this divergence may allow circadian disruption to affect many behaviors mediated by glutamatergic and dopaminergic signaling but only tangentially related to the circadian clock. Importantly, these neurotransmitter systems as well as the molecular clock itself are coordinated by the activity of both neurons and astrocytes, which directly contribute to circadian synaptic signaling and may be involved in the pathophysiology of AUD.

NEURON-ASTROCYTE INTERACTIONS IN THE REGULATION OF CIRCADIAN RHYTHMS AND ETHANOL RESPONSE

Although we typically consider synaptic transmission in terms of its presynaptic and post-synaptic neuronal components, a third player is critically important to neuromodulation and synaptic signaling. Astrocytes are multifunctional cells that play a significant role in neurogenesis, bioenergetics, neurotransmission, immune response, amino acid neurotransmitter clearance, and ionic homeostasis (Ransom and Ransom, 2012). These glial cells are 10 times more abundant than neurons within the CNS and have heterogeneous morphology and gene expression profiles dependent upon their location, neuronal contacts, and overall microenvironment (Pannasch and Rouach, 2013; Heller and Rusakov, 2015). This variability enables astrocytes to function effectively in a variety of roles throughout the brain. Synaptic transmission is regulated by a "tripartite synapse" consisting of a presynaptic neuron, post-synaptic neuron, and perisynaptic astrocyte (Araque et al., 1999). Astrocytic processes in close proximity to the synaptic milieu remove glutamate and other neurotransmitters that spill over from the synaptic cleft, protecting neurons from excitotoxicity, and inducing a wide variety of gliotransmission events that may release glutamate, ATP, adenosine, and other signaling molecules (Peters et al., 1991; Diamond, 2005; Rusakov et al., 2011; Ransom and Ransom, 2012). Furthermore, recent evidence suggests that astrocytes are at least partially responsible for regulating molecular circadian rhythms both within the SCN as well as within other regions of the CNS more directly involved in behavioral output (Jackson, 2011; Marpegan et al., 2011; Brancaccio et al., 2017). Importantly, ethanol has also been demonstrated to modulate both neuron and astrocyte-mediated purinergic and glutamatergic signaling (Rossetti and Carboni, 1995; Dahchour and De Witte, 1999; Choi et al., 2004; Nam et al., 2011; Wu et al., 2011). Thus, accounting for astrocytic influence on neurotransmission may permit a more comprehensive understanding of the synaptic and extrasynaptic signaling events that contribute to circadian disruption and neuropsychiatric conditions such as AUD.

As previously explained, long-term imaging studies revealed the presence of circadian oscillations of intracellular calcium in astrocytes, peaking during the circadian night and reaching a trough during daytime (Brancaccio et al., 2017). These calcium oscillations were found to be in phase with circadian rhythms of extracellular glutamate, which were abolished following pharmacological inhibition of astrocytic glutamate catabolism or genetic ablation of astrocytes (Brancaccio et al., 2017).

Moreover, disruption of astrocytic glutamate release altered neuronal Per2 expression and adversely affected circadian rhythmicity within the SCN (Brancaccio et al., 2017). Conversely, glutamate uptake and mRNA levels of the glutamate-aspartate transporter (GLAST) were significantly reduced in both *Clock* and *Per2* mutant astrocytes (Beaule et al., 2009). Interestingly, astrocytes also undergo circadian oscillations of ATP release, the phase of which approximates the phase of astrocyte-mediated glutamate release (Womac et al., 2009; Burkeen et al., 2011; Marpegan et al., 2011). These ATP cycles are dependent upon the circadian clock and are abolished by mutation of the *Clock* or *Per2* genes (Marpegan et al., 2011). Other studies found that astrocytic deletion of the clock component *Bmal* lengthened the period of the neuronal molecular clock and induced parallel changes in the free-running period of mutant mice (Tso et al., 2017). These studies indicate that astrocytes and the astrocytic circadian clock are instrumental for regulating the neuronal circadian clock and may mediate some forms of circadian behavior. Moreover, astrocytes are intricately involved in the regulation of glutamatergic and purinergic neurotransmission, which converge to regulate and are reciprocally regulated by the circadian clock.

Although some glial cells may release glutamate into the synaptic environment via calcium-dependent gliotransmission, astrocytes primarily regulate glutamatergic transmission by clearing glutamate from the synaptic cleft (Ransom and Ransom, 2012). Approximately 90% of extracellular glutamate is taken up by the type 2 excitatory amino acid transporter (EAAT2; known as GLT1 in rodents), which is primarily expressed on the astrocytic plasma membrane (Bergles and Jahr, 1997; Rusakov et al., 2011). EAATs can transport both glutamate and aspartate. When active, they exchange one glutamate molecule as well as three sodium ions and one proton for a single ion of potassium (Yernool et al., 2004). As the primary EAAT subtype expressed in the striatum, EAAT2 has become a transporter of interest in the field of addiction research, especially since changes in EAAT2 surface expression have been shown to influence neurotransmission (Murphy-Royal et al., 2015). Following EAAT2-mediated synaptic clearance, glutamate is converted to glutamine by glutamine synthetase, which is primarily expressed in astrocytic processes that abut glutamatergic synapses (Derouiche and Frotscher, 1991). This glutamine is subsequently released from astrocytes and taken back into presynaptic neurons, where it is converted back to glutamate and repackaged into synaptic vesicles (Coulter and Eid, 2012). Alternatively, astrocytic glutamate may be packaged into vesicles and released by astrocytes as a synaptic gliotransmitter, or be utilized as a bioenergetic substrate of the TCA cycle following conversion to alpha-ketoglutarate by glutamate dehydrogenase (Ransom and Ransom, 2012). Importantly, both glutamate uptake and GS activity display circadian variability within the SCN, peaking during the light phase and reaching a trough during the circadian night (Leone et al., 2015). Moreover, ethanol has been demonstrated to induce the expression of EAAT1 and EAAT2 in organotypic cortical slice cultures (Zink et al., 2004). This suggests that the normal circadian variation in glutamate uptake and GS activity, which may modulate the pool of astrocytic glutamate and thereby control the concentration of glutamate within astrocytes as well as the relative proportion utilized for gliotransmission, energy production, and neuronal glutamine supply, may be disrupted or altered by ethanol. Such disruption may result in altered glutamatergic signaling or dysfunctional circadian glutamate rhythms, which may contribute to the behavioral manifestations of AUD.

Astrocytic regulation of extracellular glutamate is critical for the maintenance of synaptic function. Acutely, ethanol affects GABAergic and glutamatergic neurotransmission. As the primary inhibitory neurotransmitter in the brain, GABA typically inhibits the firing of other neurons, preventing the release of their stored transmitter pools. Acute ethanol exposure potentiates GABAergic transmission and directly alters glutamatergic signaling by inhibiting the activity of NMDA receptors (Ferre and O'brien, 2011). In alcohol withdrawal syndrome (AWS) there is a rebound from suppressed glutamatergic activity and a disruption of glutamate release regulation, resulting in increased extracellular glutamate and increased potential for excitotoxicity. For example, during alcohol withdrawal, extracellular glutamate levels are increased in the striatum and hippocampus (Rossetti and Carboni, 1995; Dahchour and De Witte, 1999). Furthermore, increased extracellular glutamate in the nucleus accumbens has been shown to increase ethanol drinking in ethanol dependent mice (Griffin et al., 2014). Although not circadian in nature, this creates a maladaptive cycle of glutamatergic unbalance and behavioral resolution. More specifically, ethanol withdrawal induces a hyperglutamatergic state that is resolved by ethanol drinking, which suppresses glutamate release by enhancing GABAergic and suppressing glutamatergic signaling until ethanol is metabolized and withdrawal returns.

Adenosine is an important regulator of glutamate signaling in both acute and chronic ethanol exposure (Lindberg et al., 2015). Astrocytes are a notable source of extracellular adenosine, which may regulate transporter-mediated adenosine release from both astrocytes and neurons (**Figure 4**). Furthermore, adenosine signaling via A1Rs has been shown to alter the expression and activity of EAAT2 and to influence ethanol consumption via downstream effects on NMDA receptors and ENT1 (Nam et al., 2011; Wu et al., 2011). Interestingly, inhibition or downregulation of ENT1 reduces the expression of EAAT2, while ENT1 overexpression increases the expression of EAAT2 and enhances glutamate uptake (Wu et al., 2010). Astrocytes abundantly express ENT1, which facilitates the diffusion of nucleosides across the plasma membrane. Although acute ethanol exposure inhibits ENT1-mediated adenosine uptake, chronic ethanol exposure down-regulates ENT1 expression and impairs nucleoside transport (Asatryan et al., 2011). Importantly, ENT1 null mice exhibit increased ethanol preference as well as reduced ethanol-induced ataxia and diminished hypnotic effects (Choi et al., 2004). These results suggest that purinergic and glutamatergic signaling mechanisms converge to control drinking behavior, perhaps by modulating the sensitivity to the intoxicating effects of ethanol. Therefore, as important regulators of purinergic signaling, astrocytes are uniquely poised to modulate multiple pathways of neurotransmission. Furthermore, astrocytic modulation of rhythmic synaptic transmission and

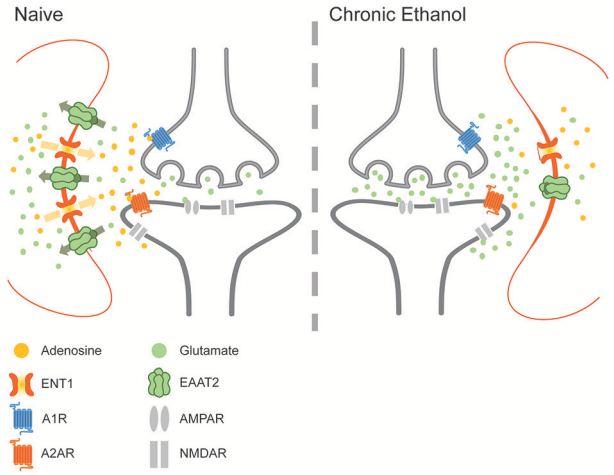

FIGURE 4 | The effects of chronic ethanol exposure on ENT1-mediated adenosine signaling. Ethanol downregulates ENT1 and decreases adenosine in the synaptic cleft, impairing adenosine-mediated inhibition of glutamate release and increasing the concentration of glutamate within the synaptic environment during withdrawal.

purinergic modulation suggests that these cells may play an important role in the development and manifestation of addiction and withdrawal.

CLINICAL PERSPECTIVES AND CONCLUSION

Substance use disorder (SUD) can be viewed as robust maladaptive cycles of use and withdrawal. By disrupting circadian oscillations of neurotransmission and astrocytic modulation, drugs of abuse may hijack the powerful endogenous rhythms that mediate our daily thoughts and actions. Moreover, ethanol may induce novel persistent oscillations of GABAergic, dopaminergic, glutamatergic, and purinergic neurotransmission, which contribute to the intermittent craving, anticipation, and impulsivity underlying AUD. As such, effective pharmacological interventions for AUD may help to bolster or reset natural circadian rhythms in order to more effectively compete with the aberrant artificial oscillations induced by ethanol. Alternatively, these interventions could specifically disrupt ethanol-induced rhythms or incite global disruption of oscillatory transmission in the hopes that natural rhythms will be restored when the cycles resume.

Current treatment options for AUD are limited, and span the spectrum from archaic to experimental. Disulfiram, sold under the trade name Antabuse, was first approved by the FDA more than 50 years ago (Suh et al., 2006). Its pharmacologic efficacy is based upon its inhibition of the acetaldehyde dehydrogenase enzyme, resulting in the accumulation of the emetic acetaldehyde (Brewer, 1984). Accordingly, the neural mechanisms underlying disulfiram treatment may be similar to those responsible for aversion therapy. Namely, oscillating anticipatory cycles mediated by glutamate and dopamine may be disrupted and replaced by a novel negative association, which blunts the exaggerated amplitude of neurotransmitter rhythms and thereby facilitates their normalization. Alternatively, some studies have demonstrated that disulfiram may also be effective in treating cocaine addiction through off-target inhibition of dopamine-beta-hydroxylase, increasing the ratio of dopaminergic to noradrenergic tone and effectively attenuating drug-primed reinstatement of cocaine seeking (Schroeder et al., 2010).

Like disulfiram, naltrexone acts to reduce the reinforcing properties of ethanol and other drugs of abuse (Gianoulakis et al., 1996). In the CNS, naltrexone acts as an antagonist to mu and kappa opioid receptors, and is known to modulate both dopaminergic and glutamatergic signaling within the mesolimbic pathway (Spanagel et al., 1992; Lee et al., 2005; Chartoff and Connery, 2014). Interestingly, some studies have demonstrated that naltrexone may also upregulate the expression of A1Rs (Bailey et al., 2003). This suggests that naltrexone and other opioid receptor antagonists may effectively modulate the phase or amplitude of glutamatergic, dopaminergic, and purinergic oscillations in the striatum. Such phasic realignment may alter the neurological response to rewarding stimuli and reduce the neurological drive to drink.

Acamprosate is a synthetic analog of the endogenous amino acid taurine (Dahchour and De Witte, 2000). Although its precise mechanism of action is unknown, acamprosate has been demonstrated to alter GABAergic transmission as well as modulate the activity of the NMDA receptor (Dahchour and De Witte, 2000). Acamprosate is known to reduce withdrawal-induced excitotoxicity, and may also affect the action other neurotransmitters such as dopamine (Dahchour and De Witte, 2000).

Topiramate is an anticonvulsant medication that has demonstrated some efficacy for the treatment of AUD (Kenna et al., 2009). This sulfate-substituted monosaccharide is thought to primarily act by modulating the action of voltage-gated sodium channels, but has also been demonstrated to affect high-voltage activated calcium channels, GABA-A receptors, and AMPA receptors in the CNS (Johnson and Ait-Daoud, 2010). These channels and receptors are instrumental for the function of all neurons, and may cause diverse effects on multiple neural circuits, potentially correcting neurotransmitter imbalances or maladaptive phasic interference between different oscillatory signaling systems.

A common theme among AUD treatments is the modulation of multiple neurotransmitter systems at both the neuronal and astrocytic levels. This is the case even for purely experimental treatments such as ceftriaxone and fibrates, which may alter transcriptional regulation of multiple proteins involved in neurotransmission and synaptic plasticity (Sari et al., 2011; Abulseoud et al., 2014; Ferguson et al., 2014). As discussed, chronic alcohol abuse broadly disrupts synaptic signaling, altering dopaminergic, glutamatergic, GABAergic, and purinergic systems, and inducing maladaptive changes in the phase and amplitude of circadian oscillations. This may create phasic interference between oscillatory neurotransmitter systems and thereby promote pathologic changes in synaptic plasticity and behavior. Such magnitude of global disruption may make

it unfeasible to target any single receptor or neurotransmitter system in order to effectively treat AUD, perhaps justifying the use of medications or treatments which more broadly affect diverse signaling systems or the circadian clock. This logic has proven moderately useful for treatment of severe depression, which is significantly although transiently mitigated by sleep deprivation therapy (Dopierała and Rybakowski, 2015). Similar treatment options targeting broad behavioral patterns of sleep and activity or largely interrupting or arresting circadian and non-circadian rhythms of multiple forms of neurotransmission may act as effective "neurologic defibrillators" that cease maladaptive or misaligned neurochemical oscillations and permit the restoration of healthy rhythms of neurotransmission and behavior. Alternatively, specific targeting of adenosine or other neurotransmitters that are highly integrated with multiple signaling systems and affect both neuronal and astrocytic function may effectively reset and realign molecular and signaling rhythms adversely affected in AUD. Moreover, the efficacy of these targeted or untargeted treatments may be dependent upon the time of administration. This is because any targeted therapy may have variable affects depending on the current phase of the targeted signaling pathway. This is especially true for signaling systems that are highly dependent upon the circadian clock such as ATP and adenosine. Thus, experimental treatment options for AUD must take into consideration the extraordinary integration of purinergic signaling with neuronal and astrocytic function as well as the time-dependent oscillations of these signaling systems and aim to mitigate the robust and all-consuming cycles of neurotransmission and behavioral cravings that underlie AUD.

AUTHOR CONTRIBUTIONS

DL, LA-B, Y-FJ, SK, and D-SC wrote the manuscript. DL and SK prepared figures. D-SC reviewed and edited the manuscript.

ACKNOWLEDGMENTS

This work was supported by the Samuel C. Johnson for Genomics of Addiction Program at Mayo Clinic, the Ulm Foundation, the Godby Foundation, the David Lehr Research Award from American Society for Pharmacology and Experimental Therapeutics, and National Institute on Alcohol Abuse and Alcoholism (AA018779).

REFERENCES

Abarca, C., Albrecht, U., and Spanagel, R. (2002). Cocaine sensitization and reward are under the influence of circadian genes and rhythm. *Proc. Natl. Acad. Sci. U.S.A.* 99, 9026–9030. doi: 10.1073/pnas.142039099

Abulseoud, O. A., Camsari, U. M., Ruby, C. L., Kasasbeh, A., Choi, S., and Choi, D. S. (2014). Attenuation of ethanol withdrawal by ceftriaxone-induced upregulation of glutamate transporter EAAT2. *Neuropsychopharmacology* 39, 1674–1684. doi: 10.1038/npp.2014.14

Ahnaou, A., Raeymaekers, L., Steckler, T., and Drinkenbrug, W. H. (2015). Relevance of the metabotropic glutamate receptor (mGluR5) in the regulation of NREM-REM sleep cycle and homeostasis: evidence from mGluR5 (-/-) mice. *Behav. Brain Res.* 282, 218–226. doi: 10.1016/j.bbr.2015.01.009

Albrecht, U. (2013). Circadian clocks and mood-related behaviors. *Handb. Exp. Pharmacol.* 217, 227–239. doi: 10.1007/978-3-642-25950-0_9

Andretic, R., Chaney, S., and Hirsh, J. (1999). Requirement of circadian genes for cocaine sensitization in Drosophila. *Science* 285, 1066–1068. doi: 10.1126/science.285.5430.1066

Angeles-Castellanos, M., Salgado-Delgado, R., Rodríguez, K., Buijs, R. M., and Escobar, C. (2008). Expectancy for food or expectancy for chocolate reveals timing systems for metabolism and reward. *Neuroscience* 155, 297–307. doi: 10.1016/j.neuroscience.2008.06.001

Antle, M. C., Steen, N. M., and Mistlberger, R. E. (2001). Adenosine and caffeine modulate circadian rhythms in the Syrian hamster. *Neuroreport* 12, 2901–2905. doi: 10.1097/00001756-200109170-00029

Araque, A., Parpura, V., Sanzgiri, R. P., and Haydon, P. G. (1999). Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci.* 22, 208–215. doi: 10.1016/S0166-2236(98)01349-6

Asatryan, L., Nam, H. W., Lee, M. R., Thakkar, M. M., Saeed Dar, M., Davies, D. L., et al. (2011). Implication of the purinergic system in alcohol use disorders. *Alcohol. Clin. Exp. Res.* 35, 584–594. doi: 10.1111/j.1530-0277.2010.01379.x

Bailey, A., Hawkins, R. M., Hourani, S. M., and Kitchen, I. (2003). Quantitative autoradiography of adenosine receptors in brains of chronic naltrexone-treated mice. *Br. J. Pharmacol.* 139, 1187–1195. doi: 10.1038/sj.bjp.0705340

Baranger, D. A., Ifrah, C., Prather, A. A., Carey, C. E., Corral-Frias, N. S., Drabant Conley, E., et al. (2016). PER1 rs3027172 genotype interacts with early life stress to predict problematic alcohol use, but not reward-related ventral striatum activity. *Front. Psychol.* 7:464. doi: 10.3389/fpsyg.2016.00464

Bass, J., and Takahashi, J. S. (2010). Circadian integration of metabolism and energetics. *Science* 330, 1349–1354. doi: 10.1126/science.1195027

Beaule, C., Swanstrom, A., Leone, M. J., and Herzog, E. D. (2009). Circadian modulation of gene expression, but not glutamate uptake, in mouse and rat cortical astrocytes. *PLoS ONE* 4:e7476. doi: 10.1371/journal.pone.0007476

Bergles, D. E., and Jahr, C. E. (1997). Synaptic activation of glutamate transporters in hippocampal astrocytes. *Neuron* 19, 1297–1308. doi: 10.1016/S0896-6273(00)80420-1

Blomeyer, D., Buchmann, A. F., Lascorz, J., Zimmermann, U. S., Esser, G., Desrivieres, S., et al. (2013). Association of PER2 genotype and stressful life events with alcohol drinking in young adults. *PLoS ONE* 8:e59136. doi: 10.1371/journal.pone.0059136

Brancaccio, M., Patton, A. P., Chesham, J. E., Maywood, E. S., and Hastings, M. H. (2017). Astrocytes control circadian timekeeping in the suprachiasmatic nucleus via glutamatergic signaling. *Neuron 93*, 1420 e1425–1435 e1425. doi: 10.1016/j.neuron.2017.02.030

Brewer, C. (1984). How effective is the standard dose of disulfiram? A review of the alcohol-disulfiram reaction in practice. *Br. J. Psychiatry* 144, 200–202. doi: 10.1192/bjp.144.2.200

Brower, K. J. (2001). Alcohol's effects on sleep in alcoholics. *Alcohol Res. Health* 25, 110–125.

Brown, R. M., Duncan, J. R., Stagnitti, M. R., Ledent, C., and Lawrence, A. J. (2012). mGlu5 and adenosine A2A receptor interactions regulate the conditioned effects of cocaine. *Int. J. Neuropsychopharmacol.* 15, 995–1001. doi: 10.1017/S146114571100126X

Burgess, H. J., Rizvydeen, M., Fogg, L. F., and Keshavarzian, A. (2016). A single dose of alcohol does not meaningfully alter circadian phase advances and phase delays to light in humans. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 310, R759–R765. doi: 10.1152/ajpregu.00001.2016

Burke, T. M., Markwald, R. R., Mchill, A. W., Chinoy, E. D., Snider, J. A., Bessman, S. C., et al. (2015). Effects of caffeine on the human circadian clock *in vivo* and *in vitro*. *Sci. Transl. Med.* 7, 305ra146. doi: 10.1126/scitranslmed.aac5125

Burkeen, J. F., Womac, A. D., Earnest, D. J., and Zoran, M. J. (2011). Mitochondrial calcium signaling mediates rhythmic extracellular ATP accumulation in suprachiasmatic nucleus astrocytes. *J. Neurosci.* 31, 8432–8440. doi: 10.1523/JNEUROSCI.6576-10.2011

Burnstock, G. (2007). Physiology and pathophysiology of purinergic neurotransmission. *Physiol. Rev.* 87, 659–797. doi: 10.1152/physrev.00043.2006

Burnstock, G. (2008). Purinergic signalling and disorders of the central nervous system. *Nat. Rev. Drug Discov.* 7, 575–590. doi: 10.1038/nrd2605

Chagoya de Sánchez, V. (1995). Circadian variations of adenosine and of its metabolism. Could adenosine be a molecular oscillator for circadian rhythms? *Can. J. Physiol. Pharmacol.* 73, 339–355. doi: 10.1139/y95-044

Chartoff, E. H., and Connery, H. S. (2014). It's MORe exciting than mu: crosstalk between mu opioid receptors and glutamatergic transmission in the mesolimbic dopamine system. *Front. Pharmacol.* 5:116. doi: 10.3389/fphar.2014.00116

Chen, C. P., Kuhn, P., Advis, J. P., and Sarkar, D. K. (2004). Chronic ethanol consumption impairs the circadian rhythm of pro-opiomelanocortin and period genes mRNA expression in the hypothalamus of the male rat. *J. Neurochem.* 88, 1547–1554. doi: 10.1046/j.1471-4159.2003.02300.x

Choi, D. S., Cascini, M. G., Mailliard, W., Young, H., Paredes, P., McMahon, T., et al. (2004). The type 1 equilibrative nucleoside transporter regulates ethanol intoxication and preference. *Nat. Neurosci.* 7, 855–861. doi: 10.1038/nn1288

Coulter, D. A., and Eid, T. (2012). Astrocytic regulation of glutamate homeostasis in epilepsy. *Glia* 60, 1215–1226. doi: 10.1002/glia.22341

Dahchour, A., and De Witte, P. (1999). Effect of repeated ethanol withdrawal on glutamate microdialysate in the hippocampus. *Alcohol. Clin. Exp. Res.* 23, 1698–1703. doi: 10.1111/j.1530-0277.1999.tb04063.x

Dahchour, A., and De Witte, P. (2000). Ethanol and amino acids in the central nervous system: assessment of the pharmacological actions of acamprosate. *Prog. Neurobiol.* 60, 343–362. doi: 10.1016/S0301-0082(99)00031-3

Damaggio, A. S., and Gorman, M. R. (2014). The circadian timing system in ethanol consumption and dependence. *Behav. Neurosci.* 128, 371–386. doi: 10.1037/a0036408

Dasgupta, S., Ferré, S., Kull, B., Hedlund, P. B., Finnman, U. B., Ahlberg, S., et al. (1996). Adenosine A2A receptors modulate the binding characteristics of dopamine D2 receptors in stably cotransfected fibroblast cells. *Eur. J. Pharmacol.* 316, 325–331. doi: 10.1016/S0014-2999(96)00665-6

Derouiche, A., and Frotscher, M. (1991). Astroglial processes around identified glutamatergic synapses contain glutamine synthetase: evidence for transmitter degradation. *Brain Res.* 552, 346–350. doi: 10.1016/0006-8993(91)90103-3

Diamond, J. S. (2005). Deriving the glutamate clearance time course from transporter currents in CA1 hippocampal astrocytes: transmitter uptake gets faster during development. *J. Neurosci.* 25, 2906–2916. doi: 10.1523/JNEUROSCI.5125-04.2005

Dong, L., Bilbao, A., Laucht, M., Henriksson, R., Yakovleva, T., Ridinger, M., et al. (2011). Effects of the circadian rhythm gene period 1 (per1) on psychosocial stress-induced alcohol drinking. *Am. J. Psychiatry* 168, 1090–1098. doi: 10.1176/appi.ajp.2011.10111579

Dopierała, E., and Rybakowski, J. (2015). Sleep deprivation as a method of chronotherapy in the treatment of depression. *Psychiatr. Pol.* 49, 423–433. doi: 10.12740/PP/30455

Eckel-Mahan, K., and Sassone-Corsi, P. (2013). Metabolism and the circadian clock converge. *Physiol. Rev.* 93, 107–135. doi: 10.1152/physrev.00016.2012

Elmenhorst, D., Mertens, K., Kroll, T., Oskamp, A., Ermert, J., Elmenhorst, E. M., et al. (2016). Circadian variation of metabotropic glutamate receptor 5 availability in the rat brain. *J. Sleep Res.* 25, 754–761. doi: 10.1111/jsr.12432

Ferguson, L. B., Most, D., Blednov, Y. A., and Harris, R. A. (2014). PPAR agonists regulate brain gene expression: relationship to their effects on ethanol consumption. *Neuropharmacology* 86, 397–407. doi: 10.1016/j.neuropharm.2014.06.024

Ferré, S., Ciruela, F., Quiroz, C., Luján, R., Popoli, P., Cunha, R. A., et al. (2007). Adenosine receptor heteromers and their integrative role in striatal function. *ScientificWorldJournal.* 7, 74–85. doi: 10.1100/tsw.2007.211

Ferré, S., Karcz-Kubicha, M., Hope, B. T., Popoli, P., Burgueño, J., Gutiérrez, M. A., et al. (2002). Synergistic interaction between adenosine A2A and glutamate mGlu5 receptors: implications for striatal neuronal function. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11940–11945. doi: 10.1073/pnas.172393799

Ferre, S., and O'brien, M. C. (2011). Alcohol and caffeine: the perfect storm. *J. Caffeine Res.* 1, 153–162. doi: 10.1089/jcr.2011.0017

Ferré, S., O'connor, W. T., Fuxe, K., and Ungerstedt, U. (1993). The striopallidal neuron: a main locus for adenosine-dopamine interactions in the brain. *J. Neurosci.* 13, 5402–5406.

Ferre, S., von Euler, G., Johansson, B., Fredholm, B. B., and Fuxe, K. (1991). Stimulation of high-affinity adenosine A2 receptors decreases the affinity of dopamine D2 receptors in rat striatal membranes. *Proc. Natl. Acad. Sci. U.S.A.* 88, 7238–7241. doi: 10.1073/pnas.88.16.7238

Ferris, M. J., España, R. A., Locke, J. L., Konstantopoulos, J. K., Rose, J. H., Chen, R., et al. (2014). Dopamine transporters govern diurnal variation in extracellular dopamine tone. *Proc. Natl. Acad. Sci. U.S.A.* 111, E2751–2759. doi: 10.1073/pnas.1407935111

Gallardo, C. M., Darvas, M., Oviatt, M., Chang, C. H., Michalik, M., Huddy, T. F., et al. (2014). Dopamine receptor 1 neurons in the dorsal striatum regulate food anticipatory circadian activity rhythms in mice. *Elife* 3:e03781. doi: 10.7554/eLife.03781

Gamble, K. L., Motsinger-Reif, A. A., Hida, A., Borsetti, H. M., Servick, S. V., Ciarleglio, C. M., et al. (2011). Shift work in nurses: contribution of phenotypes and genotypes to adaptation. *PLoS ONE* 6:e18395. doi: 10.1371/journal.pone.0018395

Gianoulakis, C., de Waele, J. P., and Thavundayil, J. (1996). Implication of the endogenous opioid system in excessive ethanol consumption. *Alcohol* 13, 19–23. doi: 10.1016/0741-8329(95)02035-7

Gillman, A. G., Kosobud, A. E., and Timberlake, W. (2008). Pre- and post-nicotine circadian activity rhythms can be differentiated by a paired environmental cue. *Physiol. Behav.* 93, 337–350. doi: 10.1016/j.physbeh.2007.09.013

Ginés, S., Hillion, J., Torvinen, M., Le Crom, S., Casadó, V., Canela, E. I., et al. (2000). Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes. *Proc. Natl. Acad. Sci. U.S.A.* 97, 8606–8611. doi: 10.1073/pnas.150241097

Griffin, W. C. III., Haun, H. L., Hazelbaker, C. L., Ramachandra, V. S., and Becker, H. C. (2014). Increased extracellular glutamate in the nucleus accumbens promotes excessive ethanol drinking in ethanol dependent mice. *Neuropsychopharmacology* 39, 707–717. doi: 10.1038/npp.2013.256

Hasler, B. P., Soehner, A. M., and Clark, D. B. (2014). Circadian rhythms and risk for substance use disorders in adolescence. *Curr. Opin. Psychiatry* 27, 460–466. doi: 10.1097/YCO.0000000000000107

Heller, J. P., and Rusakov, D. A. (2015). Morphological plasticity of astroglia: understanding synaptic microenvironment. *Glia* 63, 2133–2151. doi: 10.1002/glia.22821

Hillion, J., Canals, M., Torvinen, M., Casado, V., Scott, R., Terasmaa, A., et al. (2002). Coaggregation, cointernalization, and codesensitization of adenosine A2A receptors and dopamine D2 receptors. *J. Biol. Chem.* 277, 18091–18097. doi: 10.1074/jbc.M107731200

Huang, M. C., Ho, C. W., Chen, C. H., Liu, S. C., Chen, C. C., and Leu, S. J. (2010). Reduced expression of circadian clock genes in male alcoholic patients. *Alcohol. Clin. Exp. Res.* 34, 1899–1904. doi: 10.1111/j.1530-0277.2010.01278.x

Jackson, F. R. (2011). Glial cell modulation of circadian rhythms. *Glia* 59, 1341–1350. doi: 10.1002/glia.21097

Johnson, B. A., and Ait-Daoud, N. (2010). Topiramate in the new generation of drugs: efficacy in the treatment of alcoholic patients. *Curr. Pharm. Des.* 16, 2103–2112. doi: 10.2174/138161210791516404

Kenna, G. A., Lomastro, T. L., Schiesl, A., Leggio, L., and Swift, R. M. (2009). Review of topiramate: an antiepileptic for the treatment of alcohol dependence. *Curr. Drug Abuse Rev.* 2, 135–142. doi: 10.2174/1874473710902020135

Kosobud, A. E., Gillman, A. G., Leffel, J. K. II., Pecoraro, N. C., Rebec, G. V., and Timberlake, W. (2007). Drugs of abuse can entrain circadian rhythms. *ScientificWorldJournal.* 7, 203–212. doi: 10.1100/tsw.2007.234

Kosobud, A. E., Pecoraro, N. C., Rebec, G. V., and Timberlake, W. (1998). Circadian activity precedes daily methamphetamine injections in the rat. *Neurosci. Lett.* 250, 99–102. doi: 10.1016/S0304-3940(98)00439-X

Kovanen, L., Saarikoski, S. T., Haukka, J., Pirkola, S., Aromaa, A., Lönnqvist, J., et al. (2010). Circadian clock gene polymorphisms in alcohol use disorders and alcohol consumption. *Alcohol Alcohol.* 45, 303–311. doi: 10.1093/alcalc/agq035

Krueger, J. M., Taishi, P., De, A., Davis, C. J., Winters, B. D., Clinton, J., et al. (2010). ATP and the purine type 2 X7 receptor affect sleep. *J. Appl. Physiol. (1985)* 109, 1318–1327. doi: 10.1152/japplphysiol.00586.2010

Kull, B., Ferré, S., Arslan, G., Svenningsson, P., Fuxe, K., Owman, C., et al. (1999). Reciprocal interactions between adenosine A2A and dopamine D2 receptors in Chinese hamster ovary cells co-transfected with the two receptors. *Biochem. Pharmacol.* 58, 1035–1045. doi: 10.1016/S0006-2952(99)00184-7

Lee, Y. K., Park, S. W., Kim, Y. K., Kim, D. J., Jeong, J., Myrick, H., et al. (2005). Effects of naltrexone on the ethanol-induced changes in the rat central dopaminergic system. *Alcohol Alcohol.* 40, 297–301. doi: 10.1093/alcalc/agh163

Leone, M. J., Beaule, C., Marpegan, L., Simon, T., Herzog, E. D., and Golombek, D. A. (2015). Glial and light-dependent glutamate metabolism in the suprachiasmatic nuclei. *Chronobiol. Int.* 32, 573–578. doi: 10.3109/07420528.2015.1006328

Lindberg, D., Shan, D., Ayers-Ringler, J., Oliveros, A., Benitez, J., Prieto, M., et al. (2015). Purinergic signaling and energy homeostasis in psychiatric disorders. *Curr. Mol. Med.* 15, 275–295. doi: 10.2174/1566524015666150330163724

Logan, R. W., Williams, W. P. III., and McClung, C. A. (2014). Circadian rhythms and addiction: mechanistic insights and future directions. *Behav. Neurosci.* 128, 387–412. doi: 10.1037/a0036268

Marpegan, L., Swanstrom, A. E., Chung, K., Simon, T., Haydon, P. G., Khan, S. K., et al. (2011). Circadian regulation of ATP release in astrocytes. *J. Neurosci.* 31, 8342–8350. doi: 10.1523/JNEUROSCI.6537-10.2011

Mazzoccoli, G., Pazienza, V., and Vinciguerra, M. (2012). Clock genes and clock-controlled genes in the regulation of metabolic rhythms. *Chronobiol. Int.* 29, 227–251. doi: 10.3109/07420528.2012.658127

McCarthy, M. J., Fernandes, M., Kranzler, H. R., Covault, J. M., and Welsh, D. K. (2013). Circadian clock period inversely correlates with illness severity in cells from patients with alcohol use disorders. *Alcohol. Clin. Exp. Res.* 37, 1304–1310. doi: 10.1111/acer.12106

McClung, C. A., Sidiropoulou, K., Vitaterna, M., Takahashi, J. S., White, F. J., Cooper, D. C., et al. (2005). Regulation of dopaminergic transmission and cocaine reward by the Clock gene. *Proc. Natl. Acad. Sci. U.S.A.* 102, 9377–9381. doi: 10.1073/pnas.0503584102

Melendez, R. I., McGinty, J. F., Kalivas, P. W., and Becker, H. C. (2012). Brain region-specific gene expression changes after chronic intermittent ethanol exposure and early withdrawal in C57BL/6J mice. *Addict. Biol.* 17, 351–364. doi: 10.1111/j.1369-1600.2011.00357.x

Morioka, N., Sugimoto, T., Sato, K., Okazaki, S., Saeki, M., Hisaoka-Nakashima, K., et al. (2015). The induction of Per1 expression by the combined treatment with glutamate, 5-hydroxytriptamine and dopamine initiates a ripple effect on Bmal1 and Cry1 mRNA expression via the ERK signaling pathway in cultured rat spinal astrocytes. *Neurochem. Int.* 90, 9–19. doi: 10.1016/j.neuint.2015.06.013

Mukherjee, S., Coque, L., Cao, J. L., Kumar, J., Chakravarty, S., Asaithamby, A., et al. (2010). Knockdown of Clock in the ventral tegmental area through RNA interference results in a mixed state of mania and depression-like behavior. *Biol. Psychiatry* 68, 503–511. doi: 10.1016/j.biopsych.2010.04.031

Murphy-Royal, C., Dupuis, J. P., Varela, J. A., Panatier, A., Pinson, B., Baufreton, J., et al. (2015). Surface diffusion of astrocytic glutamate transporters shapes synaptic transmission. *Nat. Neurosci.* 18, 219–226. doi: 10.1038/nn.3901

Nakazato, R., Takarada, T., Yamamoto, T., Hotta, S., Hinoi, E., and Yoneda, Y. (2011). Selective upregulation of Per1 mRNA expression by ATP through activation of P2X7 purinergic receptors expressed in microglial cells. *J. Pharmacol. Sci.* 116, 350–361. doi: 10.1254/jphs.11069FP

Nam, H. W., Lee, M. R., Zhu, Y., Wu, J., Hinton, D. J., Choi, S., et al. (2011). Type 1 equilibrative nucleoside transporter regulates ethanol drinking through accumbal N-methyl-D-aspartate receptor signaling. *Biol. Psychiatry* 69, 1043–1051. doi: 10.1016/j.biopsych.2011.02.013

Nishi, A., Liu, F., Matsuyama, S., Hamada, M., Higashi, H., Nairn, A. C., et al. (2003). Metabotropic mGlu5 receptors regulate adenosine A2A receptor signaling. *Proc. Natl. Acad. Sci. U.S.A.* 100, 1322–1327. doi: 10.1073/pnas.0237126100

Ozburn, A. R., Falcon, E., Mukherjee, S., Gillman, A., Arey, R., Spencer, S., et al. (2013). The role of clock in ethanol-related behaviors. *Neuropsychopharmacology* 38, 2393–2400. doi: 10.1038/npp.2013.138

Pannasch, U., and Rouach, N. (2013). Emerging role for astroglial networks in information processing: from synapse to behavior. *Trends Neurosci.* 36, 405–417. doi: 10.1016/j.tins.2013.04.004

Parekh, P. K., Ozburn, A. R., and McClung, C. A. (2015). Circadian clock genes: effects on dopamine, reward and addiction. *Alcohol* 49, 341–349. doi: 10.1016/j.alcohol.2014.09.034

Partch, C. L., Green, C. B., and Takahashi, J. S. (2014). Molecular architecture of the mammalian circadian clock. *Trends Cell Biol.* 24, 90–99. doi: 10.1016/j.tcb.2013.07.002

Perreau-Lenz, S., Zghoul, T., de Fonseca, F. R., Spanagel, R., and Bilbao, A. (2009). Circadian regulation of central ethanol sensitivity by the mPer2 gene. *Addict. Biol.* 14, 253–259. doi: 10.1111/j.1369-1600.2009.00165.x

Peters, A., Josephson, K., and Vincent, S. L. (1991). Effects of aging on the neuroglial cells and pericytes within area 17 of the rhesus monkey cerebral cortex. *Anat. Rec.* 229, 384–398. doi: 10.1002/ar.1092290311

Pohl, J. B., Ghezzi, A., Lew, L. K., Robles, R. B., Cormack, L., and Atkinson, N. S. (2013). Circadian genes differentially affect tolerance to ethanol in Drosophila. *Alcohol. Clin. Exp. Res.* 37, 1862–1871. doi: 10.1111/acer.12173

Popoli, P., Pèzzola, A., Torvinen, M., Reggio, R., Pintor, A., Scarchilli, L., et al. (2001). The selective $mGlu_5$ receptor agonist CHPG inhibits quinpirole-induced turning in 6-hydroxydopamine-lesioned rats and modulates the binding characteristics of dopamine D_2 receptors in the rat striatum: interactions with adenosine A_{2a} receptors. *Neuropsychopharmacology* 25, 505–513. doi: 10.1016/S0893-133X(01)00256-1

Porkka-Heiskanen, T., Strecker, R. E., and McCarley, R. W. (2000). Brain site-specificity of extracellular adenosine concentration changes during sleep deprivation and spontaneous sleep: an *in vivo* microdialysis study. *Neuroscience* 99, 507–517. doi: 10.1016/S0306-4522(00)00220-7

Porkka-Heiskanen, T., Strecker, R. E., Thakkar, M., Bjorkum, A. A., Greene, R. W., and McCarley, R. W. (1997). Adenosine: a mediator of the sleep-inducing effects of prolonged wakefulness. *Science* 276, 1265–1268. doi: 10.1126/science.276.5316.1265

Pritchett, D., Jagannath, A., Brown, L. A., Tam, S. K., Hasan, S., Gatti, S., et al. (2015). Deletion of metabotropic glutamate receptors 2 and 3 (mGlu2 & mGlu3) in mice disrupts sleep and wheel-running activity, and increases the sensitivity of the circadian system to light. *PLoS ONE* 10:e0125523. doi: 10.1371/journal.pone.0125523

Rajakrishnan, V., Subramanian, P., Viswanathan, P., and Menon, V. P. (1999). Effect of chronic ethanol ingestion on biochemical circadian rhythms in Wistar rats. *Alcohol* 18, 147–152. doi: 10.1016/S0741-8329(98)00077-9

Ransom, B. R., and Ransom, C. B. (2012). Astrocytes: multitalented stars of the central nervous system. *Methods Mol. Biol.* 814, 3–7. doi: 10.1007/978-1-61779-452-0_1

Reichert, C. F., Maire, M., Schmidt, C., and Cajochen, C. (2016). Sleep-wake regulation and its impact on working memory performance: the role of adenosine. *Biology (Basel)* 5:E11. doi: 10.3390/biology5010011

Rosenwasser, A. M. (2010). Circadian clock genes: non-circadian roles in sleep, addiction, and psychiatric disorders? *Neurosci. Biobehav. Rev.* 34, 1249–1255. doi: 10.1016/j.neubiorev.2010.03.004

Rosenwasser, A. M., Fecteau, M. E., and Logan, R. W. (2005). Effects of ethanol intake and ethanol withdrawal on free-running circadian activity rhythms in rats. *Physiol. Behav.* 84, 537–542. doi: 10.1016/j.physbeh.2005.01.016

Rosenwasser, A. M., and Fixaris, M. C. (2013). Chronobiology of alcohol: studies in C57BL/6J and DBA/2J inbred mice. *Physiol. Behav.* 110–111, 140–147. doi: 10.1016/j.physbeh.2013.01.001

Rosenwasser, A. M., Fixaris, M. C., and McCulley, W. D. III. (2015). Photoperiodic modulation of voluntary ethanol intake in C57BL/6 mice. *Physiol. Behav.* 147, 342–347. doi: 10.1016/j.physbeh.2015.05.011

Rossetti, Z. L., and Carboni, S. (1995). Ethanol withdrawal is associated with increased extracellular glutamate in the rat striatum. *Eur. J. Pharmacol.* 283, 177–183. doi: 10.1016/0014-2999(95)00344-K

Roybal, K., Theobold, D., Graham, A., DiNieri, J. A., Russo, S. J., Krishnan, V., et al. (2007). Mania-like behavior induced by disruption of CLOCK. *Proc. Natl. Acad. Sci. U.S.A.* 104, 6406–6411. doi: 10.1073/pnas.0609625104

Ruby, C. L., Brager, A. J., DePaul, M. A., Prosser, R. A., and Glass, J. D. (2009). Chronic ethanol attenuates circadian photic phase resetting and alters nocturnal activity patterns in the hamster. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 297, R729–R737. doi: 10.1152/ajpregu.00268.2009

Ruby, C. L., O'connor, K. M., Ayers-Ringler, J., and Choi, D. S. (2014a). Adenosine and glutamate in neuroglial interaction: implications for circadian disorders and alcoholism. *Adv. Neurobiol.* 11, 103–119. doi: 10.1007/978-3-319-08894-5_6

Ruby, C. L., Vadnie, C. A., Hinton, D. J., Abulseoud, O. A., Walker, D. L., O'connor, K. M., et al. (2014b). Adenosinergic regulation of striatal clock gene expression and ethanol intake during constant light. *Neuropsychopharmacology* 39, 2432–2440. doi: 10.1038/npp.2014.94

Rusakov, D. A., Zheng, K., and Henneberger, C. (2011). Astrocytes as regulators of synaptic function: a quest for the Ca^{2+} master key. *Neuroscientist* 17, 513–523. doi: 10.1177/1073858410387304

Sari, Y., Sakai, M., Weedman, J. M., Rebec, G. V., and Bell, R. L. (2011). Ceftriaxone, a beta-lactam antibiotic, reduces ethanol consumption in alcohol-preferring rats. *Alcohol Alcohol.* 46, 239–246. doi: 10.1093/alcalc/agr023

Schroeder, J. P., Cooper, D. A., Schank, J. R., Lyle, M. A., Gaval-Cruz, M., Ogbonmwan, Y. E., et al. (2010). Disulfiram attenuates drug-primed reinstatement of cocaine seeking via inhibition of dopamine beta-hydroxylase. *Neuropsychopharmacology* 35, 2440–2449. doi: 10.1038/npp.2010.127

Seggio, J. A., Fixaris, M. C., Reed, J. D., Logan, R. W., and Rosenwasser, A. M. (2009). Chronic ethanol intake alters circadian phase shifting and free-running period in mice. *J. Biol. Rhythms* 24, 304–312. doi: 10.1177/0748730409338449

Sjöholm, L. K., Kovanen, L., Saarikoski, S. T., Schalling, M., Lavebratt, C., and Partonen, T. (2010). CLOCK is suggested to associate with comorbid alcohol use and depressive disorders. *J. Circadian Rhythms* 8, 1. doi: 10.1186/1740-3391-8-1

Spanagel, R., Herz, A., and Shippenberg, T. S. (1992). Opposing tonically active endogenous opioid systems modulate the mesolimbic dopaminergic pathway. *Proc. Natl. Acad. Sci. U.S.A.* 89, 2046–2050. doi: 10.1073/pnas.89.6.2046

Spanagel, R., Pendyala, G., Abarca, C., Zghoul, T., Sanchis-Segura, C., Magnone, M. C., et al. (2005a). The clock gene Per2 influences the glutamatergic system and modulates alcohol consumption. *Nat. Med.* 11, 35–42. doi: 10.1038/nm1163

Spanagel, R., Rosenwasser, A. M., Schumann, G., and Sarkar, D. K. (2005b). Alcohol consumption and the body's biological clock. *Alcohol. Clin. Exp. Res.* 29, 1550–1557. doi: 10.1097/01.alc.0000175074.70807.fd

Strömberg, I., Popoli, P., Müller, C. E., Ferré, S., and Fuxe, K. (2000). Electrophysiological and behavioural evidence for an antagonistic modulatory role of adenosine A2A receptors in dopamine D2 receptor regulation in the rat dopamine-denervated striatum. *Eur. J. Neurosci.* 12, 4033–4037. doi: 10.1046/j.1460-9568.2000.00288.x

Suh, J. J., Pettinati, H. M., Kampman, K. M., and O'Brien, C. P. (2006). The status of disulfiram: a half of a century later. *J. Clin. Psychopharmacol.* 26, 290–302. doi: 10.1097/01.jcp.0000222512.25649.08

Summa, K. C., Jiang, P., Fitzpatrick, K., Voigt, R. M., Bowers, S. J., Forsyth, C. B., et al. (2015). Chronic alcohol exposure and the circadian clock mutation exert tissue-specific effects on gene expression in mouse hippocampus, liver, and proximal colon. *Alcohol. Clin. Exp. Res.* 39, 1917–1929. doi: 10.1111/acer.12834

Summa, K. C., Voigt, R. M., Forsyth, C. B., Shaikh, M., Cavanaugh, K., Tang, Y., et al. (2013). Disruption of the circadian clock in mice increases intestinal permeability and promotes alcohol-induced hepatic pathology and inflammation. *PLoS ONE* 8:e67102. doi: 10.1371/journal.pone.0067102

Takahashi, J. S. (2015). Molecular components of the circadian clock in mammals. *Diabetes Obes Metab* 17(Suppl. 1), 6–11. doi: 10.1111/dom.12514

Trinkoff, A. M., and Storr, C. L. (1998). Work schedule characteristics and substance use in nurses. *Am. J. Ind. Med.* 34, 266–271. doi: 10.1002/(SICI)1097-0274(199809)34:3<266::AID-AJIM9>3.0.CO;2-T

Tso, C. F., Simon, T., Greenlaw, A. C., Puri, T., Mieda, M., and Herzog, E. D. (2017). Astrocytes regulate daily rhythms in the suprachiasmatic nucleus and behavior. *Curr. Biol.* 27, 1055–1061. doi: 10.1016/j.cub.2017.02.037

Urry, E., and Landolt, H. P. (2015). Adenosine, caffeine, and performance: from cognitive neuroscience of sleep to sleep pharmacogenetics. *Curr. Top. Behav. Neurosci.* 25, 331–366. doi: 10.1007/7854_2014_274

Videnovic, A., and Golombek, D. (2013). Circadian and sleep disorders in Parkinson's disease. *Exp. Neurol.* 243, 45–56. doi: 10.1016/j.expneurol.2012.08.018

Videnovic, A., Lazar, A. S., Barker, R. A., and Overeem, S. (2014). 'The clocks that time us'–circadian rhythms in neurodegenerative disorders. *Nat. Rev. Neurol.* 10, 683–693. doi: 10.1038/nrneurol.2014.206

Walsh, J. K., Humm, T., Muehlbach, M. J., Sugerman, J. L., and Schweitzer, P. K. (1991). Sedative effects of ethanol at night. *J. Stud. Alcohol* 52, 597–600. doi: 10.15288/jsa.1991.52.597

Weber, M., Lauterburg, T., Tobler, I., and Burgunder, J. M. (2004). Circadian patterns of neurotransmitter related gene expression in motor regions of the rat brain. *Neurosci. Lett.* 358, 17–20. doi: 10.1016/j.neulet.2003.12.053

White, W., Feldon, J., Heidbreder, C. A., and White, I. M. (2000). Effects of administering cocaine at the same versus varying times of day on circadian activity patterns and sensitization in rats. *Behav. Neurosci.* 114, 972–982. doi: 10.1037/0735-7044.114.5.972

Womac, A. D., Burkeen, J. F., Neuendorff, N., Earnest, D. J., and Zoran, M. J. (2009). Circadian rhythms of extracellular ATP accumulation in suprachiasmatic nucleus cells and cultured astrocytes. *Eur. J. Neurosci.* 30, 869–876. doi: 10.1111/j.1460-9568.2009.06874.x

Wu, J., Lee, M. R., Choi, S., Kim, T., and Choi, D. S. (2010). ENT1 regulates ethanol-sensitive EAAT2 expression and function in astrocytes. *Alcohol. Clin. Exp. Res.* 34, 1110–1117. doi: 10.1111/j.1530-0277.2010.01187.x

Wu, J., Lee, M. R., Kim, T., Johng, S., Rohrback, S., Kang, N., et al. (2011). Regulation of ethanol-sensitive EAAT2 expression through adenosine A1 receptor in astrocytes. *Biochem. Biophys. Res. Commun.* 406, 47–52. doi: 10.1016/j.bbrc.2011.01.104

Wurts, S. W., and Edgar, D. M. (2000). Caffeine during sleep deprivation: sleep tendency and dynamics of recovery sleep in rats. *Pharmacol. Biochem. Behav.* 65, 155–162. doi: 10.1016/S0091-3057(99)00173-2

Yernool, D., Boudker, O., Jin, Y., and Gouaux, E. (2004). Structure of a glutamate transporter homologue from Pyrococcus horikoshii. *Nature* 431, 811–818. doi: 10.1038/nature03018

Zink, M., Schmitt, A., Vengeliene, V., Henn, F. A., and Spanagel, R. (2004). Ethanol induces expression of the glutamate transporters EAAT1 and EAAT2 in organotypic cortical slice cultures. *Alcohol. Clin. Exp. Res.* 28, 1752–1757. doi: 10.1097/01.ALC.0000145810.12545.B3

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 30 October 2017
doi: 10.3389/fphys.2017.00864

Circadian Alterations in a Murine Model of Hypothalamic Glioma

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: *10 August 2017*
Accepted: *16 October 2017*
Published: *30 October 2017*

Citation:
Duhart JM, Brocardo L, Caldart CS, Marpegan L and Golombek DA (2017) Circadian Alterations in a Murine Model of Hypothalamic Glioma. Front. Physiol. 8:864. doi: 10.3389/fphys.2017.00864

The mammalian circadian system is controlled by a central oscillator located in the suprachiasmatic nuclei (SCN) of the hypothalamus, in which glia appears to play a prominent role. Gliomas originate from glial cells and are the primary brain tumors with the highest incidence and mortality. Optic pathway/hypothalamic gliomas account for 4–7% of all pediatric intracranial tumors. Given the anatomical location, which compromises both the circadian pacemaker and its photic input pathway, we decided to study whether the presence of gliomas in the hypothalamic region could alter circadian behavioral outputs. Athymic nude mice implanted with LN229 human glioma cells showed an increase in the endogenous period of the circadian clock, which was also less robust in terms of sustaining the free running period throughout 2 weeks of screening. We also found that implanted mice showed a slower resynchronization rate after an abrupt 6 h advance of the light-dark (LD) cycle, advanced phase angle, and a decreased direct effect of light in general activity (masking), indicating that hypothalamic tumors could also affect photic sensitivity of the circadian clock. Our work suggests that hypothalamic gliomas have a clear impact both on the endogenous pacemaking of the circadian system, as well as on the photic synchronization of the clock. These findings strongly suggest that the observation of altered circadian parameters in patients might be of relevance for glioma diagnosis.

Keywords: circadian, suprachiasmatic, glioma, glioblastoma

INTRODUCTION

Gliomas, a type of tumor arising from glial cells, are the most common type of primary brain tumors and are classified in four types (I-IV), according to their malignancy (Wen and Kesari, 2008). Patients suffering from type IV gliomas (also characterized as glioblastomas) account for approximately 45% of all malignant CNS tumors, with a median survival of less than 15 months (Ostrom et al., 2013; Lee, 2017). Among the common symptoms leading to glioma diagnosis, neurological distress appears as one of the earliest reasons for consultation (Weller et al., 2017). Previous reports have shown that two of the most common factors affecting the life quality of glioma patients are sleep disturbances and fatigue (Rooney et al., 2013). Brain and CNS tumors are the most common form of solid tumors in children and among them, anatomical location of hypothalamic and optic tract tumors represent 5–7% of all cases (Massimi et al., 2007; Ostrom et al., 2013). These gliomas tend to be nonaggressive low-grade tumors, with pilocytic astrocytoma being the most common pathology in young adults. However, chiasmatic-hypothalamic masses in adults are challenging to diagnose and tend to have an aggressive clinical course (Raelson and Chiang, 2015).

The hypothalamus also hosts the mammalian circadian clock, led by a master oscillator that resides in the suprachiasmatic nuclei (SCN), which receives photic entrainment cues through a retinohypothalamic tract. This clock is able to sustain oscillations with a period close to 24-h, even in the absence of external stimulation, and it can adapt to changes in the light-dark cycle through a specific synchronization pathway (Golombek and Rosenstein, 2010). Several physiological and behavioral variables, including hormone levels and neurological functions, are regulated by the circadian clock, which also plays a key role in the regulation of sleep-wake cycles (Musiek and Holtzman, 2016). Moreover, circadian disruption and misalignment between the internal clock and the environmental cycles have been associated with mood disorders and fatigue (Golombek et al., 2013; Bedrosian and Nelson, 2017).

Within the SCN, glial cells are involved in circadian timekeeping (Barca-Mayo et al., 2017; Brancaccio et al., 2017; Tso et al., 2017) and synchronization mechanisms (Lavialle et al., 2001; Becquet et al., 2008; Girardet et al., 2010; Duhart et al., 2013b), and are also considered as mediators between proinflammatory signals and the circadian pacemaker (Leone et al., 2006; Duhart et al., 2013a). Among the mechanisms proposed for glial modulation of the circadian pacemaker, the regulation of glutamate levels by SCN astrocytes is of key importance for the proper functioning of the clock (Beaulé et al., 2009; Leone et al., 2015; Brancaccio et al., 2017). Noteworthy, increased glutamate levels are characteristic in gliomas (reviewed in Robert and Sontheimer, 2014), suggesting that a dysregulation of proper glial function occurring in this malignant tissue may impact on both timekeeping and synchronization mechanisms of the clock. In addition, molecules involved in immune responses, such as TNF-α, IL-1β, and CCL2 have been shown to affect the master circadian oscillator (Nygård et al., 2009; Leone et al., 2012; Duhart et al., 2016). Gliomas drastically alter the microenvironment in which they develop, and molecules involved in the immune response have been shown to have an important role in tumor progression (Reviewed in Christofides et al., 2015).

Considering the prevalence of sleep alterations and fatigue as a common symptom of gliomas, the anatomical compromise of the master clock and optic tract in hypothalamic gliomas, and the alteration of molecules relevant for the circadian pacemaker in the tumor microenvironment, we hypothesized that this pathology could impact circadian timekeeping, and such alterations could be a valuable tool at the time of diagnosis. In the present work we analyzed the effects of hypothalamic gliomas in the endogenous characteristics of the circadian clock and its ability to synchronize to light. In mice bearing hypothalamic gliomas we found alterations in the synchronization to light-dark cycles, as well as a clear effect on the endogenous period and its stability.

MATERIALS AND METHODS

Animals

Adult Foxn1$^{(\Delta/\Delta)}$ male mice were purchased from Universidad Nacional de La Plata animal facilities (La Plata, Argentina), and were housed individually in cages, supplied with water and food *ad libitum* and kept under sterile air ventilation and 12:12 light-dark cycles (12 h of light, 12 h of darkness, LD). Animal manipulations and experimental protocols performed in this work were supervised and approved by the National University of Quilmes Institutional Animal Care and Use Committee, in accordance with the National Institutes of Health guide for the care and use of laboratory animals. After tumor implantation, animals were monitored daily in search for hunched or abnormal posture, lack of grooming, weight loss exceeding 20% of body weight, anorexia, or abnormal ambulation. The presence of any of these findings was considered an endpoint and mice were euthanized using a CO_2 chamber.

Cell Lines

Human glioblastoma cell line LN-229 (ATCC: CRL-2611) was cultured in DMEM medium (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (PAA, Germany), glutamine and antibiotic-antimicotic (Gibco).

Tumor Implantation

Two month-old animals were deeply anesthetized with 2% isofluorane and implanted with 200,000 glioma LN-229 cells in 1 μl of DMEM by stereotactic surgery. The injection was aimed unilaterally to the suprachiasmatic nucleus of the hypothalamus (coordinates from Bregma: +0.3 mm from midline; −0.1 mm anterior-posterior; −5.3 mm dorso-ventral). Control animals were treated with a Sham surgery in which the same volume of DMEM, without cells, was injected.

Behavioral Analyses

General activity of animals was continuously registered by infrared sensors with a system designed in our laboratory. Activity counts were collected every 5 min. In LD conditions, time is described as Zeitgeber Time (ZT): ZT0 refers to the time of lights on and ZT12 refers to the time of lights off. In constant darkness (DD) conditions, time is expressed as Circadian Time (CT), with CT 12 referring to the time of activity onset.

Prior to tumor implantation, mice were synchronized to 12:12 LD cycles and kept under these conditions for 25 days. After implantation, animals continued under LD 12:12 cycles for 18 days. Afterwards, animals were divided in two groups. One group, aimed to study the effects on circadian synchronization, was exposed to a 6-h advance in the LD cycle (experimental Jet-lag) by advancing the light phase and shortening of the dark phase. This group continued in LD conditions for 32 days, received a second 6-h advance in the LD cycle and continued in LD until endpoint. The second group, aimed to study the endogenous circadian parameters, was kept in DD conditions until endpoint. After sacrifice, brains were collected and processed for Hematoxilyn-Eosin (HE) staining.

Data Analysis

Circadian parameters were calculated with El Temps program (Antoni Díez Nogueira, University of Barcelona). In DD conditions, free-running endogenous period (τ) was analyzed using Sokolove-Bushell (SB) periodograms. For period stability,

a wavelet-based analysis was performed on custom-made Matlab (Mathworks) scripts. Heat maps referring to instant period were generated for each activity pattern, assessing the variability of endogenous period. Statistical significance for periods calculated by wavelet analysis were performed as described in Torrence and Compo (1998). A *variability index (V)* was defined by quantifying the complexity of the histogram of the periods obtained from the wavelet analysis by a Shannon entropy estimation (Masè et al., 2005). We first obtained the instant periods from the wavelet transform and chose a bin size for the histogram construction (in this case we used twice the sample size). To compute the Shannon entropy (SE) of the period distribution we used the following formula:

$$\mathrm{SE} = -\sum_{i=i}^{m} p\,(i) \ln p\,(i)$$

Where m is the number of bins with non-zero probability and $p(i)$ is the probability of assuming the ith value (frequency of occurrence of i in N observations). To relate the dispersion of the period distribution with the stability of the system, the V index was defined as follows:

$$V = 1 - \frac{SE}{\ln N}$$

The V index ranges from 0, when the spreading of the period is maximal, to 1, when the probability distribution have a single period.

In addition, Non-Parametric Circadian Rhythm Analysis (NPCRA) was performed to analyze the interdaily stability (IS index), and intradaily variability (IV index) of locomotor activity rhythms in constant darkness (Van Someren et al., 1999). The IS index was defined as the most significant period value from the chi-square periodogram, normalized for the number of data, and can be calculated as the ratio between the variance around the mean of the most significant period and the overall variance:

$$IS = \frac{n \sum_{h=1}^{p} (\bar{x}_h - \bar{x})^2}{p \sum_{i=1}^{n} (x_i - \bar{x})^2}$$

Where n is the total number of data, p is the number of data per day (according to the period), $\bar{x}_\mathrm{h}$ are the hourly means, $\bar{x}$ is the mean of all data, and x_i represents the individual data points. The IS tends to zero with Gaussian Noise and 1 for a perfect coupling. The IV index was calculated as the ratio of the mean square of the difference between all the successive hours (first derivate) and the same computation around the overall mean:

$$IV = \frac{n \sum_{i=2}^{n} (x_i - x_{i-1})^2}{(n-1) \sum_{i=1}^{n} (x_i - \bar{x})^2}$$

The IV value is zero for a perfect sinusoidal wave, and is around 2 for Noise (Gaussian Noise).

For subjective night duration (α), an hourly waveform was generated for the average activity of 15 days in DD conditions, and time was calculated as time length covered by the portion of the curve surpassing the mean of activity baseline. Conversely, subjective day duration (ρ) was calculated as the time length where the curve was under the mean activity baseline. Activity during α was calculated as the area under the curve during subjective night phase, normalized to total activity.

In LD conditions, 3 stages of the experiment were analyzed: Before-Implantation (BI), Post-Implantation (PI) and Post-jetlag (P-JL). Each period was analyzed separately: BI was the synchronization stage prior to glioma implantation, PI was considered from 4 days after surgery until the phase shift of the cycle, and P-JL stage corresponded to the days after the animals had resynchronized to the new LD cycle, until the end of experiment. Activity onset was calculated using individual waveforms of mean activity as the time at which the activity curve overpassed the mean baseline for more than 2 h. Phase angle was defined as the difference between the time of lights off (ZT 12) and the time of activity onset, with positive values referring to activity onset occurring before lights off. LD period was calculated with SB periodograms. Diurnal activity was calculated as the area under the curve that was below the mean activity baseline of individual waveforms for each stage, normalized to total mean activity. Resynchronization speed was calculated as described in Kiessling et al. (2010). Briefly, PS_{50}, which describes the amount of time in days needed to achieve 50% of the resynchronization after the Jet-lag shift, was calculated by adjusting a sigmoid dose-response curve with variable slope to the onset of locomotor activity time points for each group, with the following equation:

$$y = Bottom + \frac{(Top - Bottom)}{1 + 10^{(\log PS50 - x)\ .\ HillSlope}}$$

Differences between the best-fit PS_{50} values were compared by extra sum-of-squares F-test.

In addition, the difference between the phase of activity onset in the last 10 days of LD cycles and the first 10 days in DD conditions ($\Delta\psi$) was analyzed with circular statistics by Rayleigh tests. First, individual Rayleigh tests for LD and DD conditions were applied for each data set using the onset of activity as a phase (ψ) marker. For LD Rayleigh plots, a 24 h period was used, and for DD Rayleigh plots we applied the endogenous period. For each animal, the average $\Delta\psi_{\mathrm{LD-DD}}$ was calculated and used as entry data for a group Rayleigh analysis, using a 24 h period.

Data is presented as Mean ± SEM. Statistical analysis was performed with GraphPad Prism 6 program. P-values minor to 0.05 were considered as statistically significant.

RESULTS

Hypothalamic Glioma Model

In order to analyze the effects of hypothalamic gliomas on the circadian clock, we performed a unilateral implantation of LN-229 human glioblastoma cells in the region of the SCN. This resulted in 100% implantation efficiency and a mean survival of 54 ± 4 days ($n = 15$). Although we did not find an identical distribution of the tumors among different animals, all of them showed masses compromising the ipsilateral hypothalamus, optic

chiasm and part of the third ventricle, as well as vascularization of the tumor (**Figure 1**).

Effects of Tumor Implantation on Endogenous Circadian Timekeeping

The ability of the circadian clock to sustain proper endogenous timekeeping is fundamental in order to adjust an appropriate phase relation of clock-controlled outputs to the environment. To study the implications of hypothalamic gliomas on the endogenous properties of the circadian clock, we analyzed different circadian parameters under DD conditions, where rhythmicity is sustained only by the circadian clock, independent of environmental cues. **Table 1** summarizes our findings in this approach.

One of the most important parameters of the endogenous circadian rhythm is the period at which the clock runs without external cues. Animals implanted with hypothalamic gliomas presented a small, albeit significant, alteration in the endogenous period of the circadian clock, with larger τ values than sham controls (**Figure 2**; 23.92 $\pm$ 0.06 h, $n = 9$ and 23.58 $\pm$ 0.07, $n = 6$, for implanted and control animals, respectively; $p < 0.01$, Student's *t*-test).

Besides affecting the endogenous period, we hypothesized that hypothalamic gliomas would alter the ability of the clock to sustain robust and stable endogenous rhythms. This hypothesis was assessed by analyzing activity consolidation during the subjective night and the presence of a stable τ throughout several days. We found no difference in the distribution of the activity, identified as α and ρ, during DD conditions between implanted and control animals (**Table 1**). However, examination of instant periods across the whole temporal series by means of wavelet analysis suggested that the endogenous period was less stable in implanted mice (**Figure 3**). Although analysis of τ variability through wavelet-based computations, using the V index, did not show statistically significant differences, a more stringent analysis through NPCRA demonstrated that implanted animals presented less robust rhythms under DD conditions. We calculated the IV index which is an indicator of the fragmentation of the locomotor activity, describing the duration and repetition of transition between rest and activity, and the IS index which quantifies the invariability between the days, that is, the strength of coupling of the activity to the τ that best describes the rhythms. We found that animals implanted with hypothalamic tumors showed weaker stability of the rhythm related to the coupling to a specific τ, and higher fragmentation of the activity pattern (**Figure 3** and **Table 1**; for IS, $P < 0.01$; for IV $P < 0.05$; Student's *t*-test, $n = 6$ for controls, $n = 9$ for implanted mice).

Effect of Tumor Implantation on Synchronization to Light-Dark Cycles

Besides alteration of endogenous timekeeping, hypothalamic gliomas might affect entrainment to environmental cues. In order to study the effects of hypothalamic gliomas in the ability of the circadian clock to synchronize to light-dark schedules, we analyzed the characteristics of activity rhythms both at a stable LD schedule and during resynchronization to an abrupt shift in the LD schedule (i.e., Jet Lag). The results of this set of experiments are summarized in **Table 2**. It should be noted that since animals were subjected to a Jet Lag protocol, the analysis was divided in three stages, before implantation (BI), post-implantation (PI) and after Jet Lag protocol (P-JL). For the P-JL stage, circadian parameters were only calculated after each animal reached full resynchronization to the new LD schedule.

TABLE 1 | Summary of endogenous circadian parameters.

Parameter	Sham	Implanted	*p*
Period τ (hours)	23.58 ± 0.07	23.92 ± 0.06	<0.01
α (hours)	13.43 ± 0.90	13.08 ± 0.75	>0.05
Activity in α %	85.13 ± 1.95	83.31 ± 1.89	>0.05
α/ρ	1.31 ± 0.28	1.17 ± 0.13	>0.05
V index	0.69 ± 0.08	0.66 ± 0.04	>0.05
IS index	0.44 ± 0.03	0.31 ± 0.02	<0.01
IV index	0.68 ± 0.08	1.04 ± 0.1	<0.05

Circadian rhythms of general activity in DD conditions. α, length of the subjective day; ρ, length of the subjective night. See methods for details on V, IS, and IV index calculation. Groups were compared by Student's t-test.

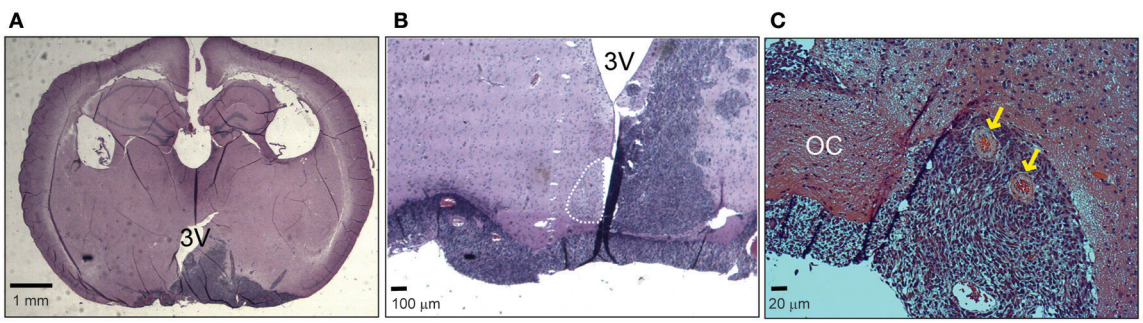

FIGURE 1 | Hypothalamic LN229 glioblastoma cells implantation. **(A)** An example of a coronal slice of brain tissue where implanted cells resulted in tumor masses compromising hypothalamic, ventricular and optic tract structures. **(B)** Another example showing a brain where the tumor affected the ipsilateral SCN and the optic chiasm. **(C)** Tumor implantation resulted in vascularization of the tissue (yellow arrows). OC, Optic chiasm; 3V, third ventricle.

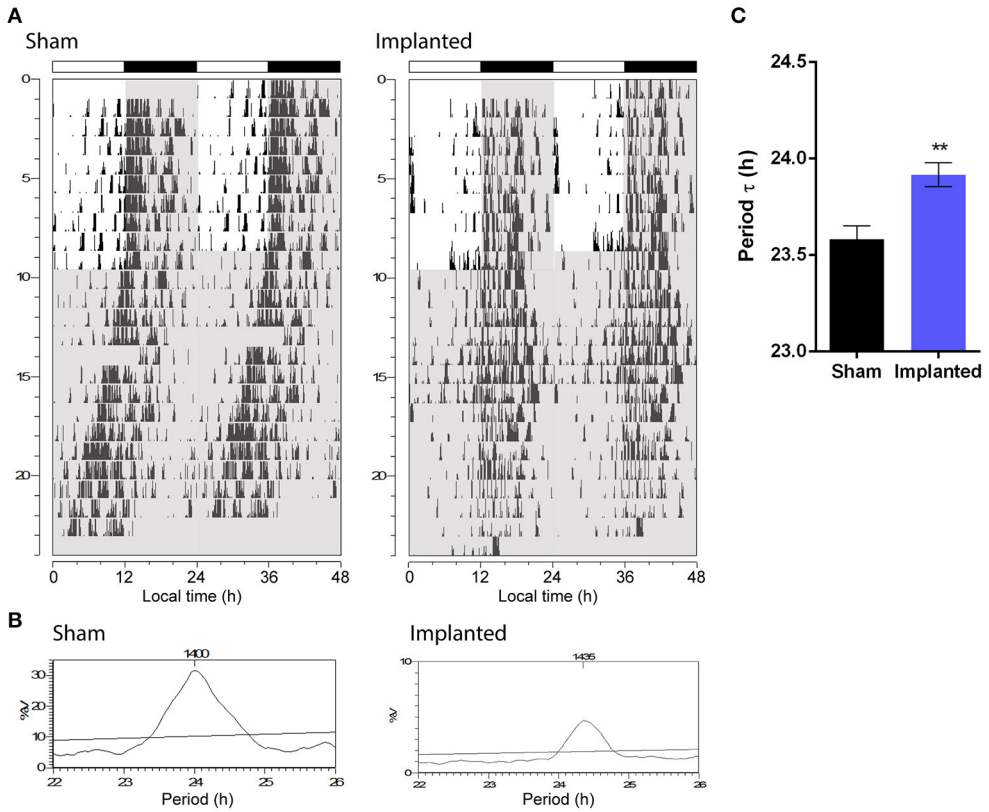

FIGURE 2 | Hypothalamic glioma alters endogenous circadian timekeeping. **(A)** Representative actograms of control (sham) and implanted animals. Gray background indicates lights-off. **(B)** Representative Sokolove-Bushell's periodograms for sham and implanted animals showing the endogenous circadian period. **(C)** Hypothalamic tumors increase the endogenous circadian period (23.92 ± 0.06 h, $n = 9$ and 23.58 ± 0.07, $n = 6$, for implanted and control (sham) animals, respectively; $^{**}p < 0.01$, Student's *t*-test).

We first characterized whether animals were able to effectively synchronize to the period imposed by an LD cycle. The period observed under LD conditions did not vary between control and implanted animals (**Table 2**), suggesting that hypothalamic gliomas do not impair the clock in terms of following an environmental cycle. Similarly, the amount of activity during the day did not vary significantly between implanted and control animals (**Table 2**). Next, when analyzing the rate of reentrainment to an abrupt phase change of the LD cycle, we found that implanted animals presented a slower resynchronization to a 6-h advance, with an average PS_{50} of 3.96 ± 0.21 days, as compared with the average PS_{50} of 2.78 ± 0.21 days for the control animals (**Figures 4A,B** and **Table 2**; extra sum-of-squares *F*-test between PS_{50} for Implanted and Sham mice, $p < 0.001$, $n = 6$ for control and implanted animals).This result points to an impairment in the ability of the circadian clock to adapt to changes in the Zeitgeber cycle in animals with hypothalamic gliomas.

Phase angle is defined as the difference between the phase of the circadian clock and the phase of its corresponding Zeitgeber, and is important in setting the relation between animal physiology and external environmental cues. We analyzed if the phase angle between the clock and the LD cycle was affected in animals bearing hypothalamic tumors. We found that implanted animals showed a clear advance in the phase angle between the activity onset and the time of light off, as compared with the sham controls, which became more pronounced with the progression of the tumor (**Figure 4** and **Table 2**; repeated measures Two-Way ANOVA followed by Holm-Sidak's *post-hoc* test for stage and treatment factors, $p < 0.05$ for the interaction. Stage effects: $p < 0.01$ for implanted animals in PI vs. BI stage, $p < 0.01$ for implanted animals in P-JL vs. PI stage, and $p < 0.001$ for implanted animals in P-JL vs. BI stage, $p > 0.05$ for all Sham contrasts. Implantation effects: $p < 0.001$ for Sham vs. Implanted animals in the P-JL stage, $p > 0.05$ for all other contrasts; $n = 6$ for control and implanted animals). This finding indicates a persistent decline in the ability of the clock to set a proper phase relation with environmental cue as the hypothalamic tumor grows.

Finally, to characterize more subtle effects of hypothalamic gliomas in the synchronization to LD cycles, we analyzed the difference between the phase of general activity during the first days of DD and compared it with the previous phase during LD conditions. If an animal is properly synchronized to the LD schedule, it can be expected that the phase in LD conditions represents a good predictor of the phase once the animal is transferred to constant DD conditions. We calculated the individual phase difference between the average phase both

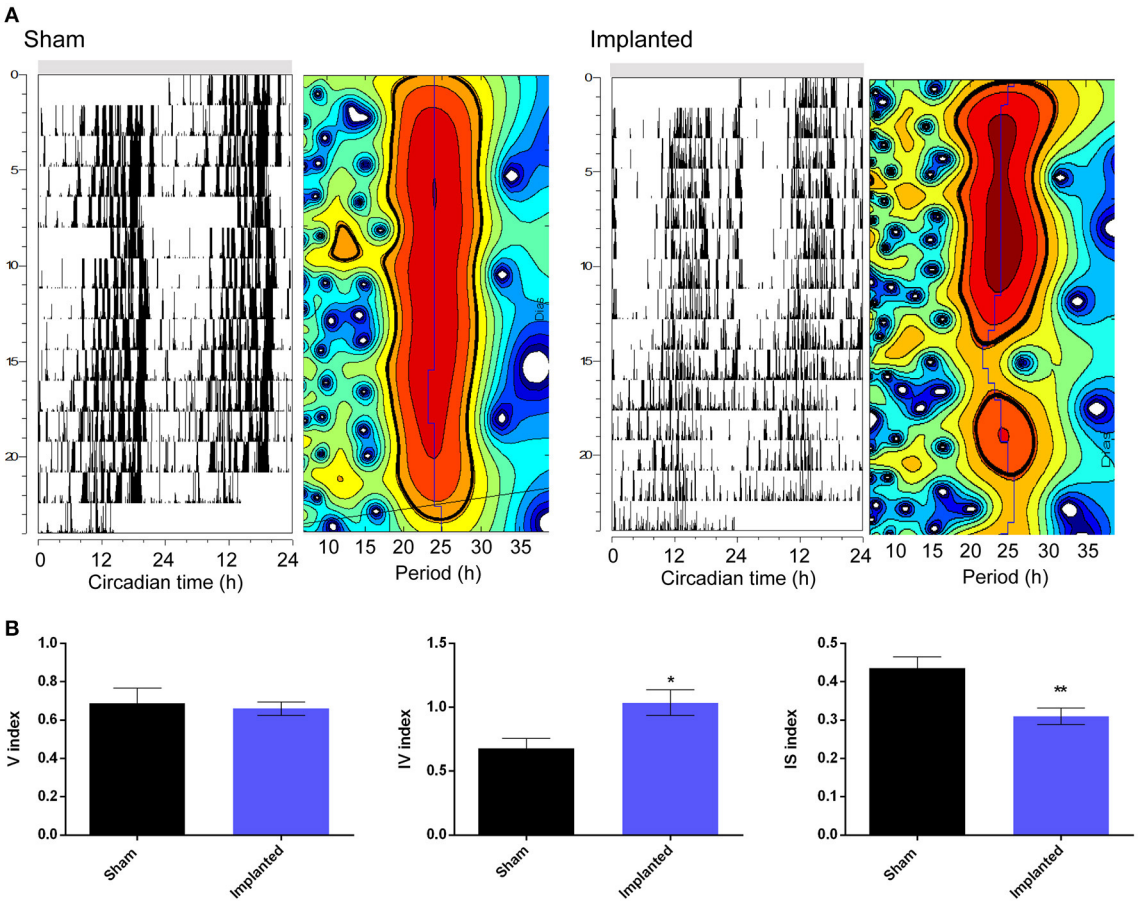

FIGURE 3 | Hypothalamic gliomas affect endogenous period stability. **(A)** Representative actograms and their corresponding instantaneous period calculation of control (sham) and implanted animals in constant darkness conditions. For instant periods, heatmaps plots refer to the significance of the endogenous period calculated by wavelet analysis. The vertical axis represent the successive days of activity, with the same scale of the corresponding actograms. The horizontal axis indicates the period in hours. The blue line shows the period of maximum significance across days. Black lines delimitate periods that are statistically significant (Torrence and Compo, 1998). **(B)** Tumor-implanted animals showed higher fragmentation of locomotor activity rhythms, as well as less strength of coupling of the activity to the τ that best describes the rhythms, although no effect was found by analysis of τ variability through wavelet-based computations (described by IV, IS, and V indexes, respectively; Student's *t*-test, $^{*}p < 0.05$ for IV, $^{**}p < 0.01$ for IS and $p > 0.05$ for V indexes, $n = 6$ for control and $n = 9$ for implanted animals).

in LD and in DD conditions and, surprisingly, we found that sham animals presented a bigger phase difference than animals carrying hypothalamic gliomas (**Figure 5**, $p < 0.05$, Student's *t*-test, $n = 6$ for controls, $n = 9$ for implanted mice).

DISCUSSION

The mammalian circadian clock is able to generate a coherent output through a complex combination of molecular and network mechanisms that can be synchronized by light inputs. Since pathologies affecting hypothalamic function can disrupt circadian timekeeping (Giustina and Braunstein, 2016), the presence of anomalous cells, such as glia-derived tumor cells could then modify circadian output clock, and be of relevance in the diagnosis of this tumors. Therefore, we aimed to study the effects of hypothalamic tumors on the circadian system, using an animal model suitable both for human tumor implantation and characterization of circadian parameters (Paladino et al., 2013).

The implantation of intracranial gliomas in the suprachiasmatic hypothalamic region resulted in both alteration of endogenous circadian timekeeping and in subtle changes in the ability of the clock to synchronize to environmental cycles. These phenomena should be interpreted in an integrative way, since the synchronization process also depends on the endogenous characteristics of the oscillator. Our results point to three important alterations in animals carrying hypothalamic gliomas: longer and unstable endogenous circadian period, alteration in the phase relation between the clock and the Zeitgeber, and a possible decrease of masking mechanisms.

The endogenous period of the circadian clock depends on individual molecular oscillations that occur in each cell, as well as on the coupling between different cells and regions of the SCN (Evans, 2016). The pace of the molecular clock can be modulated by the alteration of the transcription of clock genes, or by post-transcriptional or post-translational modifications on the products of the genes, affecting their stability or subcellular

TABLE 2 | Summary of circadian parameters under LD conditions.

Parameter	Group	BI	PI	P-JL	p^a
Phase angle ψ (min)	Sham	−15.88 ± 5.6	−1.49 ± 3.43	3 ± 8.16	>0.05
	Implanted	−16.67 ± 17.3	33.66 ± 7.64	92.33 ± 15.28	<0.05
	p^b	>0.05	>0.05	<0.001	
LD period (h)	Sham	24.08 ± 0.03	24.05 ± 0.02	24.05 ± 0.04	>0.05
	Implanted	23.99 ± 0.01	24.06 ± 0.02	24.06 ± 0.04	>0.05
	p^b	>0.05	>0.05	>0.05	
Diurnal activity %	Sham	24.54 ± 1.91	26.73 ± 2.6	33.46 ± 2	>0.05
	Implanted	34.64 ± 2.22	34.00 ± 2.40	29.23 ± 2.98	>0.05
	p^b	>0.05	>0.05	>0.05	
P_{50}	Sham	2.78 ± 0.21			
	Implanted	3.96 ± 0.21			
	p^c	<0.001			

For phase angle, data was analyzed by repeated measures two-way ANOVA, with $p < 0.05$ for the interaction effect. Effects of treatment (tumor implantation) and stage were assessed by Holm-Sidak's multiple comparisons test. For LD period and diurnal activity percentage, data was analyzed by repeated measures two-way ANOVA with $p > 0.05$ for treatment, stage and interaction effects. For reentrainment rate, data was adjusted to a sigmoid dose-response curve with variable slope and differences between best-fit PS_{50} for Sham and Implanted groups were analyzed by extra sum-of-squares F-test.

[a] *Post-test for stage factor.*

[b] *Post-test for treatment factor.*

[c] *Student's t-test.*

localization (Lowrey and Takahashi, 2011). Gliomas impose a deep alteration on the environment where they develop, secreting a variety of factors, increasing the vascularization, and remodeling the extracellular matrix, among others. Within the molecules that are increased in the tumor microenvironment, glutamate and immune factors such as TNF-α, IL-1α, and CCL2 should be taken into special consideration. Glioma cells increases extracellular glutamate levels both by means of glutamate release and by silencing of EAAT genes; indeed, increases in glutamate levels are proposed to favor neuronal death through excitotoxicity generating a physical niche for tumor growth (Watkins and Sontheimer, 2012). Glutamate is released in the SCN in response to light, activating a signaling cascade that induces clock gene expression (Golombek and Rosenstein, 2010). High glutamate levels in DD conditions could modulate clock gene expression through signaling pathways normally activated by light, inducing the alterations in τ found in implanted animals. Regarding the alteration of immune-related signaling molecules, TNF-α, IL-1β, and CCL2 have been shown to modulate the circadian clock, at the SCN level (Leone et al., 2012; Duhart et al., 2016). These, and/or other cytokines/chemokines, which are increased in the tumor microenvironment (Christofides et al., 2015; Vakilian et al., 2017) could modulate either the pace of the molecular clock or the coupling among different neurons.

Our results also showed that the proper phase relation between the oscillator outputs and the environmental cues is impaired in animals carrying hypothalamic gliomas, as evidenced by the increased phase angle between general activity rhythm onset and the time of lights-off. The alterations in the characteristics of the clock synchronization to LD cycles could be affected by the same mechanisms that lead to changes in endogenous pacemaking, which could also alter the shape and amplitude of the phase response curve (i.e., the differential effect of light on the phase of circadian rhythms according to the time of stimulation). In particular, abnormal glutamate levels in the SCN could affect synchronization to light, since this neurotransmitter is normally released preferentially during the day in this region, and induces phases adjustments if released during the night (Gillette and Mitchell, 2002). Also, TGF-α is elevated in the glioma microenvironment (Schlegel et al., 1990), and this molecule has been described to mediate the relation between the SCN and different clock outputs (Kramer et al., 2001). A dysregulation of TGF-α at the SCN level could thus lead to alterations in the coupling between the clock and the control of activity output.

Circadian entrainment was also evaluated by comparing the differences between the phase of general activity in LD and in the first days of DD conditions (**Figure 5**). If the oscillator is truly synchronized to the LD cycle, the phase during the first days in DD is predicted to be similar to the previous phase under LD. We found that control animals showed a bigger difference between the phase in LD and in DD conditions, which could be interpreted as a signal of a masking phenomenon in which the output (general activity) is directly modulated by the Zeitgeber. Masking might serve as a fine adjustment mechanism in the synchronization process; although the specific anatomical structure sustaining this phenomenon has not been identified. It has been has been suggested that masking might rely on TGF-α acting on the ventral subparaventricular zone (vSPZ, a region adjacent to the SCN) of the hypothalamus (Kramer et al., 2001). As stated before, TGF-α has been detected in human glioblastomas, correlating with the proliferative aggressiveness of the tumors (von Bossanyi et al., 1998). Alteration of this signal in the hypothalamic tumor microenvironment could impair the mechanism by which masking modulates activity rhythms, and this could be evidenced in the smaller phase difference between LD and DD conditions in animal bearing hypothalamic gliomas.

It should also be noted that gliomas have a strong influence in the physiology of the normal glial cells compromised in the tumor microenvironment. Astrocytes have recently been shown to be active players in setting the endogenous timekeeping of the circadian clock (Brancaccio et al., 2017; Tso et al., 2017). In addition, activation of these cells by immune factors induces changes in their molecular clock and, in response, astrocytes can modulate clock gene expression in the SCN (Duhart et al., 2013a). Gliomas have been shown to alter the coupling between astrocytes and the vasculature (Watkins et al., 2014), and to render astrocytes in an activated state (Lee et al., 2011; Raore et al., 2011). These or other changes in astroglial functioning could have an impact in the circadian clock, contributing to the effects seen in animals bearing hypothalamic tumors.

In summary, the glioma microenvironment greatly alters the levels of different molecules that can modulate clock gene expression, activate the photic synchronization pathway, and/or mediate the mechanisms underlying masking to light. These phenomena were evidenced in our murine model of

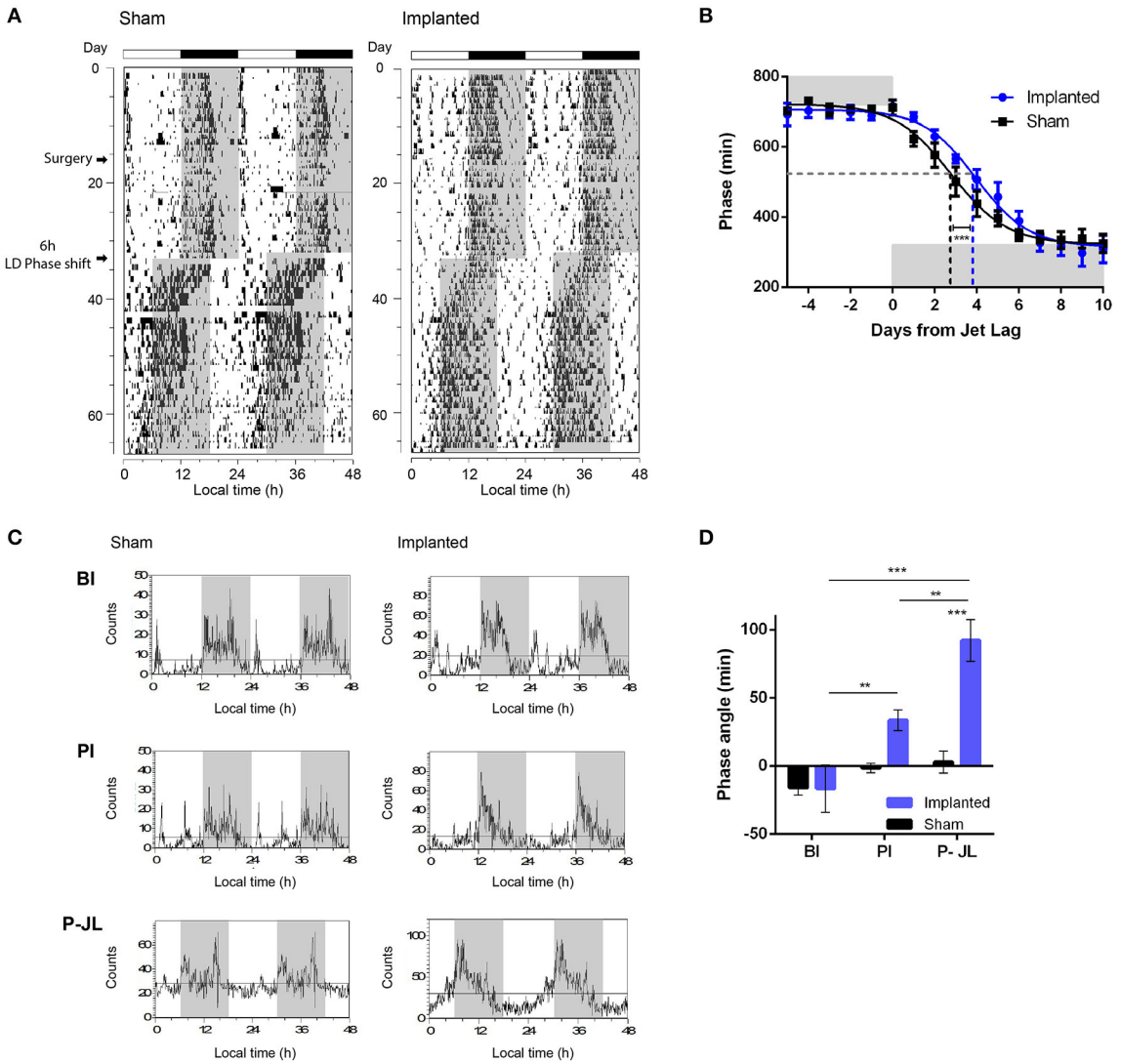

FIGURE 4 | Synchronization to LD cycles is altered by hypothalamic gliomas. **(A)** Representative actograms of control (sham) and implanted animals under LD cycles and a simulated Jet-lag protocol (6-h advance in the LD cycle). **(B)** Tumor implantation resulted in slower resynchronization to abrupt shifts in the LD cycle. The phase of activity onset, in minutes, is plotted for each day before and after the Jet-lag protocol. Gray background indicates the time of lights-off. A continuous line indicates the adjusted sigmoid dose-response curve with variable slope for each group. The dashed gray line indicates the 50% phase resynchronization, and vertical dashed lines indicate the PS_{50} for each group. Control animals showed shorter PS_{50} adjusted value, indicating faster resynchronization (extra sum-of-squares *F*-test, $^{***}p < 0.001$, $n = 6$ for control and implanted animals). **(C)** Representative waveforms of general activity of control (sham) and implanted animals in three different stages of the protocol (BI, Before implantation; AI, After implantation; P-JL, Post Jet Lag). **(D)** Tumor-implanted animals exhibited an advanced phase angle between the circadian rhythm in general activity and the LD cycle. (repeated measures Two-Way ANOVA followed by Holm-Sidak's *post-hoc* test for stage and treatment-tumor implantation-factors, $p < 0.05$ for the interaction. Stage effects: $^{**}p < 0.01$ for implanted animals in PI vs. BI stage, $^{**}p < 0.01$ for implanted animals in P-JL vs. PI stage, and $^{***}p < 0.001$ for implanted animals in P-JL vs. BI stage, $p > 0.05$ for all Sham contrasts. Implantation effects: $^{***}p < 0.001$ for Sham vs. Implanted animals in the P-JL stage, $p > 0.05$ for all other contrasts; $n = 6$ for control and implanted animals).

hypothalamic gliomas. While the effects of gliomas located in the hypothalamic/optic tract region on circadian rhythms have not been extensively analyzed in patients yet, one report from an hypothalamic astrocytoma case compromising the SCN region described, among other symptoms, an inversion in the wake-sleep cycle (Haugh and Markesbery, 1983). Also, pediatric patients carrying craniopharyngiomas, which usually compromises the optic tract and produce injury in the hypothalamus, present daytime sleepiness and alterations in the melatonin secretion rhythm (Müller et al., 2002). Recently, adult patients suffering from craniopharyngiomas were also described to present impaired 24 h sleep-wake and temperature circadian rhythms (Foschi et al., 2017). It has been previously reported that sleep disturbances are related to high-grade glioma progression (Yavas et al., 2012), although the availability of data regarding the effects of solid brain tumors on sleep/wake cycle is still limited

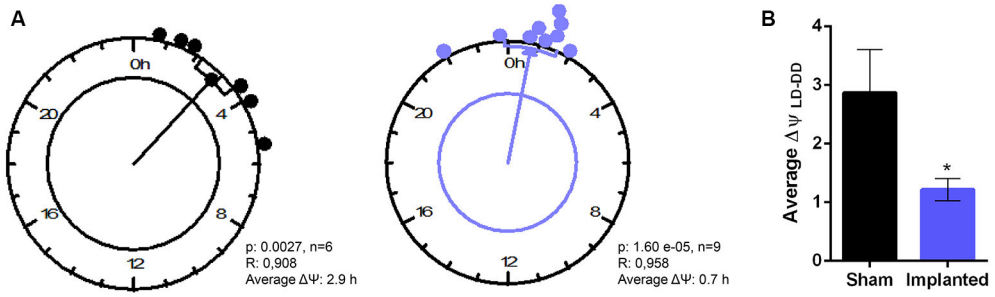

FIGURE 5 | Hypothalamic gliomas alter the relation between clock outputs and LD cycles. **(A)** Rayleigh analysis of average phase differences between LD and DD conditions for individual animals. **(B)** Tumor implantation resulted in a smaller difference between the phase of general activity in LD and DD conditions (*$p < 0.05$ Student's *t*-test, $n = 6$ for controls, $n = 9$ for implanted).

(Armstrong et al., 2017). Our work shows that hypothalamic gliomas can alter the endogenous properties of the clock, as well as the relation between the pacemaker, environment and output variables, suggesting that assessing simple circadian parameters, such as body core temperature, as well as sleep/wake cycle analysis, might be of importance during the clinical assessment and track of this disease. Further research in the characterization of the influence of hypothalamic/optic tract tumors on circadian rhythms could also be of importance in developing new factors or parameters that help both diagnosis and monitoring the progression of these diseases.

AUTHOR CONTRIBUTIONS

JD and LB helped in the design of the research, performed all experiments and analyzed data. LM and CC helped in technical aspects of the research, as well as in the analysis. DG supervised all research. All authors co-wrote the paper.

ACKNOWLEDGMENTS

This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) to LM and DG and from Universidad Nacional de Quilmes (UNQ) to DG. JD was supported by graduate fellowships from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and UNQ. CC was supported by graduate fellowships from ANPCyT and CONICET. LB was supported by an undergraduate fellowship from Consejo Interuniversitario Nacional (CIN). LM and DG are members of CONICET.

REFERENCES

Armstrong, T. S., Shade, M. Y., Breton, G., Gilbert, M. R., Mahajan, A., Scheurer, M. E., et al. (2017). Sleep-wake disturbance in patients with brain tumors. *Neuro. Oncol.* 19, 323–335. doi: 10.1093/neuonc/now119

Barca-Mayo, O., Pons-Espinal, M., Follert, P., Armirotti, A., Berdondini, L., and De Pietri Tonelli, D. (2017). Astrocyte deletion of Bmal1 alters daily locomotor activity and cognitive functions via GABA signalling. *Nat. Commun.* 8:14336. doi: 10.1038/ncomms14336

Beaulé, C., Swanstrom, A., Leone, M. J., and Herzog, E. D. (2009). Circadian modulation of gene expression, but not glutamate uptake, in mouse and rat cortical astrocytes. *PLoS ONE* 4:e7476. doi: 10.1371/journal.pone.0007476

Becquet, D., Girardet, C., Guillaumond, F., François-Bellan, A.-M., and Bosler, O. (2008). Ultrastructural plasticity in the rat suprachiasmatic nucleus. Possible involvement in clock entrainment. *Glia* 56, 294–305. doi: 10.1002/glia.20613

Bedrosian, T. A., and Nelson, R. J. (2017). Timing of light exposure affects mood and brain circuits. *Transl. Psychiatry* 7:e1017. doi: 10.1038/tp.2016.262

Brancaccio, M., Patton, A. P., Chesham, J. E., Maywood, E. S., and Hastings, M. H. (2017). Astrocytes control circadian timekeeping in the suprachiasmatic nucleus via glutamatergic signaling. *Neuron* 93, 1420–1435.e5. doi: 10.1016/j.neuron.2017.02.030

Christofides, A., Kosmopoulos, M., and Piperi, C. (2015). Pathophysiological mechanisms regulated by cytokines in gliomas. *Cytokine* 71, 377–384. doi: 10.1016/j.cyto.2014.09.008

Duhart, J. M., Brocardo, L., Mul Fedele, M. L., Guglielmotti, A., and Golombek, D. A. (2016). CCL2 mediates the circadian response to low dose endotoxin. *Neuropharmacology* 108, 373–381. doi: 10.1016/j.neuropharm.2016.05.005

Duhart, J. M., Leone, M. J., Paladino, N., Evans, J. A., Castanon-Cervantes, O., Davidson, A. J., et al. (2013a). Suprachiasmatic astrocytes modulate the circadian clock in response to TNF-. *J. Immunol.* 191, 4656–4664. doi: 10.4049/jimmunol.1300450

Duhart, J. M., Marpegán, L., Leone, M. J., and Golombek, D. A. (2013b). Role of astrocytes in the immune-circadian signaling. *Adv. Neuroimmune Biol.* 4, 85–96. doi: 10.3233/NIB-130058

Evans, J. A. (2016). Collective timekeeping among cells of the master circadian clock. *J. Endocrinol.* 230, R27–R49. doi: 10.1530/JOE-16-0054

Foschi, M., Sambati, L., Zoli, M., Pierangeli, G., Cecere, A., Mignani, F., et al. (2017). Site and type of craniopharyngiomas impact differently on 24-hour circadian rhythms and surgical outcome. A neurophysiological evaluation. *Auton. Neurosci.* doi: 10.1016/j.autneu.2017.08.006. [Epub ahead of print].

Gillette, M. U., and Mitchell, J. W. (2002). Signaling in the suprachiasmatic nucleus: selectively responsive and integrative. *Cell Tissue Res.* 309, 99–107. doi: 10.1007/s00441-002-0576-1

Girardet, C., Becquet, D., Blanchard, M. P., François-Bellan, A. M., and Bosler, O. (2010). Neuroglial and synaptic rearrangements associated with photic entrainment of the circadian clock in the suprachiasmatic nucleus. *Eur. J. Neurosci.* 32, 2133–2142. doi: 10.1111/j.1460-9568.2010.07520.x

Giustina, A., and Braunstein, G. D. (2016). "Hypothalamic syndromes," in *Endocrinology: Adult and Pediatric,* eds J. L. Jameson, L. J. De Groot, D. de Kretser, L. C. Giudice, A. Grossman, S. Melmed, J. T. Potts Jr, and G. C. Weir (Philadelphia, PA: Elsevier), 174–187.e5.

Golombek, D. A., Casiraghi, L. P., Agostino, P. V., Paladino, N., Duhart, J. M., Plano, S. A., et al. (2013). The times they're a-changing: effects of circadian desynchronization on physiology and disease. *J. Physiol. Paris* 107, 310–322. doi: 10.1016/j.jphysparis.2013.03.007

Golombek, D. A., and Rosenstein, R. E. (2010). Physiology of circadian entrainment. *Physiol. Rev.* 90, 1063–1102. doi: 10.1152/physrev.00009.2009

Haugh, R. M., and Markesbery, W. R. (1983). Hypothalamic astrocytoma. Syndrome of hyperphagia, obesity, and disturbances of behavior and endocrine and autonomic function. *Arch. Neurol.* 40, 560–563. doi: 10.1001/archneur.1983.04050080060011

Kiessling, S., Eichele, G., and Oster, H. (2010). Adrenal glucocorticoids have a key role in circadian resynchronization in a mouse model of jet lag. *J. Clin. Invest.* 120, 2600–2609. doi: 10.1172/JCI41192

Kramer, A., Yang, F. C., Snodgrass, P., Li, X., Scammell, T. E., Davis, F. C., et al. (2001). Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling. *Science* 294, 2511–2515. doi: 10.1126/science.1067716

Lavialle, M., Begue, A., Papillon, C., and Vilaplana, J. (2001). Modifications of retinal afferent activity induce changes in astroglial plasticity in the hamster circadian clock. *Glia* 34, 88–100. doi: 10.1002/glia.1044

Lee, C. Y. (2017). Strategies of temozolomide in future glioblastoma treatment. *Onco. Targets. Ther.* 10, 265–270. doi: 10.2147/OTT.S120662

Lee, J., Borboa, A. K., Baird, A., and Eliceiri, B. P. (2011). Non-invasive quantification of brain tumor-induced astrogliosis. *BMC Neurosci.* 12:9. doi: 10.1186/1471-2202-12-9

Leone, J., Marpegan, L., and Bekinschtein, A. (2006). Suprachiasmatic astrocytes as an interface for immune-circadian signalling. *J. Neurosci. Res.* 1527, 1521–1527. doi: 10.1002/jnr.21042

Leone, M. J., Beaule, C., Marpegan, L., Simon, T., Herzog, E. D., and Golombek, D. A. (2015). Glial and light-dependent glutamate metabolism in the suprachiasmatic nuclei. *Chronobiol. Int.* 32, 1–6. doi: 10.3109/07420528.2015.1006328

Leone, M. J., Marpegan, L., Duhart, J. M., and Golombek, D. A. (2012). Role of proinflammatory cytokines on lipopolysaccharide-induced phase shifts in locomotor activity circadian rhythm. *Chronobiol. Int.* 29, 715–723. doi: 10.3109/07420528.2012.682681

Lowrey, P. L., and Takahashi, J. S. (2011). Genetics of circadian rhythms in Mammalian model organisms. *Adv. Genet.* 74, 175–230. doi: 10.1016/B978-0-12-387690-4.00006-4

Masè, M., Faes, L., Antolini, R., Scaglione, M., and Ravelli, F. (2005). Quantification of synchronization during atrial fibrillation by Shannon entropy: validation in patients and computer model of atrial arrhythmias. *Physiol. Meas.* 26, 911–923. doi: 10.1088/0967-3334/26/6/003

Massimi, L., Tufo, T., and Di Rocco, C. (2007). Management of optic-hypothalamic gliomas in children: still a challenging problem. *Expert Rev. Anticancer Ther.* 7, 1591–1610. doi: 10.1586/14737140.7.11.1591

Müller, H. L., Handwerker, G., Wollny, B., Faldum, A., and Sörensen, N. (2002). Melatonin secretion and increased daytime sleepiness in childhood craniopharyngioma patients. *J. Clin. Endocrinol. Metab.* 87, 3993–3996. doi: 10.1210/jcem.87.8.8751

Musiek, E. S., and Holtzman, D. M. (2016). Mechanisms linking circadian clocks, sleep, and neurodegeneration. *Science* 354, 1004–1008. doi: 10.1126/science.aah4968

Nygård, M., Lundkvist, G. B., Hill, R. H., and Kristensson, K. (2009). Rapid nitric oxide-dependent effects of tumor necrosis factor-alpha on suprachiasmatic nuclei neuronal activity. *Neuroreport* 20, 213–217. doi: 10.1097/WNR.0b013e32831f1ca2

Ostrom, Q. T., Gittleman, H., Farah, P., Ondracek, A., Chen, Y., Wolinsky, Y., et al. (2013). CBTRUS statistical report : primary brain and central nervous system tumors diagnosed in the united states in 2006-2010. *Neouro-Oncology* 12, 28–36. doi: 10.1093/neuonc/not151

Paladino, N., Duhart, J. M., Mul Fedele, M. L., and Golombek, D. A. (2013). Characterization of locomotor activity circadian rhythms in athymic nude mice. *J. Circadian Rhythms* 11:2. doi: 10.1186/1740-3391-11-2

Raelson, C., and Chiang, G. (2015). Chiasmatic-hypothalamic masses in adults: a case series and review of the literature. *J. Neuroimaging* 25, 361–364. doi: 10.1111/jon.12132

Raore, B., Schniederjan, M., Prabhu, R., Brat, D. J., Shu, H. K., and Olson, J. J. (2011). Metastasis infiltration: an investigation of the postoperative brain–tumor interface. *Int. J. Radiat. Oncol.* 81, 1075–1080. doi: 10.1016/j.ijrobp.2010.07.034

Robert, S. M., and Sontheimer, H. (2014). Glutamate transporters in the biology of malignant gliomas. *Cell. Mol. Life Sci.* 71, 1839–1854. doi: 10.1007/s00018-013-1521-z

Rooney, A. G., McNamara, S., MacKinnon, M., Fraser, M., Rampling, R., Carson, A., et al. (2013). The frequency, longitudinal course, clinical associations, and causes of emotional distress during primary treatment of cerebral glioma. *Neuro. Oncol.* 15, 635–643. doi: 10.1093/neuonc/not009

Schlegel, U., Moots, P. L., Rosenblum, M. K., Thaler, H. T., and Furneaux, H. M. (1990). Expression of transforming growth factor alpha in human gliomas. *Oncogene* 5, 1839–1842.

Torrence, C., and Compo, G. P. (1998). A practical guide to wavelet analysis. *Bull. Am. Meteorol. Soc.* 79, 61–78.

Tso, C. F., Simon, T., Greenlaw, A. C., Puri, T., Mieda, M., and Herzog, E. D. (2017). Astrocytes regulate daily rhythms in the suprachiasmatic nucleus and behavior. *Curr. Biol.* 27, 1055–1061. doi: 10.1016/j.cub.2017.02.037

Vakilian, A., Khorramdelazad, H., Heidari, P., Sheikh Rezaei, Z., and Hassanshahi, G. (2017). CCL2/CCR2 signaling pathway in glioblastoma multiforme. *Neurochem. Int.* 103, 1–7. doi: 10.1016/j.neuint.2016.12.013

Van Someren, E. J., Swaab, D. F., Colenda, C. C., Cohen, W., McCall, W. V., and Rosenquist, P. B. (1999). Bright light therapy: improved sensitivity to its effects on rest-activity rhythms in Alzheimer patients by application of nonparametric methods. *Chronobiol. Int.* 16, 505–518. doi: 10.3109/07420529908998724

von Bossanyi, P., Sallaba, J., Dietzmann, K., Warich-Kirches, M., and Kirches, E. (1998). Correlation of TGF-alpha and EGF-receptor expression with proliferative activity in human astrocytic gliomas. *Pathol. Res. Pract.* 194, 141–147. doi: 10.1016/S0344-0338(98)80014-9

Watkins, S., Robel, S., Kimbrough, I. F., Robert, S. M., Ellis-Davies, G., and Sontheimer, H. (2014). Disruption of astrocyte–vascular coupling and the blood–brain barrier by invading glioma cells. *Nat. Commun.* 5:4196. doi: 10.1038/ncomms5196

Watkins, S., and Sontheimer, H. (2012). Unique biology of gliomas: challenges and opportunities. *Trends Neurosci.* 35, 546–556. doi: 10.1016/j.tins.2012.05.001

Weller, M., van den Bent, M., Tonn, J. C., Stupp, R., Preusser, M., Cohen-Jonathan-Moyal, E., et al. (2017). European Association for Neuro-Oncology (EANO) guideline on the diagnosis and treatment of adult astrocytic and oligodendroglial gliomas. *Lancet. Oncol.* 18, e315–e329. doi: 10.1016/S1470-2045(17)30194-8

Wen, P. Y., and Kesari, S. (2008). Malignant gliomas in adults. *N. Engl. J. Med.* 359, 492–507. doi: 10.1056/NEJMra0708126

Yavas, C., Zorlu, F., Ozyigit, G., Gurkaynak, M., Yavas, G., Yuce, D., et al. (2012). Health-related quality of life in high-grade glioma patients: a prospective single-center study. *Support. Care Cancer* 20, 2315–2325. doi: 10.1007/s00520-011-1340-4

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 09 February 2018
doi: 10.3389/fphys.2018.00005

Circadian Clock Proteins and Melatonin Receptors in Neurons and Glia of the *Sapajus apella* Cerebellum

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Oscillations of brain proteins in circadian rhythms are important for determining several cellular and physiological processes in anticipation of daily and seasonal environmental rhythms. In addition to the suprachiasmatic nucleus, the primary central oscillator, the cerebellum shows oscillations in gene and protein expression. The variety of local circuit rhythms that the cerebellar cortex contains influences functions such as motivational processes, regulation of feeding, food anticipation, language, and working memory. The molecular basis of the cerebellar oscillator has been demonstrated by "clock gene" expression within cells of the cerebellar layers. Genetic and epidemiological evidence suggests that disruption of circadian rhythms in humans can lead to many pathological conditions. Despite this importance, data about clock gene and protein expression in the cerebellum of diurnal (day-active) species, specifically primates, is currently poorly explored, mainly in regard to cellular identity, as well as the relationship with other molecules also involved in cerebellar functions. These studies could contribute to clarification of the possible mechanisms behind cerebellar rhythmicity. Considering that calcium binding proteins (CaBPs) play crucial roles in preserving and modulating cerebellar functions and that clock gene expression can be controlled by afferent projections or paracrine circadian signals such as the hormone melatonin, the present study aimed to describe cellular identities, distribution patterns and day/night expression changes in PER1, PER2, CaBPs, and MT_1 and MT_2 melatonin receptors in the cerebellar cortex of a diurnal primate using conventional fluorescence and peroxidase-antiperoxidase immunocytochemical techniques. PER1 and PER2 immunoreactive (IR) cells were observed in the Purkinje cells of the cerebellum, and MT_1 and MT_2 receptors were localized around Purkinje cells in the Pj layer in Bergmann cells. This identity was confirmed by the S100β-IR of these cells. The highest expression of PER seen in the daytime analysis coincided with the highest expression of melatonin receptors. CaBPs showed day/night morphological and density changes in the cerebellar cortex. The presence of the same temporal variations in the expression of PER in the Pj neurons and in MT_1 and MT_2 receptors in Bergmann cells indicates a possible relation between these cells during the rhythmic processing of the cerebellum, in addition to the CaBP temporal morphological and density changes.

Keywords: biological rhythms, melatonin, clock genes, cerebellum, motor, cognition, memory, language

OPEN ACCESS

Edited by:
Jolanta Górska-Andrzejak, Jagiellonian University, Poland

Reviewed by:
Eric M. Mintz, Kent State University, United States
Cristiano Bertolucci, University of Ferrara, Italy

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Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: *31 August 2017*
Accepted: *04 January 2018*
Published: *09 February 2018*

Citation:
Guissoni Campos LM, Hataka A, Vieira IZ, Buchaim RL, Robalinho IF, Arantes GEPS, Viégas JS, Bosso H, Bravos RM and Pinato L (2018) Circadian Clock Proteins and Melatonin Receptors in Neurons and Glia of the Sapajus apella Cerebellum. Front. Physiol. 9:5. doi: 10.3389/fphys.2018.00005

INTRODUCTION

Oscillations in circadian rhythms are important for the anticipation of daily and seasonal environmental rhythms. They are provided by the suprachiasmatic nucleus (SCN) of the hypothalamus, which controls the circadian rhythms of physiological, endocrine and behavioral processes (Reppert and Weaver, 2002). At the molecular level, this ability of the SCN consists of a self-sustained autoregulatory feedback mechanism reflected by the rhythmic expression of clock genes, i.e., *Per1, Per2, Per3, Cry1, Cry2, Clock, Arntl,* and *Nr1d1* (Lowrey and Takahashi, 2011).

In addition to the SCN, there are extrahypothalamic oscillators in other encephalic areas (Reppert and Weaver, 2002; Campos et al., 2015a,b) including a circadian oscillator in the cerebellar cortex, as demonstrated in rats and mice (Mendoza et al., 2010; Rath et al., 2012). Indeed, the cerebellar cortex contains a variety of local circuit rhythms, from the rhythms in the cerebellar cortex *per se* to those dictated from important afferents (Mendoza et al., 2010).

Predominantly involved in motor coordination and learning (Grimaldi and Manto, 2012; Buckner, 2013), the cerebellum has also been implicated in motivational processes (Caston et al., 1998), the regulation of feeding, food anticipation (Zhu and Wang, 2008; Mendoza et al., 2010), language (Verly et al., 2014), emotion regulation (Andreasen and Pierson, 2008), attention (Gottwald et al., 2003), and working memory (Ravizza et al., 2006).

The molecular basis of the cerebellar oscillator has been demonstrated by clock gene expression in the cerebellar cortex of rodents (Shieh, 2003; Mendoza et al., 2010; Rath et al., 2012; Paulus and Mintz, 2016). Some clock genes, i.e., *Per1, Per2, Per3, Arntl, Cry1, Nr1d1*, and *Dbp*, are rhythmically expressed (Takumi et al., 1998; Rath et al., 2012) while others, i.e., *Cry2* and *Clock*, are constitutively expressed (Guilding and Piggins, 2007; Rath et al., 2012).

Considering that all of these studies were carried out in rodents, nocturnal animals, a question remains whether clock proteins are expressed similarly in the cerebellum of primates, animals of diurnal habits. The characterized circadian gene expression of the human brain demonstrates a rhythmic increase and decrease in gene expression in regions outside of the suprachiasmatic nucleus (Li et al., 2013).

Another unresolved issue is, what determines the rhythmic expression of the clock genes in the cerebellum? Some results have shown that this expression is either independent of the SCN, as seen in an *in vitro* study (Mendoza et al., 2010), or dependent on the SCN, as seen in an *in vivo* study (Rath et al., 2012). Even in the latter case, since direct neuronal projections linking the SCN to the cerebellum have not been described, it has been assumed that this SCN influence on the cerebellum could be due to indirect cues in circadian physiology.

In addition to the possibility of cerebellar clockwork being reset by indirect cues via multiple neural projections between the hypothalamus and brainstem (Zhu and Wang, 2008), blood-borne signals could alternatively reach the cerebellar cortex. In fact, the cerebellum is known to be the target of the neuroendocrine system involved in circadian timing (El Messari et al., 1998; Choeiri et al., 2002), including the hormone melatonin. This target is marked by the presence of the G-protein coupled receptors named melatonin receptor type 1 (MT_1) and type 2 (MT_2) (Mazzucchelli et al., 1996; Adamah-Biassi et al., 2014; Lacoste et al., 2015; Pinato et al., 2015).

Here, we propose an investigation of cellular temporal characteristics in the cerebellar cortex of primates, by assessing the day/night expression of the PER1 and PER2 proteins, MT_1 and MT_2 melatonin receptors, and the calcium-binding proteins (CaBPs) calbindin (CB) and calretinin (CR). The CaBPs partially operate as buffers, decreasing the concentration of cytoplasmic Ca^{2+} in neurons, in addition to participating in enzyme activity and gene transcription (Barski et al., 2003). The choice for including CaBPs in this investigation was motivated by the fact that changes in the expression of CaBPs may be correlated with the neurochemical, morphological, and functional results seen in the cells (Babij et al., 2013). Alterations in CaBP levels in the cerebellum have been demonstrated in pathological situations (Soghomonian et al., 2017), but there are no data about possible day/night variations in primates under normal conditions. The relevance of exploring such temporal characteristics lies in the fact that these oscillations could have a definitive impact on the way information is processed in the cerebellum.

METHODS AND MATERIALS

Ethical Approval

The procedures involving animal use were compliant with the National Research Council (US) Committee on Guidelines for the Use of Animals in Neuroscience and Behavioral Research (2003) and were approved by the local ethics committee (FOA/UNESP process no. 00259/2013).

Animals

Brain slices were obtained from six adult male capuchin monkeys (*Sapajus apella*) (2–3 kg) from the Center of Tufted Capuchin Monkey Procreation of the São Paulo State University (UNESP), Araçatuba, SP, Brazil. Animals were housed in a room with a transparent and retractable roof in individual cages under natural light, temperature, and humidity conditions and fed with a controlled diet consisting of eggs, fruit, granulated protein and dried corn; water was provided *ad libitum*. In the experiments, 6 a.m. o'clock (sunrise time) was considered to be zeitgeber time 0 (ZT 0), and sunset time started at ∼6 p.m. (ZT 12). Following these time parameters, animals were anesthetized and perfused at ZT10 (daytime point) and ZT 19 (nighttime point, anesthetized in dim light), with an $N = 3$ per ZT. The analyze of PER expression in the cerebellum at ZT 10 and ZT 19 in the present study, was based on the possibility of relating the results of PER2 expression in the cerebellum and results of SCN in this species which had showed a peak around ZT 9 and the nadir around ZT 18 (Rocha et al., 2014).

Tissue Collection

Following the protocol described by Campos et al. (2014), after transcardiac perfusion, the brains were cryoprotected and

cryosectioned, and sections of 30 μm were stored as 10 different stepwise series in an anti-freeze solution. The coronal sections of one series, representing the entire extent of the cerebellum, were placed in rostrocaudal order. After this, five sections representing the same rostrocaudal levels were processed for each antibody.

Immunohistochemistry

Brain sections were processed using immunohistochemical techniques for CB, CR, PER1, PER2, NeuN (neuronal marker), MT_1 and MT_2. The sections were washed using a solution of TBS-TX buffer (0.05 M), incubated for 48 h at 4°C in a solution containing 0.05 M TBS-TX buffer, 2% normal serum (Vector Laboratories, CA, USA) and the appropriate primary antibody: anti-CB (1:7500, Abcam, MA, USA), anti-CR (1:7500, Abcam, MA, USA), anti-PER1 (1:200, Abcam, MA, USA), anti-PER2 (1:500, Santa Cruz, TX, USA), anti-NeuN (1:1000, Abcam, MA, USA), anti-MT_1 (1:200, Santa Cruz Biotechnology, TX, USA) or anti-MT_2 (1:200, Santa Cruz Biotechnology, TX, USA). A combination of MT_1 and NeuN or MT_2 and NeuN primary antibodies was added to separate sections for 48 h. Negative staining controls were performed by omitting the primary CB and CR antibodies.

Following the protocol described in Campos et al. (2014), primary antibodies were added to separate sections for 48 h. Next, the sections with primary antibodies against PER1, PER2, NeuN, MT_1 and MT_2 were washed in buffer and incubated for 2 h in Alexa-488 donkey-anti-rabbit (1:200, Jackson Immunoresearch, cod. 711-545-152, PA, USA) and Cy3-labeled donkey-anti-goat (1:200, Jackson Immunoresearch, cod. 705-165-147, PA, USA) fluorescent secondary antibodies specific for each species of primary antibody. The pre-adsorption of the anti-MT_1 and anti-MT_2 antibodies with the immunogenic peptides MEL-1A-R (N-20) P (Santa Cruz Biotechnology**,** sc13179 peptide, TX, USA), and MEL-1B-R (G-20) P (Santa Cruz Biotechnology, sc28453 peptide, TX, USA) eliminated any positive staining in the cerebellum sections. Coverslips were placed on the slides using glycerol buffer as the mounting medium.

Negative staining controls were performed by adding Per1 (E-8) blocking peptide (Santa Cruz Biotechnology, sc-398890 P, TX, USA) and Per2 control/blocking peptide #1 PER2 (1-P) (Alpha Diagnostic International, Inc., TX, USA) to the primary incubation solution of PER1 and PER2 antibodies, which blocked PER1 and PER2 staining.

The sections with the primary antibodies CB and CR were washed with 0.05 M TBS-TX and incubated in a biotinylated secondary antibody specific to the primary antibody species, diluted (1:200) in the same solution as the primary, for 2 h. The sections were washed again with 0.05 M TBS-TX, incubated in a solution containing avidin-biotin complex (Vector Laboratories, CA, USA) for 2 h, and washed with Tris–HCl buffer (pH 7.6). Labeling was developed using 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma—Aldrich, MO, USA) as a chromogen. Next, the sections were mounted on gelatin-coated slides and dehydrated; coverslips were placed on the slides using DPX as the mounting medium (Sigma—Aldrich, MO, USA).

To ensure that sample differences would not reflect different efficiencies of immunohistochemical labeling, brain sections from the two different ZTs were processed and incubated in the same solution at the same time.

Data Analysis

The cerebellum areas were identified using brain sections stained with Nissl and the atlases "A Stereotaxic Atlas of the Brain of Cebus Monkey" (*Cebus apella*) (Manocha et al., 1968) and "The Rhesus Monkey Brain in Stereotaxic Coordinates" (Paxinos et al., 2000). For each animal, all the coronal sections of a series, representing the full extent of the cerebellum, were placed in a rostrocaudal order. After this, five sections from each animal, similar across animals (representing the same rostrocaudal level), were processed for each antibody. The sections representing different levels of the rostrocaudal extension were adjacent among antibodies. Each coronal section was analyzed under light field and epifluorescence (Olympus BX50 microscope), and the images were acquired with cellSens software (USA). Images were acquired with adequate resolution and uniform brightness, and contrast was adjusted using Adobe Photoshop CS6, which was used similarly in all images. Five images captured randomly representing different areas of the coronal slice were then analyzed, and all visible CB-IR, CR-IR, PER1-IR, PER2-IR, MT_1-IR, and MT_2-IR neurons of the cerebellum layers were counted in each image. The optical density (O.D) of the immunoreactivity was also quantified using the digital image processing and analysis software ImageJ (McMaster Biophotonics Facility, Canada).

Statistical Analysis

For the statistical analyses, the average cell number or O.D of the five cerebellum coronal sections of each animal was obtained. The data are expressed as the mean ± standard error of the mean of the cell number or O.D from the three monkeys perfused at the same ZT. Mann Whitney tests were used to compare the two ZTs. Values of $P < 0.05$ were considered statistically significant. As demonstrated by the standard errors of the mean, the intra-individual variability was small for the analyzed parameters.

RESULTS

The clock gene proteins PER1 and PER2 along with melatonin receptors and calcium binding proteins were analyzed in the layers of the cerebellar cortex of the primate *S. apella*. The distribution of the PER1 protein in the cerebellar cortex was detected in Purkinje cell layer (Pj) but absent in the granular (Gr) and molecular (Mol) layers. The cell-IR number (PER1, ZT 10 = 16.9 ± 1.6 vs. ZT 19 = 9.1 ± 1.3, Z = 67.5, $P < 0.0001$) and IR optical density (O.D) (PER1, ZT 10 = 90.6 ± 3.6 vs. ZT 19 = 64.7 ± 3.3, Z = 66, $P < 0.0001$) quantification indicated that PER1 expression was higher at ZT10 than in the ZT 19 (**Figures 1A–F**).

The distribution of the PER2 protein in the cerebellar cortex was also detected in the Pj cell layer. The cell number (PER2, ZT 10 = 9.5 ± 0.7 vs. ZT 19 = 7.1 ± 0.7, Z = 269.5, $P = 0.01$) and IR optical density (O.D) (PER2, ZT 10 = 99.9 ± 3.5 vs. ZT 19 = 80.9 ± 6.6, Z = 293, $P = 0.03$) quantification indicated that

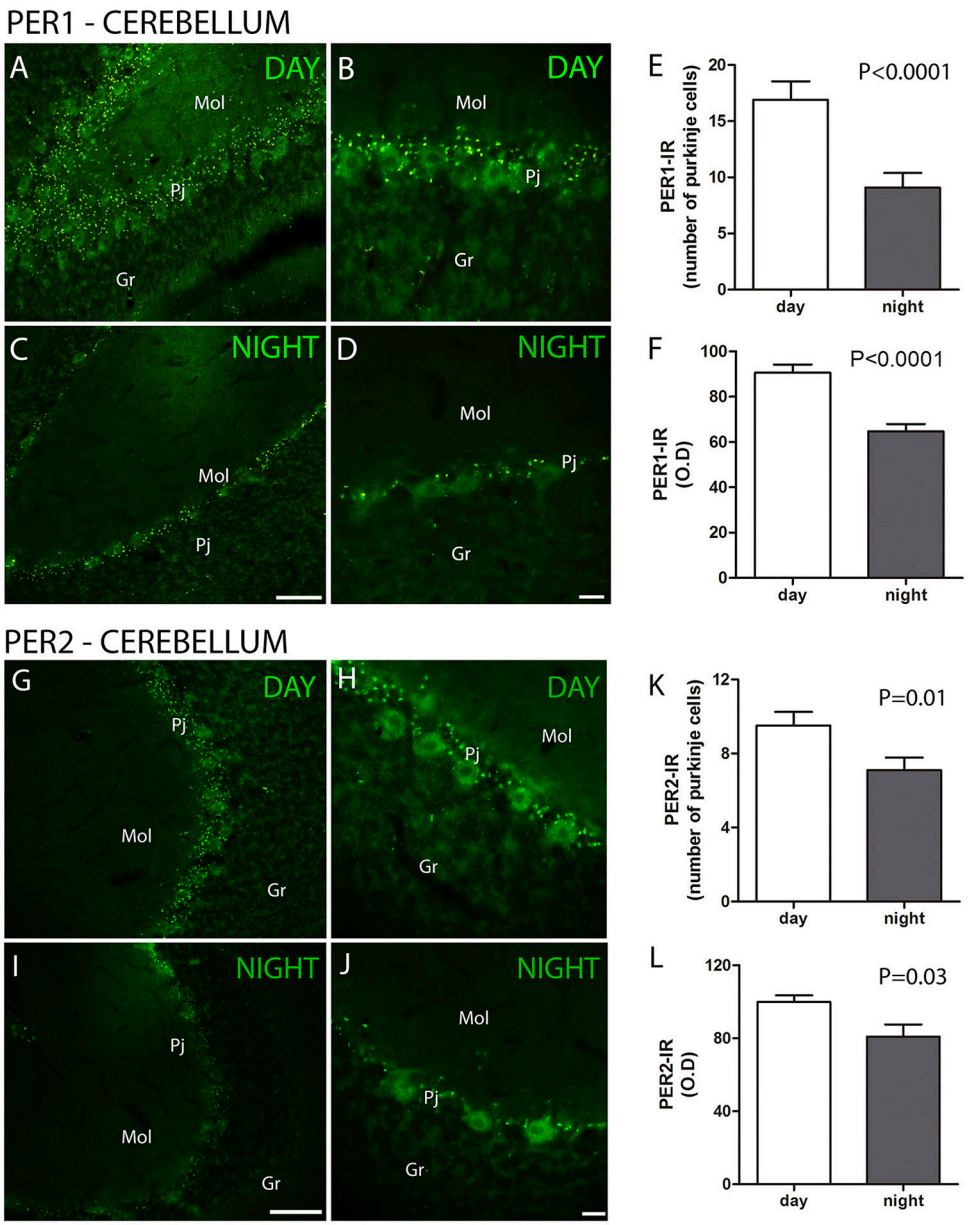

FIGURE 1 | The distribution of PER1 and PER2 proteins in the cerebellar cortex in the primate *Sapajus apella*. Photomicrographs of frontal brain sections show PER1-IR cells **(A–D)** (green) or PER2-IR cells **(G–J)** at two time points, one during the day (ZT 10) and another at night **(C,D)**. The graphs show the means ± standard error of the mean of the number **(E)** and IR intensity (O.D) quantification **(F)** of PER1-IR **(E,F)** or PER2-IR **(K,L)** cells in the Purkinje cell layers of monkeys perfused at day or nighttime points. Bar = 100 or 50 μm. Purkinje cell layer (Pj); molecular layer (mol); granular layer (Gr).

PER2 expression was higher at ZT 10 than at ZT 19, while it was absent in the Gr and Mol layers (**Figures 1G–L**).

The MT_1 and MT_2 receptors showed differences in IR intensity (O.D) between the day- and nighttime points, as well as specific patterns of distribution between the layers analyzed. The immunofluorescence analysis revealed a prevalence of both MT_1-IR (MT1, ZT 10 = 48.0 ± 2.1 vs. ZT 19 = 21.2 ± 0.8, Z = 6.0, $P < 0.0001$) (**Figures 2A–G**) and MT_2-IR (MT2, ZT 10 = 37.1 ± 2.0 vs. ZT 19 = 28.5 ± 1.1, Z = 149.5, $P < 0.0001$) (**Figures 2H–N**) at the daytime point. The higher intensity in the Pj layer and the lower intensity of MT_1 and MT_2 receptor expression in the Gr and Mol layers at the same coronal level of the cerebellum were seen at both the day- and nighttime points (**Figure 2**). Regarding the identity of the cells that showed MT_1-IR and MT_2-IR, the melatonin receptors were mainly localized around Purkinje cells in Bergmann cells, identified by S100β (**Figure 3**), and a few astrocytes (GFAP-IR) showed co-localization with these receptors (**Figure 4**).

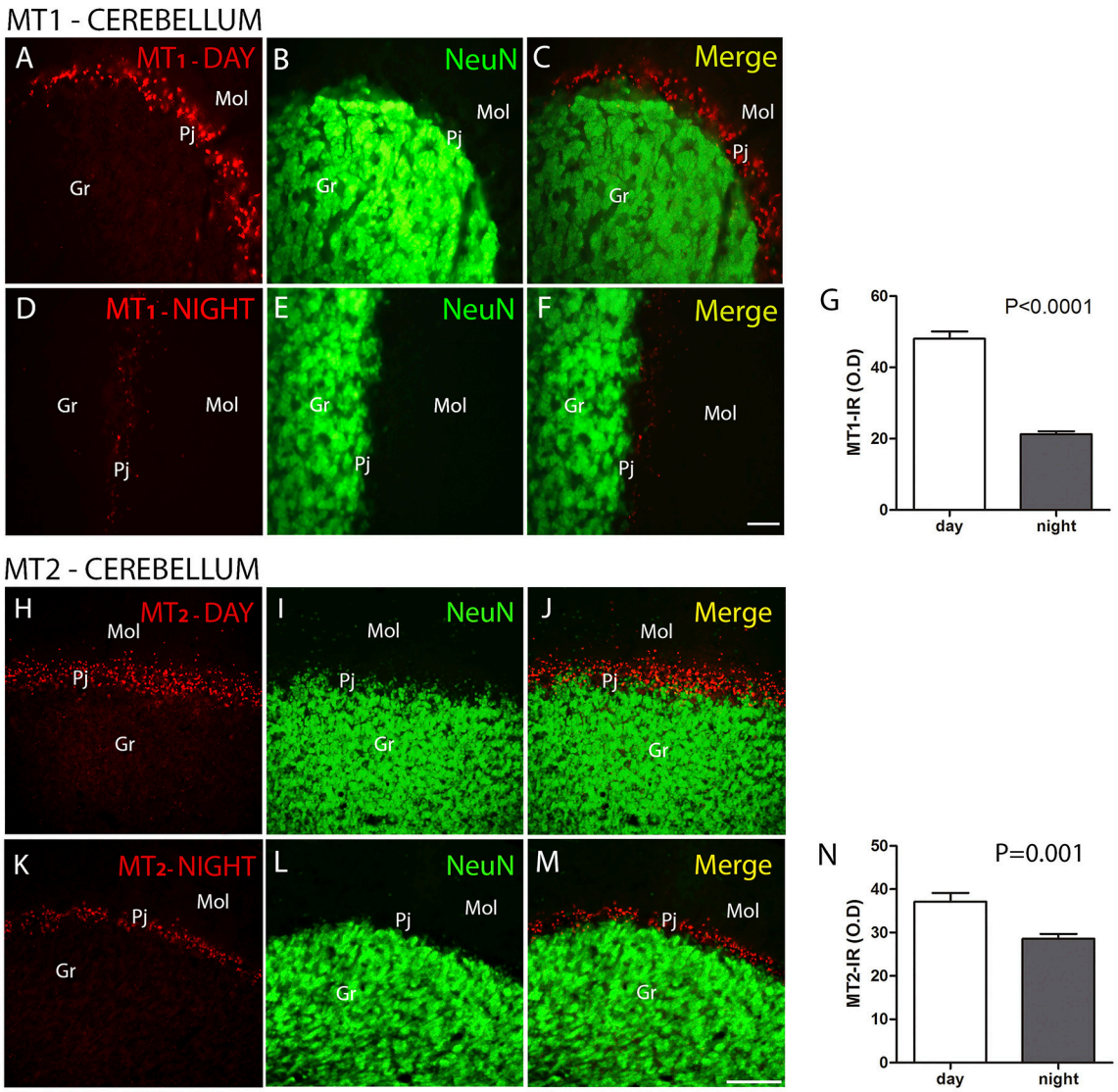

FIGURE 2 | The distribution of MT_1 and MT_2 proteins in the cerebellar cortex in the primate *Sapajus apella*. Photomicrographs of frontal brain sections show MT_1 protein **(A–F)** (red) or MT_2 protein **(H–M)** (red) and NeuN cells (green) at two time points, one during the day (ZT 10) and another at night (ZT 19). The graphs show the means ± standard error of the mean of the IR intensity (O.D) quantification of MT_1-IR cells **(G)** or MT_2-IR cells **(N)** in the Purkinje cell layers of monkeys perfused at day or nighttime points. Bar = 50 μm. Purkinje cell layer (Pj); molecular layer (mol); granular layer (Gr).

CR showed differences between the day and nighttime points in IR intensity (O.D) (CR, ZT 10 = 119.9 ± 4.6 vs. ZT 19 = 88.8 ± 4.3, $Z = 213$, $P < 0.0001$) (**Figures 5A–G**) and in cell morphology. The most significant expression of CR was in the Gr layer, where these neurons showed higher expression at the daytime point than at the nighttime point (**Figures 5A–G**). At the daytime point, the cell bodies of CR-IR neurons were more strongly stained. In addition to the increase in density of CB-IR, these neurons showed morphological differences when comparing the two time points, with a broader dendritic arborization at the daytime point (**Figures 5A–C**) than at the nighttime point (**Figures 5D–F**).

Furthermore, CB-IR and CR-IR proteins showed specific patterns of distribution between the layers analyzed (**Figure 6**). The Purkinje cells showed strong CB-IR. At both time points, they exhibited immunoreactivity in form of fine granular deposits dispersed in the cytoplasm, showing various degrees of labeling intensity (**Figure 6A**). At the daytime point, immunoreactivity was observed in the bodies and proximal segments of their main processes (**Figures 7A–C**). At the night point, these neurons showed differences in morphology, with long fibers that reached the Mol layer of the cerebellar cortex, forming a broader dendritic arborization and extension of Purkinje cell axon collateral plexus (**Figures 7D–F**).

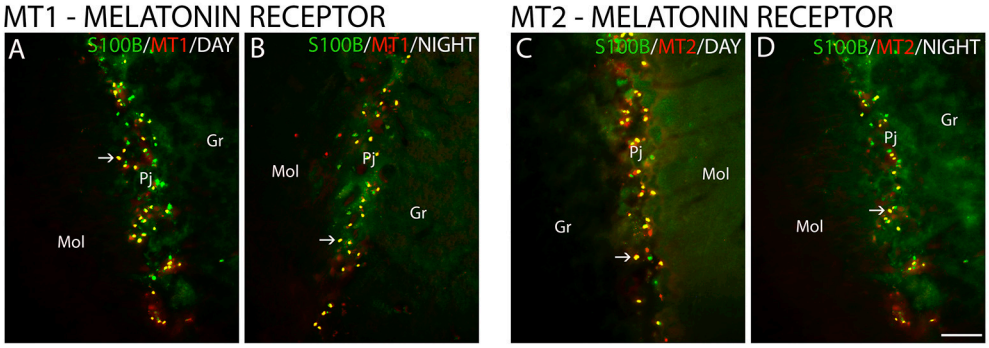

FIGURE 3 | The distribution of S100β, MT_1 and MT_2 proteins in the cerebellar cortex in the primate *Sapajus apella*. Photomicrographs of frontal brain sections show MT_1 protein **(A,B)** (red) or MT_2 protein **(C,D)** (red) and S100β (green) at two time points, one during the day (ZT 10) and another at night (ZT 19). The arrows indicate the co-localization (merge) of S100β and melatonin receptors. Bar = 100 μm.

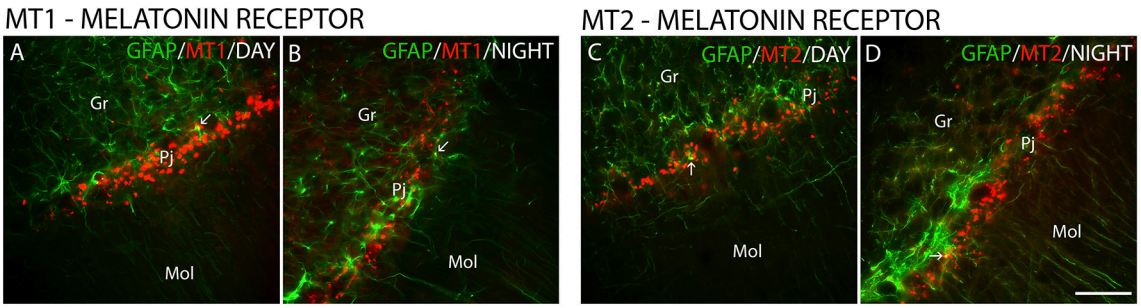

FIGURE 4 | The distribution of GFAP, MT_1 and MT_2 proteins in the cerebellar cortex in the primate *Sapajus apella*. Photomicrographs of frontal brain sections show MT_1 protein **(A,B)** (red) or MT_2 protein **(C,D)** (red) and GFAP (green) at two time points, one during the day (ZT 10) and another at night (ZT 19). The arrows indicate the co-localization (merge) of GFAP and melatonin receptors. Bar = 100 μm.

DISCUSSION

To determine if molecules associated with generation or modulation of biological rhythms and plasticity are expressed in the cerebellar cortex of diurnal animals, here we describe the pattern of expression of PER1 and PER2 proteins, melatonin receptors and CaBPs at two different time points in the cerebellar cortex of the primate *S. apella*. First, we demonstrated evidence that PER1 and PER2 are expressed in the Purkinje cell layer but are absent from the Gr and Mol layers of the cerebellar cortex of this primate and that these expressions exhibit a difference between the day- and nighttime points analyzed. This includes the cerebellar cortex up to the cerebral regions in which clock gene proteins variations have been detected in primates (Courtemanche et al., 2013; Rocha et al., 2014; Campos et al., 2015a,b).The pattern of distribution of PER2 protein found in the present study is in agreement with the expression of the PER2 protein in the Pj of the cerebellar cortex in rats (Mendoza et al., 2010). Similarly, the clock genes *Per1* and *Per2* are also described in the perikarya of the Pj of rodents, but different from the results of protein expression, the genes were also found in the Gr layer of rodents using hybridization techniques (Shieh, 2003; Rath et al., 2012).

In the present study, the expression of PER1 and PER2 in the cerebellar cortex was higher at the day (ZT 10) than at the night (ZT 19) time point analyzed. Previous studies in rats and mice had demonstrated a peak of PER1 and PER2 proteins or *Per1* and *Per2* gene expression at night (Akiyama et al., 2001; Farnell et al., 2008; Mendoza et al., 2010). Such differences may be related to the differences in the activity habits of the animals since the cerebellum is the major center of motor activity modulation (Doya, 2000). They could also be related to the differences in the availability of food among these animals. The cerebellum participates in the behavior of anticipating the time of food accessibility, which involves increased activity, behavioral arousal, and body temperature changes (Mistlberger, 1994; Mendoza et al., 2010). Thereby, the present study expands current knowledge on the rhythmic characteristics of the cerebellum, which was previously based on investigations showing clock gene expression in rodent and human studies (Shieh, 2003; Farnell et al., 2008; Mendoza et al., 2010; Rath et al., 2012; Li et al., 2013; Paulus and Mintz, 2016).These rhythmic clock gene and protein expressions are transduced into a rhythmic neuronal output signal that can influence other brain targets and play a crucial role in the anticipation of 24 h predictable environmental changes

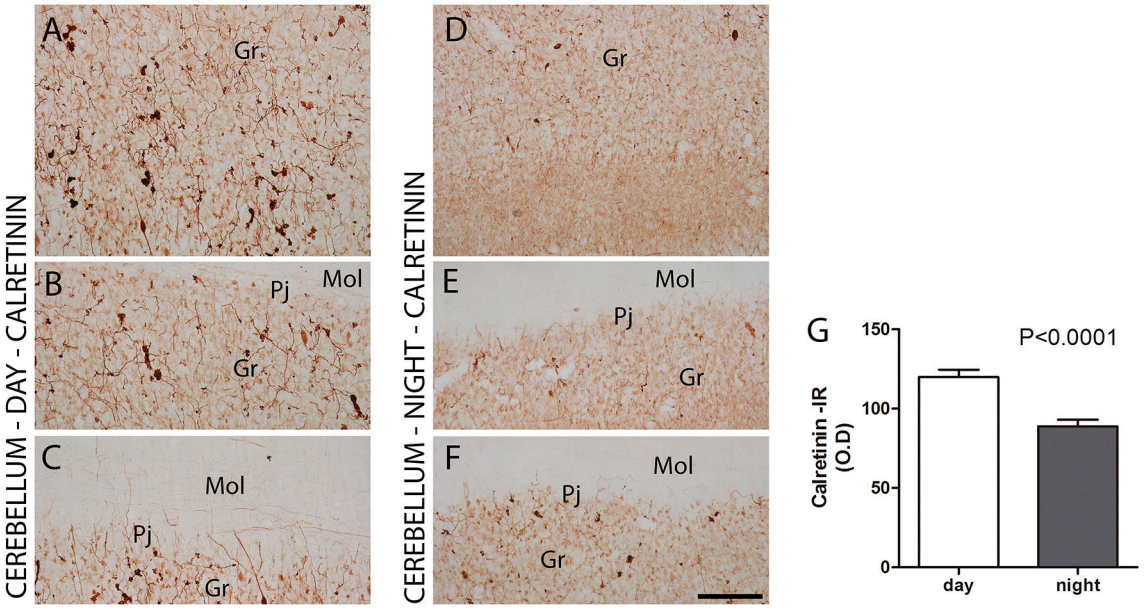

FIGURE 5 | The distribution of calretinin (CR) protein in the cerebellar cortex in the primate *Sapajus apella*. Photomicrographs of frontal brain sections show CR-IR with significant expression in the granular layer **(A–F)** at two time points, one during the day (ZT 10) **(A–C)** and another at night (ZT 19) **(D–F)**. The CR-IR showed day/night variations in IR intensity (O.D) in the cells in the granular layer **(G)**. Bar = 100 μm. Purkinje cell layer (Pj); molecular layer (mol); granular layer (Gr).

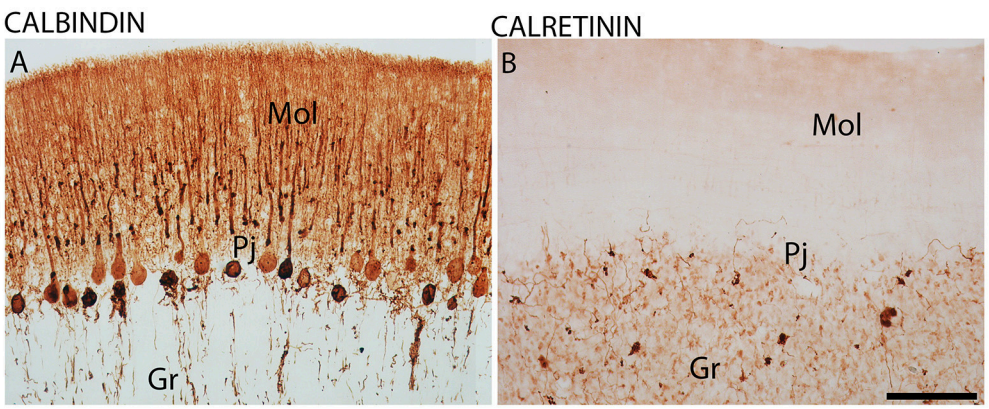

FIGURE 6 | The distribution of calbindin (CB) and calretinin (CR) proteins in the cerebellar cortex in the primate *Sapajus apella*. Photomicrographs of frontal brain sections show strongly stained CB-IR cells in the Purkinje cell layer (Pj) **(A)** and CR-IR with significant expression in the granular layer (Gr) **(B)**. Bar = 100 μm. Molecular layer (mol).

(Courtemanche et al., 2013). Once processed by the cerebellar cortex, temporal messages can be transferred via identified neural pathways to structures including the diencephalic and cortical areas of the sensorimotor system (Cavdar et al., 2001; Schnitzler and Gross, 2005; Courtemanche et al., 2013).

The origin of the rhythmicity of clock genes and proteins in the cerebellum has not been clarified. The molecular clockwork of the cerebellum could be autonomous or, to some extent, controlled by the master clock of the SCN (Mendoza et al., 2010; Rath et al., 2012).To explore whether or not the differences between the day- and nighttimes levels of PER 1 and PER2 proteins found in the *S. apella* are dependent on the SCN, it will be necessary (in future studies with more time points) to keep these animals in a free-running rhythm, or even to perform SCN lesions. At this time, we can only compare the results of the present study with previous results of the same species, where the PER2 protein was found also in the SCN during the day (Rocha et al., 2014), similar to the peak found in the cerebellum in the present study.

Although the mechanism responsible for conferring information on circadian time from the SCN to the cerebellar cortex remains enigmatic, external cues such as feeding schedules, neurotransmitters, and neurohormones have been shown to entrain extrahypothalamic oscillators in

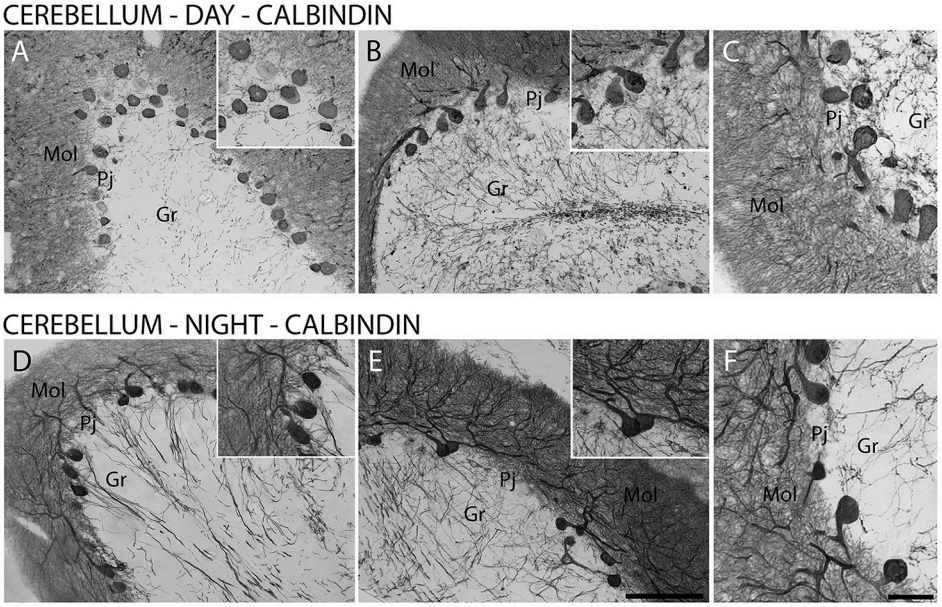

FIGURE 7 | The distribution of calbindin (CB) protein in the cerebellar cortex in the primate *Sapajus apella*. Photomicrographs of frontal brain sections show strongly stained CB-IR cells in Purkinje cell layer (Pj) **(A–F)** at two time points, one during the day (ZT 10) **(A–C)** and another at night (ZT 19) **(D–F)**. The CB-IR showed day/night variations in cell morphology in the Purkinje cell layer **(D–F)**. Bar = 100 or 50 μm. Molecular layer (mol); granular layer (Gr).

the brain (Verwey and Amir, 2009). One molecule that is implicated in circadian functions, relaying information about the environmental state, showing a circadian rhythm of its level, and acting directly on the master clock in the SCN is the pineal hormone melatonin. Once secreted by the pineal gland at night, melatonin enters into the blood circulatory system, through which it travels to act on different regions of the body to achieve desirable physiological responses (Costa et al., 1995; Gilgun-Sherki et al., 2001). Its influence occurs by binding to melatonin receptors, whose high density indicates that an area is a target for melatonin action (Dubocovich et al., 2003).

In the present study, MT_1-IR and MT_2-IR cells were mainly localized in Bergmann cells, with a higher intensity at the daytime point. Few GFAP-IR cells were colocalized with melatonin receptors independent of the time point. mRNA expression has been shown already in the human cerebellum, but without temporal information. MT_1 mRNA was expressed in granule cells and basket-stellate cells, whereas melatonin MT_2 mRNA was observed in Bergmann cells and astrocytes (Mazzucchelli et al., 1996; Al-Ghoul et al., 1998). MT_1 and MT_2 were both previously described in the cerebellum of mice (Adamah-Biassi et al., 2014) and rats (Lacoste et al., 2015). The interactions expected in this case are with melatonin acting in glial cells by modulating glutamate functions through the activation of melatonin receptors. In the cerebellum, Bergmann glial function is associated with synapses in the molecular cell layer. In the case of glutamate synapses, glial cells are thought to be involved in the re-uptake of glutamate (Ottersen et al., 1997; Miyazaki et al., 2017). Furthermore, there is evidence implicating glial cells in circadian rhythm and melatonin—related functions (Welsh and Reppert, 1996; Adachi et al., 2002). In this context, it is noteworthy that the pineal hormone melatonin, as a component of the circadian timing system, may permanently modulate circadian properties of the cerebellum through altered expression or temporal configurations of its molecular components. The possible actions of melatonin in the cerebellum could be in sensorimotor performances (Fraschini et al., 1999; Ng et al., 2017) or in neuroprotective effects (Manda et al., 2008; Pinato et al., 2015). Melatonin receptors can also be involved in the modulation of clock gene expression in the cerebellum as demonstrated in other brain areas (Dardente et al., 2003; Coelho et al., 2015; Vriend and Reiter, 2015). This result could represent an indirect pathway for the transmission of temporal cues to the cerebellar cortex.

The investigation of CR-IR and CB-IR showed cellular temporal characteristics dependent on the period of activity and rest. The subpopulations of Pj cells that appeared as strongly stained CB-IR neurons agrees with previous results in humans (Babij et al., 2013). At the nighttime point, these neurons showed morphology with longer fibers than at the daytime point, which reached the Mol layer of the cerebellar cortex. On the other hand, the most significant expression of CR was in the Gr layer, which has already been described (Ito, 1984). The present study adds the information that CR-IR neurons showed higher expression during the day than in the nighttime period, with morphological differences of dendritic arborization in the Gr layer. In other brain areas, morphological and density changes in CaBPs have also been observed (Gall et al., 2003; Campos et al., 2015a,b), i.e., in the hippocampus, which, along with the cerebellum, exercises different functions dependent on the time of day (Squire, 2007). Since the functions of CaBPs include the regulation of intracellular processes such as neuronal excitability

and release of neurotransmitters (Hof et al., 1999; Schwaller et al., 2002), these patterns may represent responses to different conditions, in this case, day- and nighttime periods.

In conclusion, our data establish several characteristics that can affect the way information is processed in the cerebellum. Day/night changes in the clock proteins PER1 and PER2 are present in the Purkinje cells of the cerebellum of the primate *S. apella*. MT_1 and MT_2 receptors are localized mainly in Bergmann cells in the Pj layer. The highest expression of PER proteins at the daytime point coincides with the highest expression of melatonin receptors. The calcium-binding proteins showed morphological and density changes in the cerebellar cortex between the two time points analyzed.

AUTHOR CONTRIBUTIONS

LMGC: conception or design of the work; analysis, interpretation of data for the work; drafting the work; AH: interpretation of data for the work, analysis, drafting the work; IZV: acquisition, analysis, interpretation of data for the work; drafting the work. RLB: acquisition, analysis, interpretation of data for the work; IFR: acquisition, analysis, interpretation of data for the work; drafting the work; GEPSA: acquisition, analysis, interpretation of data for the work; drafting the work; JSV: acquisition, analysis, interpretation of data for the work; drafting the work; HB: acquisition, analysis, interpretation of data for the work; drafting the work; RMB: acquisition, analysis, interpretation of data for the work; drafting the work; LP: conception or design of the work; analysis, interpretation of data for the work; drafting the work.

FUNDING

This work was supported by funding from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) research grant to LP (2011/51495-4) and (2018/00563-9).

ACKNOWLEDGMENTS

Professor José Américo de Oliveira, Núcleo de Procriação de Macacos Prego, Universidade Estadual Paulista -UNESP for providing capuchin monkey tissues.

REFERENCES

Adachi, A., Natesan, A. K., Whitfield-Rucker, M. G., Weigum, S. E., and Cassone, V. M. (2002). Functional melatonin receptors and metabolic coupling in cultured chick astrocytes. *Glia* 39, 268–278. doi: 10.1002/glia.10109

Adamah-Biassi, E. B., Zhang, Y., Jung, H., Vissapragada, S., Miller, R. J., and Dubocovich, M. L. (2014). Distribution of MT1 melatonin receptor promoter-driven RFP expression in the brains of BAC C3H/HeN transgenic mice. *J. Histochem. Cytochem.* 62, 70–84. doi: 10.1369/0022155413507453

Akiyama, M., Minami, Y., Nakajima, T., Moriya, T., and Shibata, S. (2001). Calcium and pituitary adenylate cyclase-activating polypeptide induced expression of circadian clock gene mPer1 in the mouse cerebellar granule cell culture. *J. Neurochem.* 78, 499–508. doi: 10.1046/j.1471-4159.2001.00452.x

Al-Ghoul, W. M., Herman, M. D., and Dubocovich, M. L. (1998). Melatonin receptor subtype expression in human cerebellum. *EuroReport* 9, 4063–4068. doi: 10.1097/00001756-199812210-00011

Andreasen, N. C., and Pierson, R. (2008). The role of the cerebellum in schizophrenia. *Biol. Psychiatry* 64, 81–88. doi: 10.1016/j.biopsych.2008.01.003

Babij, R., Lee, M., Cortés, E., Vonsattel, J. P., Faust, P. L., and Louis, E. D. (2013). Purkinje cell axonal anatomy: quantifying morphometric changes in essential tremor versus control brains. *Brain* 136, 3051–3061. doi: 10.1093/brain/awt238

Barski, J. J., Hartmann, J., Rose, C. R., Hoebeek, F., Mörl, K., Noll-Hussong, M., et al. (2003). Calbindin in cerebellar Purkinje cells is a critical determinant of the precision of motor coordination. *J. Neurosci.* 23, 3469–3477.

Buckner, R. L. (2013). The cerebellum and cognitive function: 25 years of insight from anatomy and neuroimaging. *Neuron* 80, 807–815. doi: 10.1016/j.neuron.2013.10.044

Campos, L. M., Cruz-Rizzolo, R. J., and Pinato, L. (2015a). The primate seahorse rhythm. *Brain Res.* 1613, 81–91. doi: 10.1016/j.brainres.2015.03.054

Campos, L. M., Cruz-Rizzolo, R. J., Watanabe, I. S., Pinato, L., and Nogueira, M. I. (2014). Efferent projections of the suprachiasmatic nucleus based on the distribution of vasoactive intestinal peptide (VIP) and arginine vasopressin (AVP) immunoreactive fibers in the hypothalamus of *Sapajus apella*. *J. Chem. Neuroanat.* 58, 42–53. doi: 10.1016/j.jchemneu.2014.03.004

Campos, L. M., Osório, E. C., Santos, G. L., Nogueira, M. I., Cruz-Rizzolo, R. J., and Pinato, L. (2015b). Temporal changes in calcium-binding proteins in the medial geniculate nucleus of the monkey *Sapajus apella*. *J. Chem. Neuroanat.* 68, 45–54. doi: 10.1016/j.jchemneu.2015.07.005

Caston, J., Chianale, C., Delhaye-Bouchaud, N., and Mariani, J. (1998). Role of the cerebellum in exploration behavior. *Brain Res.* 808, 232–237. doi: 10.1016/S0006-8993(98)00847-6

Cavdar, S., San, T., Aker, R., Sehirli, U., and Onat, F. (2001). Cerebellar connections to the dorsomedial and posterior nuclei of the hypothalamus in the rat. *J. Anat.* 198(Pt 1), 37–45. doi: 10.1017/S0021878200007172

Choeiri, C., Staines, W., and Messier, C. (2002). Immunohistochemical localization and quantification of glucose transporters in the mouse brain. *Neuroscience* 111, 19–34. doi: 10.1016/S0306-4522(01)00619-4

Coelho, L. A., Peres, R., Amaral, F. G., Reiter, R. J., and Cipolla-Neto, J. (2015). Daily differential expression of melatonin-related genes and clock genes in rat cumulus-oocyte complex: changes after pinealectomy. *J. Pineal Res.* 58, 490–499. doi: 10.1111/jpi.12234

Costa, E. J., Lopes, R. H., and Lamy-Freund, M. T. (1995). Permeability of pure lipid bilayers to melatonin. *J. Pineal Res.* 19, 123–126. doi: 10.1111/j.1600-079X.1995.tb00180.x

Courtemanche, R., Robinson, J. C., and Aponte, D. I. (2013). Linking oscillations in cerebellar circuits. *Front. Neural Circuits* 7:125. doi: 10.3389/fncir.2013.00125

Dardente, H., Menet, J. S., and Poirel, V. J. (2003). Melatonin induces Cry1 expression in the pars tuberalis of the rat. *Brain Res. Mol. Brain Res.* 114, 101–106. doi: 10.1016/S0169-328X(03)00134-7

Doya, K. (2000). Complementary roles of basal ganglia and cerebellum in learning and motor control. *Curr. Opin. Neurobiol.* 10, 732–739. doi: 10.1016/S0959-4388(00)00153-7

Dubocovich, M. L., Rivera-Bermudez, M. A., Gerdin, M. J., and Masana, M. I. (2003). Molecular pharmacology, regulation and function of mammalian melatonin receptors. *Front. Biosci.* 8, d1093–d1108. doi: 10.2741/1089

El Messari, S., Leloup, C., Quignon, M., Brisorgueil, M. J., Penicaud, L., and Arluison, M. (1998). Immunocytochemical localization of the insulin-responsive glucose transporter 4 (Glut4) in the rat central nervous system. *J. Comp. Neurol.* 399, 492–512. doi: 10.1002/(SICI)1096-9861(19981005)399:4<492::AID-CNE4>3.0.CO;2-X

Farnell, Y. Z., Allen, G. C., Nahm, S. S., Neuendorff, N., West, J. R., Chen, W. J., et al. (2008). Neonatal alcohol exposure differentially alters clock gene oscillations within the suprachiasmatic nucleus, cerebellum, and liver of adult rats. *Alcohol. Clin. Exp. Res.* 32, 544–552. doi: 10.1111/j.1530-0277.2007.00598.x

Fraschini, F., Cesarani, A., Alpini, D., Esposti, D., and Stankov, B. M. (1999). Melatonin influences human balance. *Biol. Signals Recept*. 8, 111–119. doi: 10.1159/000014578

Gall, D., Roussel, C., Susa, I., D'Angelo, E., Rossi, P., Bearzatto, B., et al. (2003). Altered neuronal excitability in cerebellar granule cells of mice lacking calretinin. *J. Neurosci*. 23, 9320–9327.

Gilgun-Sherki, Y., Melamed, E., and Offen, D. (2001). Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. *Neuropharmacology* 40, 959–975. doi: 10.1016/S0028-3908(01)00019-3

Gottwald, B., Mihajlovic, Z., Wilde, B., and Mehdorn, H. M. (2003). Does the cerebellum contribute to specific aspects of attention? *Neuropsychologia* 41, 1452–1460. doi: 10.1016/S0028-3932(03)00090-3

Grimaldi, G., and Manto, M. (2012). Topography of cerebellar deficits in humans. *Cerebellum* 11, 336–351. doi: 10.1007/s12311-011-0247-4

Guilding, C., and Piggins, H. D. (2007). Challenging the omnipotence of the suprachiasmatic timekeeper: are circadian oscillators present throughout the mammalian brain? *Eur. J. Neurosci*. 25, 3195–3216. doi: 10.1111/j.1460-9568.2007.05581.x

Hof, P. R., Glezer, I. I., Condé, F., Flagg, R. A., Rubin, M. B., Nimchinsky, E. A., et al. (1999). Cellular distribution of the calcium-binding proteins parvalbumin, calbindin, and calretinin in the neocortex of mammals: phylogenetic and developmental patterns. *J. Chem. Neuroanat*. 16, 77–116. doi: 10.1016/S0891-0618(98)00065-9

Ito, M. (1984). The modifiable neuronal network of the cerebellum. *Jpn. J. Physiol*. 34, 781–792. doi: 10.2170/jjphysiol.34.781

Lacoste, B., Angeloni, D., Dominguez-Lopez, S., Calderoni, S., Mauro, A., Fraschini, F., et al. (2015). Anatomical and cellular localization of melatonin MT1 and MT2 receptors in the adult rat brain. *J. Pineal Res*. 58, 397–417. doi: 10.1111/jpi.12224

Li, J. Z., Bunney, B. G., Meng, F., Hagenauer, M. H., Walsh, D. M., Vawter, M. P., et al. (2013). Circadian patterns of gene expression in the human brain and disruption in major depressive disorder. *Proc. Natl. Acad. Sci. U.S.A*. 110, 9950–9955. doi: 10.1073/pnas.1305814110

Lowrey, P. L., and Takahashi, J. S. (2011). Genetics of circadian rhythms in mammalian model organisms. *Adv. Genet*. 74, 175–230. doi: 10.1016/B978-0-12-387690-4.00006-4

Manda, K., Ueno, M., and Anzai, K. (2008). Melatonin mitigates oxidative damage and apoptosis in mouse cerebellum induced by high-LET 56Fe particle irradiation. *J. Pineal Res*. 44, 189–196. doi: 10.1111/j.1600-079X.2007.00507.x

Manocha, S. L., Shantha, T. R., and Bourne, G. H. (1968). *A Stereotaxic Atlas of the Brain of the Cebus Monkey (Cebus apella)*. Oxford: Clarendon Press.

Mazzucchelli, C., Pannacci, M., Nonno, R., Lucini, V., Fraschini, F., and Stankov, B. M. (1996). The melatonin receptor in the human brain: cloning experiments and distribution studies. *Brain Res. Mol. Brain Res*. 39, 117–126. doi: 10.1016/0169-328X(96)00017-4

Mendoza, J., Pévet, P., Felder-Schmittbuhl, M. P., Bailly, Y., and Challet, E. (2010). The cerebellum harbors a circadian oscillator involved in food anticipation. *J. Neurosci*. 30, 1894–1904. doi: 10.1523/JNEUROSCI.5855-09.2010

Mistlberger, R. E. (1994). Circadian food-anticipatory activity: formal models and physiological mechanisms. *Neurosci. Biobehav. Rev*. 18, 171–195. doi: 10.1016/0149-7634(94)90023-X

Miyazaki, T., Yamasaki, M., Hashimoto, K., Kohda, K., Yuzaki, M., Shimamoto, K., et al. (2017). Glutamate transporter GLAST controls synaptic wrapping by *Bergmann glia* and ensures proper wiring of Purkinje cells. *Proc. Natl. Acad. Sci. U.S.A*. 114, 7438–7443. doi: 10.1073/pnas.1617330114

National Research Council (US) Committee on Guidelines for the Use of Animals in Neuroscience and Behavioral Research (2003). *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research*. Washington, DC: National Academies Press.

Ng, K. Y., Leong, M. K., Liang, H., and Paxinos, G. (2017). Melatonin receptors: distribution in mammalian brain and their respective putative functions. *Brain Struct. Funct*. 222, 2921–2939. doi: 10.1007/s00429-017-1439-6

Ottersen, O. P., Chaudhry, F. A., Danbolt, N. C., Laake, J. H., Nagelhus, E. A., Storm-Mathisen, J., et al. (1997). Molecular organization of cerebellar glutamate synapses. *Prog. Brain Res*. 114, 97–107. doi: 10.1016/S0079-6123(08)63360-9

Paulus, E. V., and Mintz, E. M. (2016). Circadian rhythms of clock gene expression in the cerebellum of serotonin-deficient Pet-1 knockout mice. *Brain Res*. 1630, 10–17. doi: 10.1016/j.brainres.2015.10.044

Paxinos, G., Huang, X. F., and Toga, A. W. (2000). *The Rhesus Monkey Brain in Stereotaxic Coordinates*. San Diego, CA: Academic Press.

Pinato, L., da Silveira Cruz-Machado, S., Franco, D. G., Campos, L. M., Cecon, E., Fernandes, P. A., et al. (2015). Selective protection of the cerebellum against intracerebroventricular LPS is mediated by local melatonin synthesis. *Brain Struct. Funct*. 220, 827–840. doi: 10.1007/s00429-013-0686-4

Rath, M. F., Rohde, K., and Møller, M. (2012). Circadian oscillations of molecular clock components in the cerebellar cortex of the rat. *Chronobiol. Int*. 29, 1289–1299. doi: 10.3109/07420528.2012.728660

Ravizza, S. M., McCormick, C. A., Schlerf, J. E., Justus, T., Ivry, R. B., and Fiez, J. A. (2006). Cerebellar damage produces selective deficits in verbal working memory. *Brain* 129, 306–320. doi: 10.1093/brain/awh685

Reppert, S. M., and Weaver, D. R. (2002). Coordination of circadian timing in mammals. *Nature* 418, 935–941. doi: 10.1038/nature00965

Rocha, V. A., Frazão, R., Campos, L. M., Mello, P., Donato, J. Jr., Cruz-Rizzolo, R. J., et al. (2014). Intrinsic organization of the suprachiasmatic nucleus in the capuchin monkey. *Brain Res*. 1543, 65–72. doi: 10.1016/j.brainres.2013.10.037

Schnitzler, A., and Gross, J. (2005). Normal and pathological oscillatory communication in the brain. *Nat. Rev. Neurosci*. 6, 285–296. doi: 10.1038/nrn1650

Schwaller, B., Meyer, M., and Schiffmann, S. (2002). 'New' functions for 'old' proteins: the role of the calcium-binding proteins calbindin D-28k, calretinin and parvalbumin in cerebellar physiology, studies with knockout mice. *Cerebellum* 1, 241–258. doi: 10.1080/147342202320883551

Shieh, K. R. (2003). Distribution of the rhythm-related genes rPERIOD1, rPERIOD2, and rCLOCK, in the rat brain. *Neuroscience* 118, 831–843. doi: 10.1016/S0306-4522(03)00004-6

Soghomonian, J. J., Zhang, K., Reprakash, S., and Blatt, G. J. (2017). Decreased parvalbumin mRNA levels in cerebellar Purkinje cells in autism. *Autism Res*. 10, 1787–1796. doi: 10.1002/aur.1835

Squire, L. R. (2007). Neuroscience. Rapid consolidation. *Science* 316, 57–58. doi: 10.1126/science.1141812

Takumi, T., Taguchi, K., Miyake, S., Sakakida, Y., Takashima, N., Matsubara, C., et al. (1998). A light-independent oscillatory gene mPer3 in mouse SCN and OVLT. *EMBO J*. 17, 4753–4759. doi: 10.1093/emboj/17.16.4753

Verly, M., Verhoeven, J., Zink, I., Mantini, D., Peeters, R., Deprez, S., et al. (2014). Altered functional connectivity of the language network in ASD: role of classical language areas and cerebellum. *Neuroimage Clin*. 4, 374–382. doi: 10.1016/j.nicl.2014.01.008

Verwey, M., and Amir, S. (2009). Food-entrainable circadian oscillators in the brain. *Eur. J. Neurosci*. 30, 1650–1657. doi: 10.1111/j.1460-9568.2009.06960.x

Vriend, J., and Reiter, R. J. (2015). Melatonin feedback on clock genes: a theory involving the proteasome. *J. Pineal Res*. 58, 1–11. doi: 10.1111/jpi.12189

Welsh, D. K., and Reppert, S. M. (1996). Gap junctions couple astrocytes but not neurons in dissociated cultures of rat suprachiasmatic nucleus. *Brain Res*. 706, 30–36. doi: 10.1016/0006-8993(95)01172-2

Zhu, J. N., and Wang, J. J. (2008). The cerebellum in feeding control: possible function and mechanism. *Cell Mol. Neurobiol*. 28, 469–478. doi: 10.1007/s10571-007-9236-z

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 19 March 2018
doi: 10.3389/fphys.2018.00230

On Variations in the Level of PER in Glial Clocks of *Drosophila* Optic Lobe and Its Negative Regulation by PDF Signaling

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We show that the level of the core protein of the circadian clock Period (PER) expressed by glial peripheral oscillators depends on their location in the *Drosophila* optic lobe. It appears to be controlled by the ventral lateral neurons (LNvs) that release the circadian neurotransmitter Pigment Dispersing Factor (PDF). We demonstrate that glial cells of the distal medulla neuropil (dMnGl) that lie in the vicinity of the PDF-releasing terminals of the LNvs possess receptors for PDF (PDFRs) and express PER at significantly higher level than other types of glia. Surprisingly, the amplitude of PER molecular oscillations in dMnGl is increased twofold in PDF-free environment, that is in Pdf^0 mutants. The Pdf^0 mutants also reveal an increased level of glia-specific protein REPO in dMnGl. The photoreceptors of the compound eye (R-cells) of the PDF-null flies, on the other hand, exhibit de-synchrony of PER molecular oscillations, which manifests itself as increased variability of PER-specific immunofluorescence among the R-cells. Moreover, the daily pattern of expression of the presynaptic protein Bruchpilot (BRP) in the lamina terminals of the R-cells is changed in Pdf^0 mutant. Considering that PDFRs are also expressed by the marginal glia of the lamina that surround the R-cell terminals, the LNv pacemakers appear to be the likely modulators of molecular cycling in the peripheral clocks of both the glial cells and the photoreceptors of the compound eye. Consequently, some form of PDF-based coupling of the glial clocks and the photoreceptors of the eye with the central LNv pacemakers must be operational.

Keywords: circadian clocks, glial oscillators, neuronal pacemakers, PER, PDF, drosophila glia, *Drosophila*

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: *01 November 2017*
Accepted: *01 March 2018*
Published: *19 March 2018*

Citation:
Górska-Andrzejak J, Chwastek EM, Walkowicz L and Witek K (2018) On Variations in the Level of PER in Glial Clocks of Drosophila Optic Lobe and Its Negative Regulation by PDF Signaling. Front. Physiol. 9:230. doi: 10.3389/fphys.2018.00230

INTRODUCTION

The circadian clocks, displaying molecular oscillations of canonical clock molecules Period (PER) and Timeless (TIM) with a period of ∼24 h, are endogenous pacemakers that lay cellular foundation for biological timekeeping (Tataroglu and Emery, 2015). There are two main types of cells that express genes encoding PER and TIM (*per*, *tim*) in the brain of *Drosophila melanogaster*: the so called clock neurons and the glial cells (Siwicki et al., 1988; Zerr et al., 1990). The former constitute the central pacemaker whereas the latter play the function of peripheral oscillators, similar to photoreceptors of the compound eye and to many cells in non-neural tissues of the head and body (Hardin, 2011; Xu et al., 2011).

In *Drosophila* brain, there are about 150 clock neurons of the central pacemaker distributed in lateral and dorsal clusters that minister different circadian functions (Helfrich-Förster, 2005; Nitabach and Taghert, 2008; Hermann-Luibl and Helfrich-Förster, 2015). There are seven groups in each brain hemisphere, but the most important role in the circadian network and behavioral rhythmicity of flies plays the ventrolateral cluster of clock neurons (LNvs), which includes neurons with small and large cell bodies (Allada and Chung, 2010). The small-lateral neurons (s-LNvs) are crucial for maintaining the circadian activity rhythm (Blanchardon et al., 2001). They project toward the dorsal protocerebrum, where they form short arborizations (Helfrich-Förster, 1998) displaying prominent circadian changes of morphology (Fernández et al., 2008; Gorostiza et al., 2014). The large-lateral neurons (l-LNvs), on the other hand, are not necessary for sustaining the rhythm of activity in constant darkness (DD), but they are essential for light-mediated modulation of arousal and sleep (Sheeba et al., 2008, 2010). It is presumed that their input may be particularly robust, since they display the molecular rhythms of PER and TIM that are phase-advanced and of higher amplitude compared to other clock neurons (Rosato and Kyriacou, 2008). They send projections to the optic lobe and densely arborize on the surface of the second optic neuropil, the medulla (Helfrich-Förster, 1998; Helfrich-Förster et al., 2007). They are, therefore, anatomically well-situated to receiving the light input from the retina of the compound eye (in addition to the one they receive via activation of their photopigment–Cryptochrome; Yoshii et al., 2008) and conveying circadian signals to the optic lobe. They control the output by the paracrine release of the main circadian transmitter - the neuropeptide Pigment Dispersing Factor (PDF) (Helfrich-Förster, 1997; Park et al., 2000), and by signaling via its receptor—PDFR (Renn et al., 1999; Lin et al., 2004; Lear et al., 2005; Shafer et al., 2008; Im and Taghert, 2010; Im et al., 2011). It synchronizes different clusters of clock neurons and the whole circadian network (Lin et al., 2004; Lear et al., 2005; Nitabach et al., 2006; Shafer et al., 2008; Yoshii et al., 2009).

The glial cells, even though much less studied than the clock neurons, have already proved to be integral components of the circadian network (Zwarts et al., 2015). In *Drosophila* brain, like in vertebrates, we discern many different types of glial cells (Edwards and Meinertzhagen, 2010) based on their morphology (Carlson and Saint Marie, 1990; Cantera and Trujillo-Cenoz, 1996), gene expression, and lineage analysis (Ito et al., 1995; Giangrande, 1996; Klämbt et al., 1996; Edwards et al., 2012). The early studies on clock genes expression in *Drosophila* revealed that numerous glial cells display cyclic expression of *per* (Siwicki et al., 1988; Zerr et al., 1990) and *tim* (Peschel et al., 2006), and that the expression of *per* in glia might be sufficient to drive a weak behavioral rhythm (Ewer et al., 1992). Exciting recent works have shown that rhythmic expression of both clock proteins and glia-specific proteins, such as Ebony are involved in regulation of behavioral rhythms (Suh and Jackson, 2007; Ng et al., 2011; Ng and Jackson, 2015). Glial cells of the visual system of Diptera, on the other hand, have been shown to contribute to the circadian plasticity of flies visual system. Epithelial glial cells of the first visual neuropil or lamina display robust rhythmic changes in their volume (Pyza and Górska-Andrzejak, 2004) and in the level of expression of the catalytic subunit of sodium pump, the Na^+/K^+-ATPase α subunit (Górska-Andrzejak et al., 2009). Their modulatory input affects both the rhythm of expression of a presynaptic protein Bruchpilot in the lamina synaptic neuropil (Górska-Andrzejak et al., 2013) and the pattern of rhythmic morphological changes of L1 and L2 interneurons, which are the main postsynaptic partners of the compound eye photoreceptors (Pyza and Górska-Andrzejak, 2004; Górska-Andrzejak, 2013).

So far, the glial clocks are known to act downstream of the clock neurons (Suh and Jackson, 2007), but they signal back to them as well (Ng et al., 2011). The circadian rhythmicity, including the rhythmicity of behavior, appears therefore to depend on the glia-neuronal communication and reciprocal interactions (Zwarts et al., 2015; Ng et al., 2016). Nevertheless, the exact nature of mutual influence of the neuronal and glial clocks is far from being fully understood. It is still under investigation how much influence the neuronal pacemakers have on the peripheral glial oscillators and what are the exact neuronal and glial signals that are used in their communication (Zwarts et al., 2015).

Our results reveal heterogeneity of the optic lobe glial clocks. We show that the glial cells situated in the vicinity of the terminals of the circadian clock ventral LNvs may be the most robust molecular oscillators among the glia. We also demonstrate that the clock neurons of the ventrolateral cluster influence the level of PER (the amplitude of the clock) in the glia and in the eye photoreceptors by PDF signaling. Consequently, we propose a novel role for PDF as a potential link between the central and the peripheral clocks in glial and photoreceptor cells. Our study on Pdf^0 mutants suggests that the LNv neurons negatively influence the level of PER in the former and enhance the synchronization among the latter.

MATERIALS AND METHODS

Animals

We used the following strains of *D. melanogaster*: wild-type Canton-S (CS), *w+*; Pdf^0 mutant (referred to as Pdf^0; a kind gift from Charlotte Förster, University of Würzburg), *period* mutant (per^0) and *tim*-Gal4 transgenic strain (a kind gift from François Rouyer, Paris Saclay Institute of Neuroscience), as well as other transgenic strains from Bloomington Drosophila Stock Centre (BDSC): *repo*-Gal4 (BDSC, stock no. 7415), *pdfR*-Gal4 (BDSC, stock no. 33070), UAS-pdfR^{RNAi} (BDSC, stock no. 42508), UAS-VAL10- GFP (BDSC, stock no. 35786), UAS-S65T-GFP (BDSC, stock no. 1521), and UAS-mCD8-GFP (BDSC, stock no. 5137). The stocks were maintained on a standard yeast-cornmeal-agar medium, at 25 ± 1°C, under light/dark or day/night conditions (12 h of light and 12 h of darkness, LD 12:12; ZT0 and ZT12 denote the beginning of the day and the night, respectively, ZT—Zeitgeber Time). 7- to 10- days old males were used for each experiment. Following their eclosion, they were divided into two groups which were kept either in LD 12:12, or in DL 12:12 (reversed cycle) for 1 week prior to their decapitation at several time points during the day and night of the 24-h cycle. Flies kept in LD 12:12 were decapitated during the light phase (day)

of the cycle, at ZT24/0, ZT1, and ZT4, whereas flies kept in DL 12:12 were decapitated during the dark phase (night) of the cycle, at ZT13, ZT16, ZT19, and ZT21. Flies used for experiments in constant darkness conditions (DD) were entrained in LD 12:12 for 4 days and then transferred to DD for 2 days. On the third day of DD they were decapitated at CT1, CT4, CT13, CT16, CT19, CT21, and CT24 (CT0 and CT12 denote the beginning of the subjective day and the subjective nigh, respectively, CT-Circadian Time).

Immunolabeling

Experimental flies were immobilized with CO_2 and decapitated directly in a drop of freshly prepared fixative: the solution of 4% paraformaldehyde (PFA) in 0.1 M Phosphate Buffer (PB). Approximately 30 flies were sacrificed for each data point. After fixation and cryoprotecting infiltration in the solution of 25% sucrose in 0.01 M sodium Phosphate Buffer Saline (PBS), their heads were cut either in the frontal or horizontal plane into 20 μm thick cryosections. Following this, they were incubated with a rabbit polyclonal anti-PER serum (a gift from Ralf Stanewsky, University of Munster; Stanewsky et al., 1997) and a goat anti-rabbit Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). To visualize the clock neurons that synthetize the neuropeptide PDF, we used a polyclonal rabbit anti-β-PDH serum, which recognizes the insect PDF (a gift from Ezio Rosato, University of Leicester; Dircksen et al., 1987). We also used anti-REPO (8D12) and anti-BRP (nc82) mouse antibodies (Developmental Studies Hybridoma Bank, DSHB). The fluorescence staining was performed with Cy3-conjugated secondary antibodies. Fluorescently labeled tissue was examined using Zeiss LSM 510 Meta confocal microscope following extensive washing and mounting in a Vectashield or DAPI-containing Vectashield (Vector).

Quantification of Immunolabeling

Drosophila melanogaster heads that were collected at various time points (of the same 24-h cycle) were fixed, processed and immunolabeled in parallel, under the same conditions. Then, their images were acquired at non saturated settings, using identical image acquisition parameters for all data points (time points). The circadian changes in the intensity of PER-specific immunolabeling corresponding to the changes in the amount of PER protein, were measured in the glial cells of the optic lobe, as well as in the lateral clock neurons and in the photoreceptors of the compound eye. The level (brightness) of fluorescence represented by the Mean Gray Value (the sum of the gray values of all pixels in the selected area, divided by the number of pixels within the selection) was measured using ImageJ software (NIH, Bethesda). In this software, the range of gray values (between the *Min* and *Max*) in 8-bit images is divided into 256 bins. The background signal was subtracted.

Statistics

To allow the comparisons between data from different experiments, which revealed certain differences in the intensity of labeling, the data were presented as a percentage of the highest value (100%) that was obtained in a given experiment. All the data were statistically analyzed using the Shapiro–Wilk *W*-test for normality. The differences between experimental groups (ZT/CT time points) were calculated based on the mean of measurements obtained from 7 to 12 individuals within a group (decapitated at the particular time point). The statistical significance of differences between groups was estimated using either ANOVA, or the nonparametric counterpart of ANOVA—Kruskal–Wallis test (one-way test) followed by *post-hoc* Multiple Comparison Test. In each analysis a probability value of $p < 0.05$ was set for significant differences.

RESULTS

PER-specific immunolabeling was observed in the usual locations (Siwicki et al., 1988; Zerr et al., 1990; Ewer et al., 1992) of CS brains, as well as in brains of *repo*-Gal4/UAS-S65T-GFP transgenic flies (**Figure 1**) that were sectioned at the end of the dark phase/night (ZT24). It was detected in the nuclei of circadian pacemaker neurons (of lateral and dorsal groups; LNs and DNs), compound eye photoreceptors, and numerous glial cells (**Figure 1A**). Such a pattern of labeling in CS and *repo*-Gal4/UAS-S65T-GFP transgenic flies (**Figure 1**) confirmed the specificity of the applied serum, which was further supported by lack of labeling in per^0 mutant—a negative genetic control.

dMnGl Display the Highest Level of PER of All the Glia

The nuclei of PER-immunoreactive glial cells were found in the whole brain of *D. melanogaster* (**Figure 1A**). When checked on thin (20 μm) cryosections, the intensity of their immunolabeling (reflecting the amount of nuclear PER) was discovered to vary significantly, depending on the location of glia in the brain, in other words—on the type of glia. The largest differences were observed between glia of the first (lamina) and the second (medulla) visual neuropils (**Figure 1**). PER-specific immunofluorescence was bright in glia of the medulla, while it was barely detectable in glial cells of the lamina (LGl). Interestingly, the highest level of immunofluorescence was observed in the small nuclei of glia inhabiting the distal part of the medulla (hereafter referred to as distal medulla neuropil glia, dMnGl), whose cell bodies are located precisely on the border between the cortex and the neuropil of the medulla (**Figures 1A–C**). The average level of PER-specific immunofluorescence in the nuclei of dMnGl was 58% higher than in the lamina neuropil glia (LGl), 67% higher than in the cortex/satellite glia of medulla (McGl), and 47% higher than in the tract glia of the inner chiasm (iChGl) (Kruskal–Wallis Test: $H[3, N = 59] = 32.5$, $p = 0.00001$, followed by Multiple Comparison Test, $p = 0.0008$, $p = 0.000003$, and $p = 0.0004$, respectively; **Figure 1**). These cells also expressed the highest level of GFP reporter of *tim*, when examined in *tim*-Gal4/UAS-S65T-GFP transgenic flies (**Figures 1D–D"**). The differences in the expression level of GFP reporter of *tim* (driven by *tim*-Gal4 pan-circadian driver) and the intensity of PER-specific staining in different types of glia indicate that the population of glial clocks is heterogeneous. The particularly high levels (as far as the glial cells

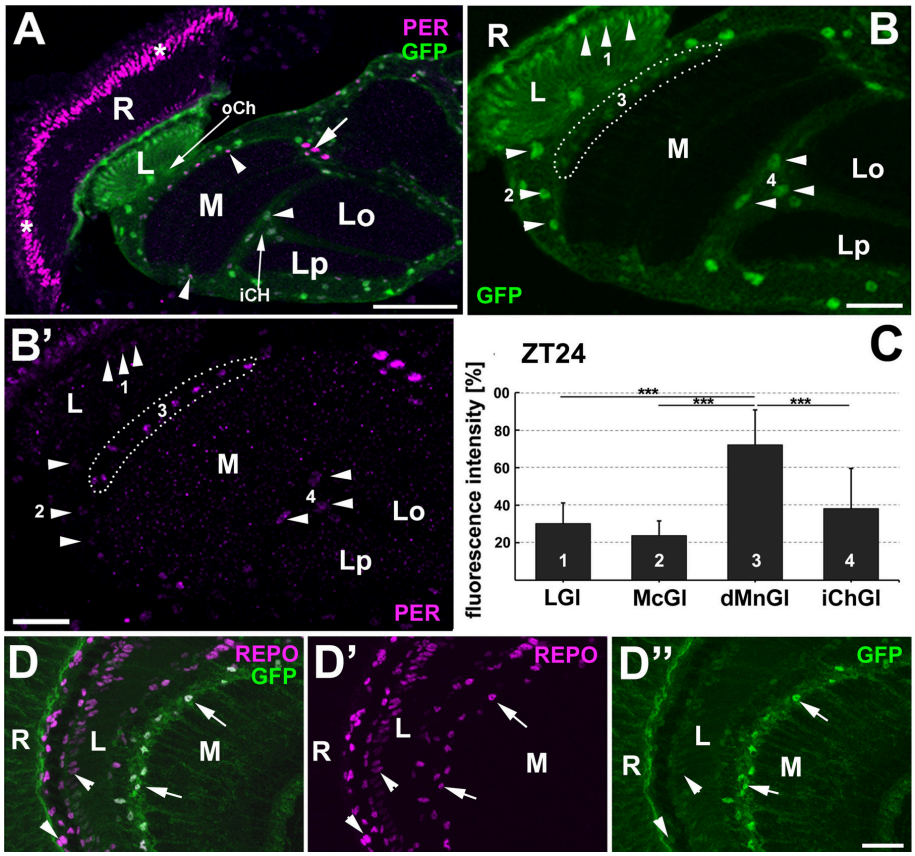

FIGURE 1 | (A) Compound eye retina and underlying optic lobe of flies with targeted expression of Green Fluorescent Protein (GFP) to glial cells (*repo*-Gal4/UAS-S65T-GFP) and immunolabeled with anti-PER serum. PER-positive nuclei (magenta) belong to the clock cells of the lateral protocerebrum, the so called ventral lateral neurons-LNvs (arrow), the compound eye photoreceptors (asterisks) and the glial cells (arrowheads). R-retina, L-lamina, M-medulla, Lo, and Lp-two parts of the lobula complex, oCh- outer chiasm and iCh-inner chiasm. Scale bar: 50 μm. **(B)** The glial cells selected for measurements of PER-specific immunofluorescence (arrowheads): the epithelial glial cells of the lamina neuropil (LGl, 1), the medula cortex glia (McGl, 2), the distal medulla glia (dMnGl, 3), and the inner chiasm glia (iChGl, 4). R-retina, L-lamina, M-medulla, Lo, and Lp- two parts of the lobula complex. Scale bar: 20 μm. **(B')** PER-specific immunofluorescence of cells shown in **(B)**. Scale bar: 20 μm. **(C)** The average level of PER-specific immunofluorescence (±*SD*) in the nuclei of LGl (1), McGl (2), dMnGl (3), and iChGl (4) in the optic lobe of CS flies at the end of the night (ZT24). The statistically significant differences are marked by asterisks (***$p \leq 0.001$). **(D–D")** The lamina and medulla of flies with targeted expression of GFP (green) to *tim* expressing cells (*tim*-Gal4/UAS-S65T-GFP) and immunolabeled using 8D12 Mab against pan-glial REPO marker (magenta). Among many glial cells that are marked by REPO-specific immunofluorescence, only dMnGl (arrows) express the GFP reporter on such a high level. The low level of GFP is seen in the nuclei of other glial cells, especially glia of the lamina cortex, or lamina neuropil (arrowheads). R, retina; L, lamina; M, medulla. Scale bar: 20 μm.

are concerned) of PER and TIM in dMnGl imply high-amplitude cycling of the clock proteins.

dMnGl Are Weaker Oscillators Than Neuronal Clocks, but Work in Phase With Them

Daily changes of nuclear PER-specific immunofluorescence (reflecting PER rhythmic accumulation in the nucleus) were generally the same in the nuclei of dMnGl as in the nuclei of clock cells of the lateral protocerebrum (LNvs), or in the photoreceptors of the eye (R-cells; **Figure 2**). The fluorescence was the most intense at the end of the night and at the beginning of the day (ZT24, ZT1), and undetectable (the same as in the surrounding cytoplasm) at the beginning of the night (ZT13; **Figure 2**), which confirmed the similarity of PER nuclear accumulation patterns in neurons and glial cells. dMnGl were in phase with rhythms of the LNvs and the R-cells, suggesting that they are coupled with the circadian timing system. Even though the oscillatory pattern of the glia was similar to that of the neuronal oscillators (cf. **Figure 2**), the average daily level of nuclear immunofluorescence in dMnGl was 52% lower than in the LNvs and 65% lower than in the R-cells (Kruskal–Wallis Test: H [2, $N = 210$] = 59.7, $p = 0.00001$, followed by Multiple Comparison Test, $p = 0.00002$ and $p = 0.000001$, respectively). The considerable differences in the level of PER-specific immunofluorescence in dMnGl and LNvs or R-cells were observed at each of the time points of LD cycle, at which the nuclear accumulation of PER can normally be observed (ZT16-ZT4; **Figure 2**).

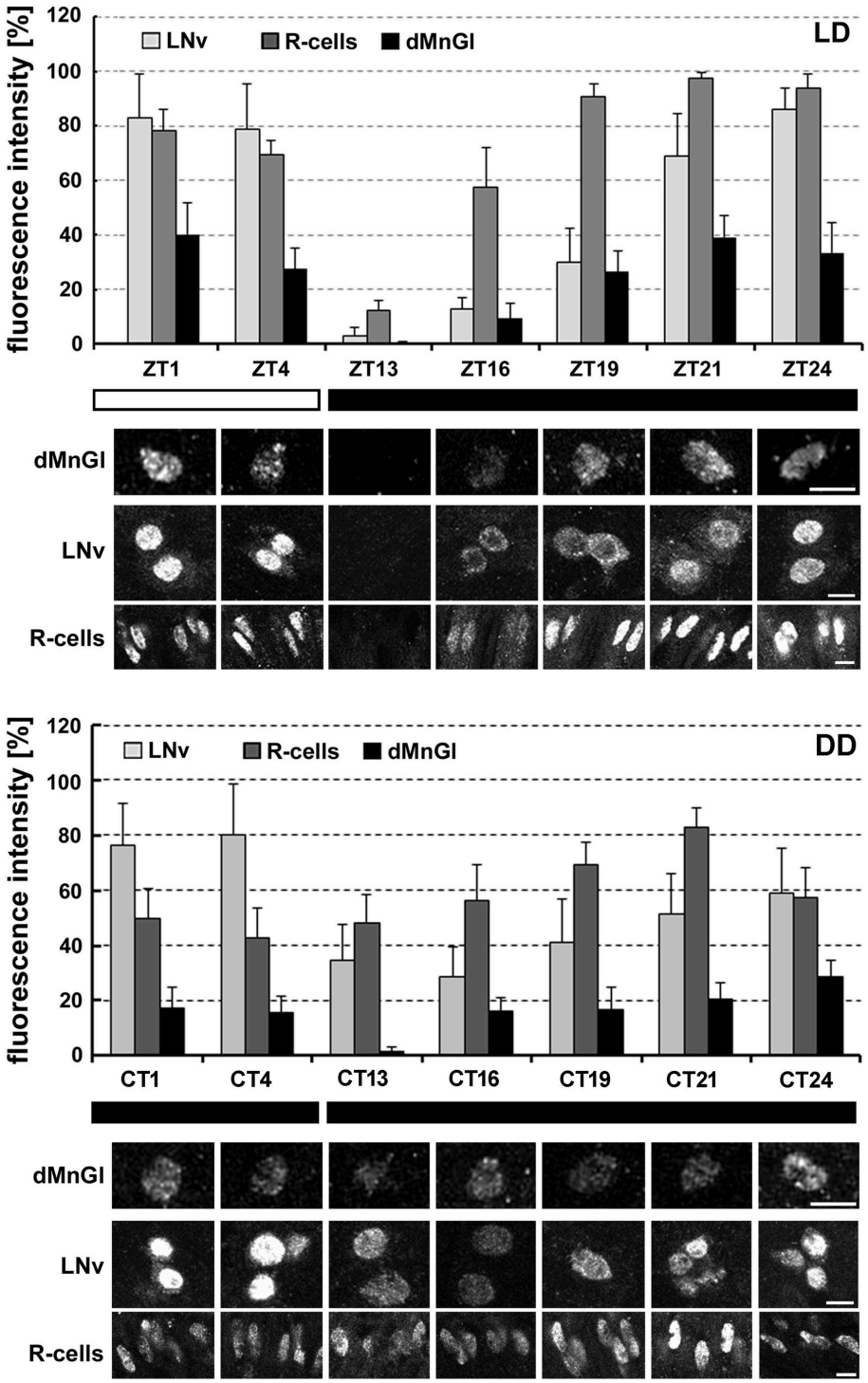

FIGURE 2 | The daily (LD) and circadian (DD) rhythms in PER-specific immunofluorescence (average ± *SD*) as the measure of PER molecular oscillations in the nuclei of dMnGl, the large-lateral ventral pacemaker neurons (LNv) and photoreceptors of the compound eye (R-cells) in the optic lobe of CS flies. Like in the LNv neurons and the R-cells, the average level of PER-specific fluorescence in dMnGl changed significantly in the course of the day in LD (dMnGl: Kruskal–Wallis Test: H [6, $N = 67$] = 48.7, $p = 0.00001$; LNvs: Kruskal–Wallis Test: H [6, $N = 73$] = 58.8, $p = 0.00001$; R-cells: Kruskal–Wallis Test: H [6, $N = 70$] = 59.8, $p = 0.00001$), and in DD (dMnGl: Kruskal–Wallis Test: H [6, $N = 69$] = 36.8, $p = 0.00001$; LNvs: Kruskal–Wallis Test: H [6, $N = 110$] = 65.8, $p = 0.00001$; R-cells: Kruskal–Wallis Test: H [6, $N = 70$] = 41.2, $p = 0.00001$), but with considerably smaller amplitude. Below the charts: exemplary images collected at different time points (ZTs or CTs), showing the peak and trough accumulation of PER in the nuclei of dMnGl, LNvs, and R-cells. Scale bar: 5 μm. White and black bars on the bottom indicate light and dark periods, respectively.

In DD conditions the amplitude of oscillations of PER expression was smaller in all three types of cells (**Figure 2**). The maximum levels of fluorescence (at CT1, CT4, CT21, and CT24) were reduced (e.g., in the R-cells and dMnGl at CT1 and CT4), while the minimum levels (e.g., in the R-cells and the LNvs at CT13) were increased with respect to LD (**Figure 2**). The elevation of fluorescence intensity at CT13, however, was not observed in dMnGl. The average daily level of fluorescence in dMnGl was on average 70% lower than in the LNvs and in the R-cells (Kruskal–Wallis Test: H [2, $N = 249$] = 130.1, $p = 0.0001$, followed by Multiple Comparison Test, $p = 0.00001$ in case of both dMnGl vs. LNvs and dMnGl vs. R-cells). Hence, the main difference between dMnGl and the neuronal oscillators (LNvs and R-cells) concerns mainly the amplitude of PER oscillations.

dMnGl Possess Receptors for PDF (PDFRs)

The distinguishing feature of dMnGl is the location of their cell bodies in the neighborhood of the optic lobe terminals of the LNv neurons, which secrete the neuropeptide PDF (**Figure 3**). This anatomical proximity enables the direct and strong influence of the LNvs on the circadian function of dMnGl. To find out whether the particularly high level of PER in dMnGl might result from this proximity (**Figures 3B,C**) and this influence, we checked if (i) dMnGl were equipped with receptors for PDF (PDFRs) and (ii) whether the level of PER was lower in dMnGl of Pdf^0 mutants (due to PDF absence) and of *repo*-Gal4 /UAS-pdfR^{RNAi} flies (due to RNAi-driven silenced expression of PDFRs in glia).

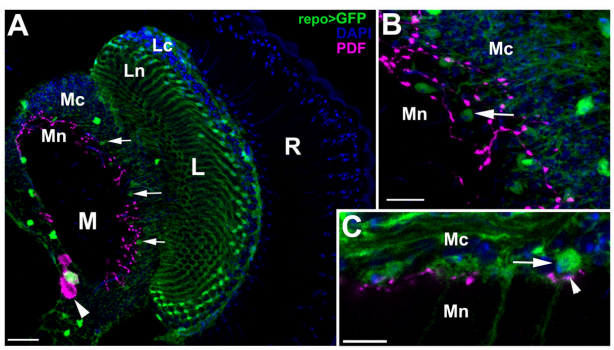

FIGURE 3 | Confocal images of the optic lobe of *repo*-Gal4/UAS-S65T-GFP transgenic flies showing cytoplasmic and nuclear expression of GFP (green) in glial cells, in combination with anti-PDF immunolabeling (magenta), and DAPI nuclear labeling (blue). **(A)** The location of dMnGl in relation to PDF-immunoreactive varicosities of the LNvs on the medulla neuropil surface in frontal section of the optic lobe. PDF-positive cell bodies of the LNvs (arrowhead) are located in the accessory medulla, whereas their optic lobe terminals reside in the distal part of the medulla neuropil, where small nuclei of dMnGl (arrows) are located. Scale bar: 20 μm. **(B)** Magnification of dense varicose arborization of PDF-positive fibers of the LNvs and the nuclei of dMnGl (arrow). Scale bar: 10 μm. **(C)** The horizontal section reveals that PDF releasing terminals (arrowhead) are located right beneath the medulla cortex, in close proximity to dMnGl cell bodies (arrow). R, retina; L, lamina; M, medulla; Lc, lamina cortex; Ln, lamina neuropil; Mc, medulla cortex; Mn, medulla neuropil. Scale bar: 50 μm.

To resolve the first issue, we investigated in detail the pattern of expression of *pdfR*-Gal4 driver (which reflects the endogenous *pdfR* expression according to Lear et al., 2009), in the region of dMnGl residence. It turned out that the cytoplasmic and nuclear GFP in *pdfR*-Gal4/UAS-S65T-GFP transgenic flies exposed dMnGl (**Figures 4A–B'**), which indicated the presence of PDFRs in these cells. The level of GFP fluorescence in their nuclei was, however, by 70–78% lower than in the nuclei of other GFP-positive cells localized in their proximity, such as the LNvs and other neurons (**Figures 4B,B'**). Even though dMnGl express relatively lower amounts of PDFR, they must belong to the LNv target cells, being not only conveniently positioned in the vicinity of the PDF releasing terminals of the LNvs (**Figure 3**), but also capable of receiving the PDF conveyed circadian information (**Figures 4B,B'**).

The GFP expression controlled by *pdfR*-Gal4 driver exposed also the varicose network of the LNv terminals on the surface of the medulla neuropil (**Figure 4A**). Their examination in the medulla of *pdfR*-Gal4/UAS-S65T-GFP (cytoplasmic GFP) and *pdfR*-Gal4/mCD8-GFP (membranous GFP) transgenic flies revealed the presence of much brighter spots of green fluorescence—presumably patches of PDFR aggregation (**Figures 4A,C**). Interestingly, these bright PDFR patches were settled right next to sites of PDF release immunolabeled with anti-PDF Ab (**Figure 4C**). The LNv terminals, which both release PDF and receive PDF-conveyed information, seem to envelope the center of each unit (column) of the medulla neuropil (**Figure 4D**). Consequently, the processes that build the medulla column (with dMnGl processes among them) must receive (directly or indirectly) the PDF-conveyed synchronizing signals sent to the optic lobe by the LNv pacemakers. Each medulla column is innervated by PDF neurons and comprises processes that possess PDFRs. It appears, however, that PDF diffuses as far as the proximal part of the first optic neuropil or lamina, since the lamina marginal glia that reside at that part of neuropil strongly express PDFRs (**Figure 4E**).

The Level of PER Increases in Glia of Pdf^0 Mutants

In order to account for the influence of PDF on the clock mechanism of dMnGl, we checked whether the level of nuclear PER in dMnGl would be lower in Pdf^0 mutants than in CS flies. Contrary to our expectations, however, in the absence of PDF the level of PER was higher (**Figures 5A,B**). In LD conditions, PER-specific immunofluorescence in dMnGl of Pdf^0 with respect to CS was 50% higher at ZT24 (t-test, $t = -4.86$, df = 18, $p = 0.0001$), 32% higher at ZT1 (t-test, $t = -3.5$, df = 18, $p = 0.003$), and 18.7% higher at ZT4 (t-test, $t = -2.2$, df = 15, $p = 0.04$). It was 50% lower in Pdf^0 than in CS only in the middle of the night, at ZT16 (t-test, $t = 2.2$, df = 19, $p = 0.04$), suggesting a delay in nuclear aggregation of PER in Pdf^0 glia (**Figure 5C**).

In DD conditions (**Figure 5D**) PER-specific immunofluorescence in dMnGl of Pdf^0 showed significant increase with respect to CS at CT1 (48%; t-test, $t = -4.6$, df = 16, $p = 0.003$) and CT13 (93%; t-test, $t = -5.5$, df = 20, p

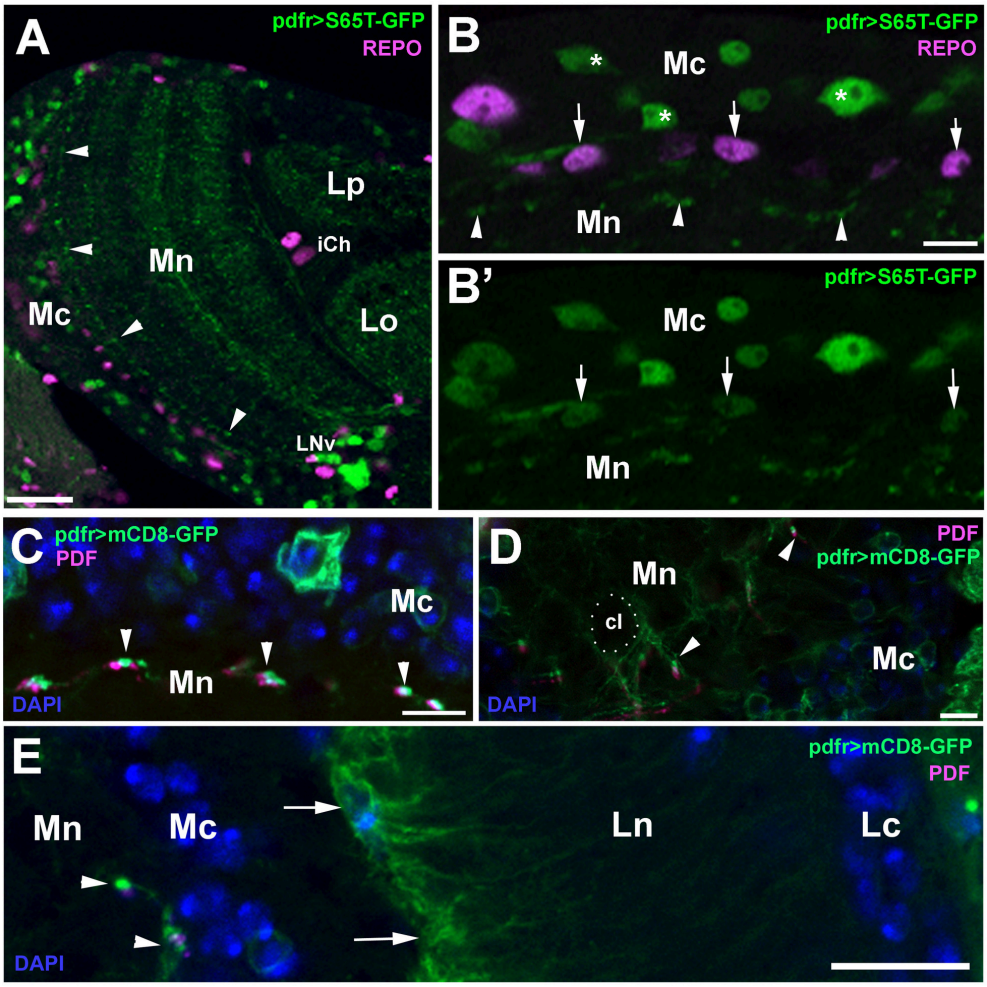

FIGURE 4 | An overview of *pdfR*-Gal4 expression pattern in *Drosophila* optic neuropils. **(A–B')** The medulla of *pdfR*-Gal4/UAS-S65T-GFP flies immunolabeled with anti-REPO Mab (magenta). **(A)** The cytoplasmic and nuclear expression of S65T-GFP reporter (green) shows numerous nuclei and processes of cells that express PDFR, the cell bodies (LNv) and terminals (arrowheads) of the LNvs among them. Lo and Lp-parts of the lobula complex, iCh-inner chiasm. Scale bar: 20 μm. **(B,B')** Higher magnification of the area at the interface of medulla cortex (Mc) and medulla neuropil (Mn) reveals the presence of S65T-GFP reporter **(B')** is REPO-positive nuclei of glia **(B)**, which due to their location must belong to dMnGl (arrows). The terminals of the LNvs are marked by patches of cytoplasmic GFP (arrowheads). GFP-positive, but REPO negative nuclei (asterisks) belong to PDFR-expressing neurons. **(C,D)** The distal medulla of *pdfR*-Gal4/UAS-mCD8-GFP flies immunolabeled using anti-PDF antibodies (magenta). Mc, medulla cortex; Mn, medulla neuropil. **(C)** Membranous expression of mCD8-GFP reporter reveals that the sites (arrowheads) of PDFR expression (green) and PDF release (magenta) are localized next to each other on the LNv terminals. **(D)** PDFR-expressing processes (green) encircle the medulla columns (cl). There are PDF releasing varicosities (magenta) visible on some of these processes (arrowheads). Scale bar for **(B–D)**: 5 μm. **(E)** Expression of mCD8-GFP in the marginal glia (arrows) localized at the margin of the lamina neuropil (Ln) in relation to the LNv terminals (arrowheads) in the distal medulla. Lc, lamina cortex; Mc, medulla cortex; Mn, medulla neuropil. Scale bar: 10 μm.

= 0.00002). The high level of fluorescence at CT13 was rather unexpected, as in CS it is usually the lowest at this time of the day in both LD and DD (**Figure 2**). This high level of fluorescence, however, was accompanied by the highest dispersion of results obtained from different individuals. It may imply that the population of Pdf^0 was not well synchronized (which typically shows up at the beginning of the day or the night).

The increase of PER at CT13 (by 73% with respect to ZT13) was also observed in dMnGl of the flies with silenced expression of PDF receptors in glia (*repo*-Gal4/UAS-pdfR^{RNAi}, **Figure 5E**). The level of PER was 64% higher than at CT1 (Kruskal–Wallis Test: H [3, N = 36] = 23.1, p = 0.0001, followed by Multiple Comparison Test: p = 0.00003 for CT13 vs. ZT13 and p = 0.004 for CT13 vs. CT1). The increase at CT13 (58%; with respect to ZT13) was also observed in the control flies (*repo*-Gal4/UAS-VAL10-GFP). In this case, however, the fluorescence at CT13 was not significantly higher than at CT1 (Kruskal–Wallis Test: H [3, N = 38] = 13.9, p = 0.003, followed by Multiple Comparison Test: p = 0.002 for CT13 vs. ZT13 and p = 0.08 for CT13 vs. CT1; **Figure 5E**). Also, the lack of statistically significant differences between the experimental and the control flies observed at all the studied

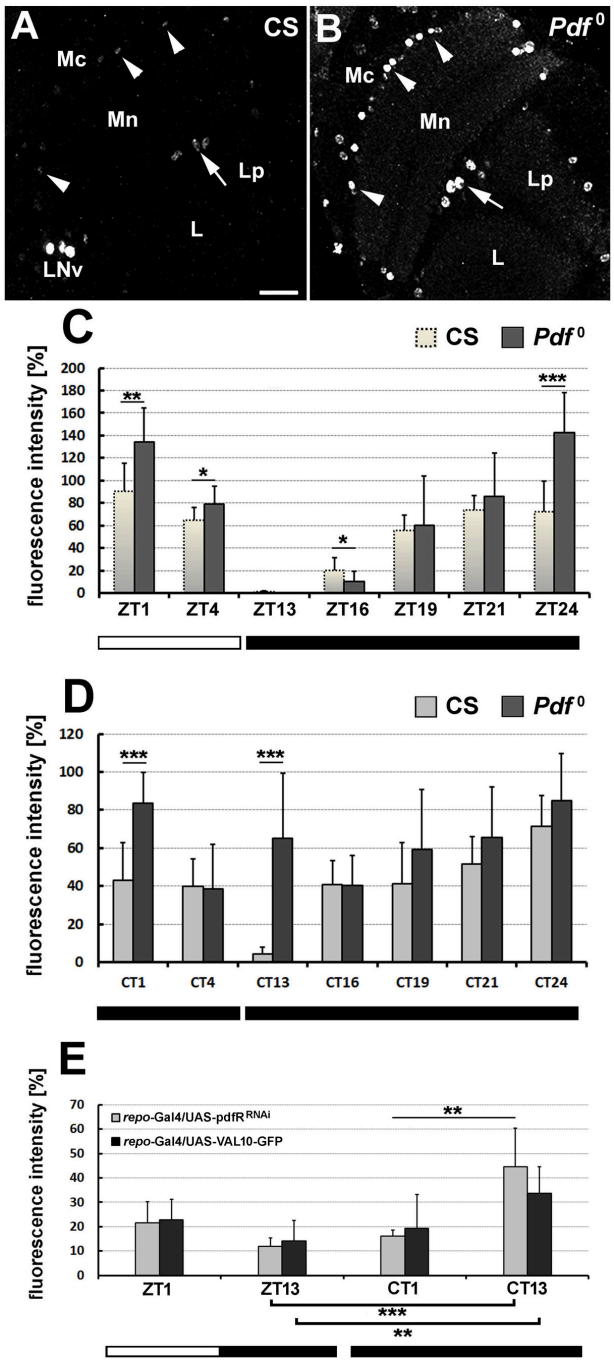

FIGURE 5 | The daily rhythm in the intensity of PER-specific immunofluorescence as the measure of PER molecular oscillations in optic lobes of CS and *Pdf*^{0} flies. **(A,B)** Horizontal sections of optic lobes of flies sacrificed at ZT24. Due to considerable differences in the level of PER immunolabeling in dMnGl (arrowheads) and iChGl (arrows) of CS and *Pdf*^{0}, the images collected at the same image acquisition parameters show the nuclei of CS glia on the verge of visibility, when the nuclei of *Pdf*^{0} glia are shown very clearly (with the signal being almost saturated). Mc, medulla cortex; Mn, medulla neuropil; L, lobula; Lp, lobula plate; LNv, ventral lateral neurons. Scale bar: 20 μm. **(C)** Daily profile of anti-PER labeling of *Pdf*^{0} dMnGl (Kruskal–Wallis Test: H [6, $N = 71$] = 55.95, $p = 0.00001$, followed by Multiple *(Continued)*

FIGURE 5 | Comparison Test: ZT13 vs. ZT1 [$p = 0.000002$], ZT4 [$p = 0.03$], ZT21 [$p = 0.004$], and ZT24 [$p = 0.00001$], also ZT16 vs. ZT1 [$p = 0.00005$], ZT21 [$p = 0.049$], and ZT24 [$p = 0.00001$]) to compare with the profile of CS dMnGl (Kruskal–Wallis Test: H [6, $N = 66$] = 48, $p = 0.00001$ followed by Multiple Comparison Test: ZT13 vs. ZT1 [$p = 0.000001$], ZT4 [$p = 0.004$], ZT21 [$p = 0.00003$], and ZT24 [$p = 0.001$], also ZT16 vs. ZT1 [$p = 0.0001$], ZT21 [$p = 0.002$], and ZT24 [$p = 0.04$], $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$). **(D)** The circadian profile of anti-PER labeling of *Pdf*^{0} dMnGl (Kruskal–Wallis Test: H [6, $N = 74$] = 22.62, $p = 0.0009$, followed by Multiple Comparison Test: CT1 vs. CT4 [$p = 0.02$] and CT16 [$p = 0.02$], as well as CT24 vs. CT4 [$p = 0.003$], and CT16 [$p = 0.005$]) to compare with the profile of CS dMnGl (Kruskal–Wallis Test: H [6, $N = 69$] = 36.8, $p = 0.00001$ followed by Multiple Comparison Test: CT13 vs. CT1 [$p = 0.008$], CT4 [$p = 0.04$], CT16 [$p = 0.03$], CT19 [$p = 0.02$], CT21 [$p = 0003$], and CT24 [$p = 0.000001$], $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$). **(E)** The average level (±*SD*) of PER-specific immunofluorescence in dMnGl of *repo*-Gal4/UAS-pdfR^{RNAi} at ZT1/ZT13 and CT1/CT13.

time points indicates that the silencing of PER expression in these flies was not strong enough to bring up visible changes of fluorescence.

Pdf^0 mutation affected the level of PER also in glia of other locations (**Figure 6A**). At the end of the night, the level of PER-specific immunofluorescence in iChGl (like in dMnGl) was twice as strong as in their counterparts of CS (*t*-Test, $t = 6.9$, $p = 0.0000001$, and $t = 3.8$, $p = 0.0008$, respectively). In the case of LGl, there was a smaller (23%), but statistically significant (Mann–Whitney Test, $U = 51$, $p = 0.03$) increase in the intensity of fluorescence in *Pdf*^{0} (**Figure 6B**). This increase considerably improved detectability of LGl in *Pdf*^{0} lamina probed for PER presence. (PER-specific staining in CS lamina was usually faint and less reproducible than in other parts of the brain). Finally, the immunofluorescence in McGl of *Pdf*^{0} was only 20% stronger than in McGl of CS (Mann–Whitney Test, $U = 58$, $p = 0.3$) (**Figure 6B**). Since PER-specific immunofluorescence increased significantly in *Pdf*^{0} glia of all considered locations in the optic lobe, it appears as though PDF-conveyed signals from the LNv pacemakers attenuated the expression of PER in glia in the wild type flies. Consequently, the CS LNv pacemakers appear to negatively influence the amplitude of molecular oscillations in glial clocks.

The comparison of PER level between different types of glia (dMnGl, LGl, McGl, and iChGl) in *Pdf*^{0} mutants (**Figure 6B**) not only confirmed the presence of type-related differences (Kruskal–Wallis Test: H [3, $N = 74$] = 53.2, $p = 0.00001$) observed initially in CS optic lobe (**Figure 1C**), but it showed them more clearly due to general increase of PER-specific immunofluorescence. Like in CS flies, the level of PER was the highest in the case of dMnGl (**Figures 6A,B**). It was 75, 83, and 50% higher in dMnGl than in LGl ($p = 0.0000001$), McGl ($p = 0.0000001$), and iChGl ($p = 0.03$), respectively (**Figure 6B**). The differences between dMnGl and LGl or McGl increased by 17 and 16% with respect to the corresponding differences in CS. The difference between the iChGl and McGl in *Pdf*^{0} was also statistically significant ($p = 0.001$). Hence, the differences in PER-labeling (PER level) observed between varied types of CS glia appear to be enhanced in *Pdf*^{0} mutants (**Figure 6B**).

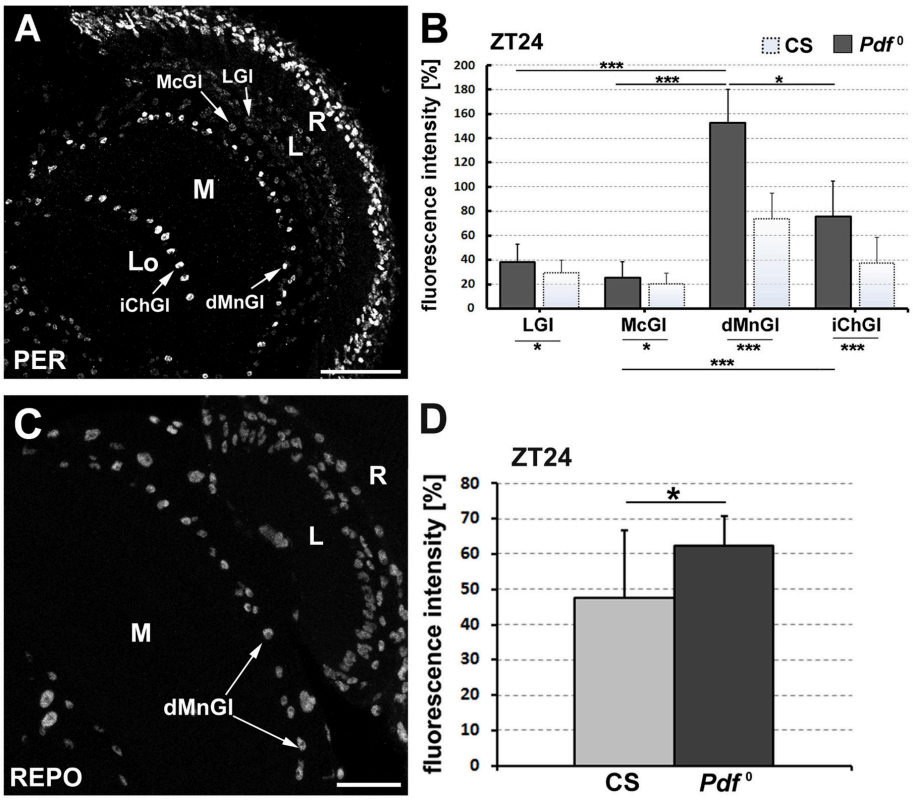

FIGURE 6 | (A) The confocal image of *Pdf*^{0} optic lobe displaying type-dependent differences in the level of PER-specific immunofluorescence in the optic lobe glial cells at the end of the night. These differences are similar to those observed in the optic lobe of CS (cf. **Figure 1B'**) but better visible. The strongest signal was observed in dMnGl and iChGl of the medulla neuropil, whereas the weakest was seen in McGl of the medulla cortex and in the lamina. R, retina; L, lamina; M, medulla; Lo, lobula complex. Scale bar: 20 μm. **(B)** The average level of PER-specific immunofluorescence (±*SD*) in the nuclei of dMnGl, LGl, McGl, and iChGl of *Pdf*^{0} flies (dark bars) at ZT24, to compare with the signal in the same types of glia in CS flies (light bars) (*$p \leq 0.05$, ***$p \leq 0.001$). **(C)** Horizontal section of the optic lobe of CS flies immunolabeled for REPO. dMnGl-distal medulla neuropil glia, R-retina, L-lamina, M-medulla. Scale bar: 20 μm. **(D)** The average level of REPO (±*SD*) in dMnGl of CS and *Pdf*^{0} at ZT24. Glia od *Pdf*^{0} mutants display higher level of REPO than glia of Canton-S (*$p \leq 0.05$).

The Level of Repo Increases in Glia of *Pdf*^{0} Mutants

Next, we wanted to find out whether differences in glial PER could affect glia-specific functions. Therefore, we checked if the elevation of glial PER in *Pdf*^{0} mutants was accompanied by alterations in the level of the major glial regulator—the glia-specific homeodomain transcription factor reversed polarity (REPO). The comparative analysis of REPO-specific immunofluorescence (**Figure 6C**) in the nuclei of dMnGl of CS and *Pdf*^{0} at ZT24 indeed revealed the 24% increase of the signal in dMnGl of *Pdf*^{0}. The difference between the levels of REPO in dMnGl of CS and in *Pdf*^{0} (**Figure 6D**) was not as big as the respective difference in the level of glial PER (50% difference in PER-specific immunofluorescence). It was, however, statistically significant (Mann–Whitney Test, $U = 26$, $p = 0.04$). Therefore it appears that the REPO-controlled glial functions are maintained at higher level in dMnGl of *Pdf*^{0} than in CS flies. Moreover, they are possibly modulated in the circadian manner by neuronal pacemakers (at least by the PDF-releasing LNv neurons).

R-Cells of *Pdf*^{0} Display De-synchrony of PER Molecular Rhythm

Further, we checked whether the absence of PDF-conveyed information influences the autonomous clock of *Pdf*^{0} photoreceptors (R-cells), which (like the glial cells) belong to the peripheral circadian oscillators. The comparative analysis revealed that daily patterns of changes (in LD) of PER-specific immunofluorescence in the nuclei of *Pdf*^{0} and CS photoreceptor cells were similar (**Figure 7A**). The average level of immunofluorescence (level of PER), however, was smaller in *Pdf*^{0}. Interestingly, this decrease resulted from higher variability in the level of fluorescence in the compound eye photoreceptors (**Figure 7B**). The standard deviation in *Pdf*^{0} was twice as big as in CS. Therefore, PER molecular oscillations in the retina photoreceptors may be regarded as less synchronized in the absence of PDF. This can be also observed in DD. The level of PER in DD was much lower in *Pdf*^{0} than in CS, showing very small amplitude (**Figure 7C**). Since we have not observed the R-cells to express PDFRs, they appear to receive this signal

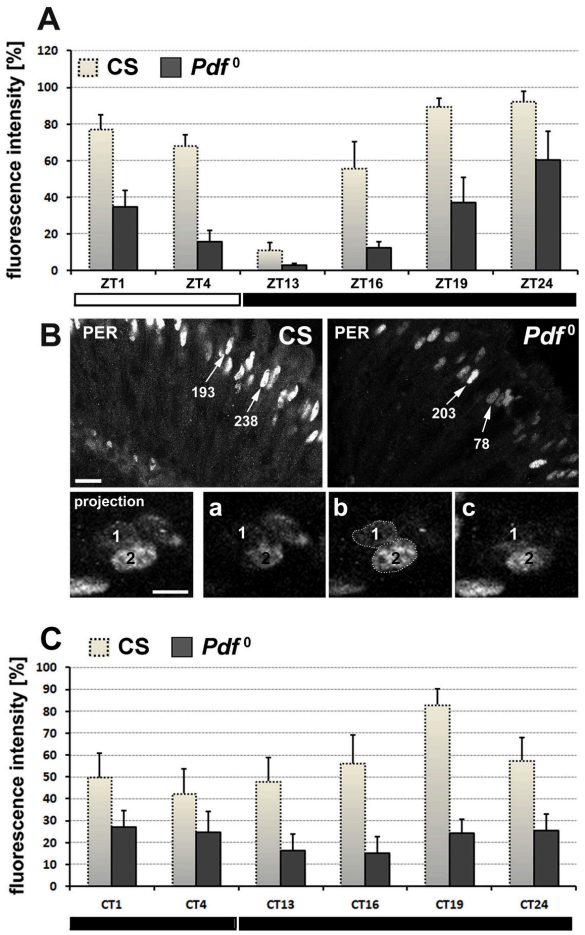

FIGURE 7 | (A) The average level (±*SD*) of PER-specific fluorescence in the nuclei of compound eye photoreceptors (R-cells) of CS and Pdf^0 at different ZTs of LD12:12. **(B)** Fragments of CS and Pdf^0 retinas immunolabeled using anti-PER serum. The differences in the level of PER-specific fluorescence among the R-cells of Pdf^0 are much bigger than among the R-cells of CS, which show the annotations of the Mean Gray Value for the nuclei indicated by arrows. Lower panel: the projection of three **(a–c)** consecutive optical sections of the two nuclei of Pdf^0 R-cells, marked as 1 and 2. They display the same level of PER-specific fluorescence (1-low and 2-high) throughout their whole depth—at all three optical sections **(a–c)**. Scale bar: 10 μm. **(C)** The average level (±*SD*) of PER-specific fluorescence in the R-cells nuclei of CS and Pdf^0 at different CTs (CS: Kruskal–Wallis Test: H [6, $N = 70$] = 41.23, $p = 0.00001$, followed by Multiple Comparison Test: CT21 vs. CT1 [$p = 0.0004$], CT4 [$p = 0.000003$], CT13 [$p = 0.02$], and CT24 [$p = 0.03$], as well as CT19 vs. CT4 [$p = 0.003$] and CT13 [0.04]); Pdf^0: Kruskal–Wallis Test: H [6, $N = 74$] = 20.70, $p = 0.002$, followed by Multiple Comparison Test: CT16 vs. CT1 [$p = 0.03$] and CT19[$p = 0.03$]).

indirectly. It may occur through the marginal glia of the proximal lamina, which strongly express PDFRs (**Figure 4E**).

Lack of PDF Changes the Daily Pattern of Abundance of BRP Protein in the Lamina Synaptic Cartridges

Since the lack of PDF in Pdf^0 flies influences PER expression in both the glial and the retinal clocks (although in different ways), we checked the impact of PDF absence on the daily pattern of expression of the presynaptic protein, Bruchpilot (BRP) in the synaptic units (cartridges) of the first visual neuropil (lamina) of Pdf^0 flies. In the lamina neuropil, the BRP daily pattern of expression (composed of two peaks-the morning peak and the evening peak, Górska-Andrzejak et al., 2013), was slightly altered in Pdf^0 flies (**Figure 8**). While the evening, clock-regulated (glia dependent) peak was firm (there was a statistically significant difference between ZT13 and ZT4; Kruskal–Wallis Test: H [3, $N = 44$] = 15.5, $p = 0.001$, followed by Multiple Comparison Test, $p = 0.0007$), the morning, light-regulated (photoreceptor-dependent) one was small and statistically insignificant (ZT1 vs. ZT4: $p = 0.2$; ZT1 vs. ZT13; $p = 0.09$; ZT1 vs. ZT16: $p = 0.9$). This seems to be consistent with the molecular rhythm strengthening in glia and with de-synchrony that can be observed among the retina photoreceptors.

DISCUSSION

Glial Oscillators Are Weaker Than Neuronal Oscillators

The abundance of PER, the prime repressor in the mechanism of circadian clock (Landskron et al., 2009; Hardin, 2011), is known to influence the pace of the clock and the phase of the circadian rhythm (Baylies et al., 1987). Therefore, flies with the lowest levels of PER have slow-running biological clocks (Baylies et al., 1987), while temporary increase in PER concentration can alter the phase of the rhythm (Edery et al., 1994). Our studies revealed lower intensity of PER staining in the optic lobe glial cells than in the l-LNv central pacemakers, as well as in the R-cells of the compound eye, which indicates that the optic lobe glia maintain lower amplitude of PER molecular oscillations. Consequently, they are weaker oscillators with respect to the neurons mentioned above and so they are not able to drive outputs in DD conditions for a longer time (Weiss et al., 2014).

Glial Oscillators Are Heterogeneous—They Express PER at Different Levels

Glial cells of *Drosophila* are known, however, to be highly diversified both morphologically and functionally (Edwards and Meinertzhagen, 2010). Our studies showed that the large population of glial cells that function as glial clocks (PER-expressing glia) appear to be heterogeneous, as the cells in different locations express different amounts of PER. Because certain oscillatory subgroups (residing in different regions of the optic lobe) display different amplitudes of their molecular rhythms, the strength of their regulatory influence must also differ. Consequently, the lower level of PER in the lamina glia and the higher level of PER in the medulla glia (which is by far the most easily noticeable difference) suggest that the circadian network of these two neuropils require different amounts of the glial circadian activity. Indeed, the epithelial glial cells of the lamina, which envelope the synaptic units (cartridges) of the lamina neuropil (Boschek, 1971; Pyza and Górska-Andrzejak, 2008), might not need to express *per* at high level, as the presynaptic terminals of photoreceptors—the main components

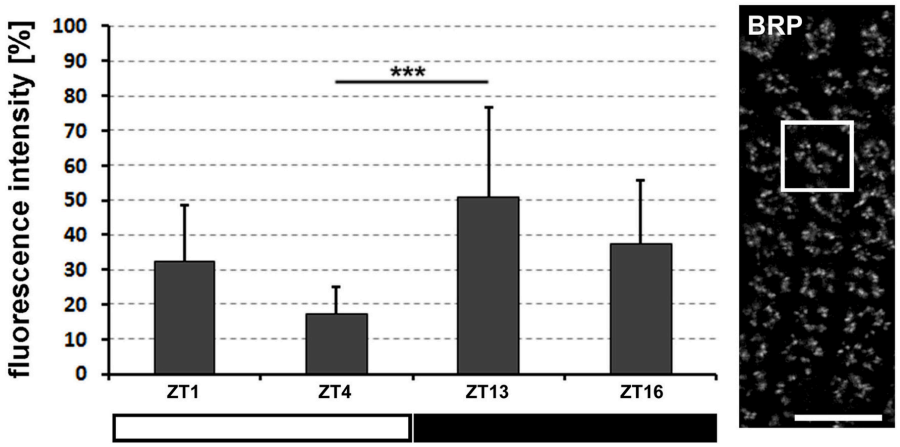

FIGURE 8 | The average level (±*SD*) of BRP-specific immunofluorescence measured in confocal images of the lamina of Pdf^0 mutants at specific time points of LD 12:12. Data represent the average score of fluorescence intensity obtained for the lamina synaptic units called cartridges, which are seen in the lamina fragment on the right (the single cartridge in the frame; ***$p \leq 0.001$). Scale bar: 10 μm.

of the cartridge—are equipped with their own, autonomous circadian mechanism (Siwicki et al., 1988; Zerr et al., 1990; Cheng and Hardin, 1998).

dMnGl Display the Highest Level of PER

The highest level of PER in dMnGl, on the other hand, implies that these cells belong to the strongest of glial oscillators. It may also support the hypothesis that PER in glia is required for the regulation of circadian light sensitivity (Zwarts et al., 2015), in which dMnGl could cooperate with their close neighbors, the l-LNv pacemakers (Yoshii et al., 2016). This agrees with the fact that the level of PER in glia, and especially in dMnGl, is elevated in Pdf^0 mutants in LD at ZT1 and in DD at CT1. It is tempting to speculate that in the absence of PDF signaling from the l-LNvs the glial cells increase their PER expression (the strength of their molecular rhythms) to compensate for that lack. In DD conditions, they appear to do so at two crucial time points, at the beginning of the circadian day (regulated predominantly by light) and at the beginning of the subjective night (clock-regulated time point).

Lack of PDF Changes the Level of PER in Glia

As already mentioned, the main function of the LNv pacemakers is coupling different pacemakers/clock centers within the fly brain (Helfrich-Förster, 1998; Renn et al., 1999). PDF synchronizes the different clock neurons that make up the *Drosophila* circadian neural circuit via PDF receptors (Renn et al., 1999; Lin et al., 2004; Lear et al., 2005; Nitabach et al., 2006; Shafer et al., 2008; Im et al., 2011). It therefore appears as a good candidate for linking up the glial oscillators to the neuronal oscillatory network. This notion agrees with the fact that glial cells take part in the output regulation, since modifications of gliotransmission, calcium stores, or glial ionic gradients result in the arrhythmic locomotor activity (Ng et al., 2011).

Assuming that the highest level of PER specifically in dMnGl results from the direct influence of PDF signaling from the LNvs (Helfrich-Förster, 1998), we checked the glial level of PER in null Pdf^0 mutants, which display reduced morning behavior and advanced evening behavior in LD conditions, as well as progressive dampening of locomotor rhythmicity and shortened period in DD (Yoshii et al., 2016). We found out that Pdf^0 mutants displayed higher level of glial PER than CS. This confirmed the notion that dMnGl belong to multiple targets of PDF but are normally (in CS) negatively influenced by PDF. We conclude this because the level of glial PER is elevated in the absence of PDF. PDF regulation of *per* and *tim* rhythms in *Drosophila* optic neuropils has been reported by Damulewicz et al. (2015). PDF has also been reported to act on PDF neurons themselves to regulate their rhythmic strength (in addition to evening activity phase and period length regulation in non-PDF clock neurons; Lear et al., 2009).

These results also show the range of PDF inhibition. In Pdf^0 mutants, all types of glia exhibit higher level of PER, maintaining differences among the glia of different locations (which are observed in CS). We therefore conclude that PDF influences the entire glial circadian system. Since the amplitude of the molecular rhythm is proportional to the level of PER, the increase in the level of PER in glia in the PDF-free tissue must result in strengthening of the glial circadian functions and, consequently, in increased impact of the glial oscillators on the whole circadian network.

The significantly higher level of PER in glia of Pdf^0 mutant also suggests that the glial cells may play a submissive role in the circadian system. Modulating their amplitude, the PDF-positive clock cells actually influence the gear of circadian clock of the glial cells, which seem to be capable of expressing PER at much higher level when allowed by the LNv pacemakers (the lack of PDF appears to be interpreted by the glial cells as green light for amplitude enhancement).

PDFR in Glia

Till now, the presence of PDFRs have been reported in the PDF and non-PDF clock neurons (Lear et al., 2009; Im and Taghert, 2010) and the non-neuronal (glial) cells situated at the base of the compound eye (Im and Taghert, 2010). Our analysis of the pattern of expression of *pdfR*-Gal4 driver using sequences upstream of the *pdfR*-gene (Lear et al., 2009) revealed that dMnGl express receptors for PDF. On this view, the LNvs can be said to communicate not only to other clock neurons but also to the glial clocks, which can be one of the components of the output of the LNv circuit. The fact that the glial clocks belong to the PDFR-responsive targets indicates their importance for the activity of the whole circadian network. It also implies that the glial clocks have to be synchronized in the same way (via PDF) as different clusters of neuronal pacemakers.

The above findings may explain the observation by Lear et al. (2009), who reported that the rescue of *Pdfr* mutant phenotypes using *Pdf*-Gal4 and *npf*-Gal4 drivers (which do not drive expression to glial cells) failed to rescue significantly both the LD and DD phenotypes. This failure may have been caused by the absence of the relevant glia-derived circadian components.

Judging by the intensity of GFP fluorescence, however, the level of PDFR in glia appears to be lower than in other cells, which may be related to the negative influence of PDF on the level of PER in glia. This mechanism would enable modulation, but at the same time it would protect against the complete switching off the glial circadian functions. The low level of PDFR expression may also explain why dMnGl were not visible when PDFR was detected by the antibodies raised against N- (Hyun et al., 2005) or C-terminus (Mertens et al., 2005). Detection might thus be restricted to the cells with relatively high level of PDFR expression.

The results assessing the level of PER-immunofluorescence in flies with silenced expression of PDFR show the tendency to increase PER level at CT13, similar to the one observed in Pdf^0. They are, however, not entirely conclusive, as the increase observed in the experimental flies is not statistically significant with respect to the control flies. It appears that the RNAi-mediated silencing did not suppress the PDFR expression in glia efficiently enough to mimic the complete lack of PDF in Pdf^0 mutants.

Glial Cells of Pdf^0 Display Higher Level of REPO, the Glia Specific Protein

We observed that Pdf^0 mutants display an increased level of REPO, a glia-specific, paired-like homeodomain transcription factor, which inhibits neuronal and activates glial differentiation during development (Xiong et al., 1994; Halter et al., 1995). The lack of PDF-conveyed signals from the LNvs influences the glial functions controlled by REPO. The function of REPO in the adult brain is still unclear. Recently it has been found, however, that REPO controls glutamate receptor clustering and synaptic physiology at *Drosophila* larval neuromuscular junction (Kerr et al., 2014). Importantly, the expression of glial *repo* is also required for the Long-Term Memory (LTM) formation, as its expression increases upon LTM induction (Matsuno et al., 2015). Artificially elevated REPO expression can also rescue mutants with LTM defects and the knockdowns of KLINGON, the cell adhesion molecule required for LTM formation, which localizes to juncture between neurons and glia. Consequently, REPO influences the KLG-mediated communication between neurons and glia (Matsuno et al., 2015). Higher levels of REPO in dMnGl may, therefore, reflect the higher level of communication. The coincidence of the elevated levels of PER and REPO in Pdf^0 mutants suggests that the circadian clock modulates both REPO-controlled glial functions and neuron-glia interactions.

R-Cells Respond to Lack of PDF in a Different Way Than Glial Cells

Photoreceptors of the compound eye (and ocelli) of the fruit fly were among the first, in which robust circadian oscillations of PER and TIM were observed (Siwicki et al., 1988; Zerr et al., 1990). Later studies confirmed that circadian oscillations in the R-cells occur autonomously, i.e., independently of the central circadian pacemaker in the brain (Cheng and Hardin, 1998), being involved in regulation of the visual system sensitivity to daily changes of light intensity (Giebułtowicz, 2000). Our results suggest that the PDF-releasing LNvs, which directly perceive light, influence the circadian oscillations of the retina photoreceptors via PDF signaling. PDF has been known to provide feedback facilitating synchronization of different groups of the clock neurons within the brain (Lin et al., 2004). The primary role of PDF may lie in enhancing and synchronizing individual clock oscillations (Hyun et al., 2005; Mertens et al., 2005). Since different R-cells of Pdf^0 retina displayed major changes in the level of PER nuclear accumulation at the beginning of the day, revealing signs of desynchronization, our results show that the eye multiple oscillators may also be coupled, at least to some extent, via PDF. The eye photoreceptors may thus depend on PDF synchronization. This also explains the necessity of the clock neurons to have direct light input through CRY. On the other hand, the compound eye CRY appears to have a minor contribution to light entrainment. Flies expressing *cry* in the eyes do not entrain significantly better than *cry* mutants (Emery et al., 2000; Yoshii et al., 2016).

Lack of PDF Influences Daily Pattern of BRP

Daily remodeling of the lamina synaptic contacts has been observed using immunohistochemistry based on Bruchpilot—specific Nc82 antibody (Górska-Andrzejak et al., 2013). The level of Bruchpilot (BRP), the large scaffold protein that is a major constituent of the presynaptic ribbons (so called T-bars) of synapses, fluctuated during the day and night (Górska-Andrzejak et al., 2013). The morning and evening peaks, observed in LD 12:12, are regulated in different ways. The morning peak depends predominantly on the light and phototransduction pathway in the R-cells of the retina and also on the clock gene *per*. The evening peak, on the other hand, is regulated endogenously, by the input from the pacemaker located in the brain. In addition, the two peaks depend on the clock gene-expressing photoreceptors and on the glial cells of the visual

system, respectively. Interestingly, in Pdf^0 mutant, the first peak was smaller and insignificant, while the second was still present. The results concerning the level of PER in the glia and the R-cells of Pdf^0 mutant, appear to explain such pattern of BRP expression in these flies. The first peak, which depends predominantly on the presence of the light stimuli and the R-cells activity may be smaller due to visible desynchronization of the compound eye photoreceptors, whereas the second peak is maintained since it is driven by the glial cells, which (judging by increased level of PER in their nuclei) appear to be more active in this mutant than in the wild type flies. Similar results were obtained by Damulewicz et al. (2013) on the expression of the α-subunit of the sodium-potassium pump in Pdf^0 mutants.

PDF-conveyed information is able to reach the R7 and R8 photoreceptors, as they project to the medulla neuropil, where they terminate in different layers of synaptic connections; R7 in layer M6, R8 in layer M3 (Kremer et al., 2017). It is also possible, however, that paracrine release of PDF can reach the proximal lamina, where the terminals of R1-R6 end. It cannot be a coincidence that the receptors for PDF are expressed both in the marginal glia of the proximal lamina (**Figure 4E**) and in the glial cells situated at the base of the compound eye (Im and Taghert, 2010). The former cells extend their processes toward the distal part of the lamina so high that they branch among processes of the epithelial glial cells and contact the terminals of R1–R6 (Edwards et al., 2012). Although they do not appear to form such intimate connections with photoreceptor terminals as the epithelial glial cells (which invaginate the terminals forming the so called capitate projections, Stark and Carlson, 1986), they do invaginate photoreceptor terminals at some sites of contacts and contain coated vesicles (as shown in EM by Edwards et al., 2012). Small, club-headed capitate projections of glial cells invaginating the terminals of R7 and R8 in the medulla (Takemura et al., 2008; Edwards and Meinertzhagen, 2009) are most probably the projections of dMnGl (Edwards et al., 2012), which we have shown to poses PDF receptors.

To sum up, our studies show for the first time that glial cells in general and dMnGl in particular, belong to PDF downstream circuits as the integral part of the LNv pacemakers output. It is the first step toward understanding how the activity of peripheral oscillators, such as the glial cells or the eye photoreceptor cells, is synchronized with the circadian network and adjusted by the central clock. Further studies should elucidate in greater detail whether other types of glia possess PDF receptors, and dissect the molecular mechanism by which PDF acts on glial cells in order to regulate their clock and circadian activity.

AUTHOR CONTRIBUTIONS

JG-A: Designed the study, performed experiments, collected and analyzed data, and prepared the manuscript; EC: Performed experiments and collected data; LW: Performed some of the experiments and revised the manuscript; KW: Performed one of the experiments.

FUNDING

The studies were supported by the grant K/ZDS/007356 and the Laboratory of Confocal Microscopy in the Institute of Zoology and Biomedical Research.

ACKNOWLEDGMENTS

The authors express their gratitude to Charlotte Förster (University of Würzburg) and François Rouyer (Paris Saclay Institute of Neuroscience) for the fly strains that the Department of Cell Biology and Imaging received from them, as well as to Bloomington Drosophila Stock Center (USA) for *repo*-Gal4 (7415), *pdfR*-Gal4 (33070), UAS-mcD8-GFP (5137), and UAS-S65T-GFP (1521) strains. The authors also wish to express their gratitude to M. Andrzejak for invaluable discussions and assistance in preparation of this manuscript.

REFERENCES

Allada, R., and Chung, B. Y. (2010). Circadian organization of behavior and physiology in Drosophila. *Annu. Rev. Physiol.* 72, 605–624. doi: 10.1146/annurev-physiol-021909-135815

Baylies, M. K., Bargiello, T. A., Jackson, F. R., and Young, M. W. (1987). Changes in abundance or structure of the per gene product can alter periodicity of the Drosophila clock. *Nature* 326, 390–392. doi: 10.1038/326390a0

Blanchardon, E., Grima, B., Klarsfeld, A., Chélot, E., Hardin, P. E., Préat, T., et al. (2001). Defining the role of Drosophila lateral neurons in the control of circadian rhythms in motor activity and eclosion by targeted genetic ablation and PERIOD protein overexpression. *Eur. J. Neurosci.* 13, 871–888. doi: 10.1046/j.0953-816x.2000.01450.x

Boschek, C. B. (1971). On the fine structure of the peripheral retina and lamina ganglionaris of the fly, *Musca domestica*. *Z. Zellforsch. Mikrosk. Anat.* 118, 369–409. doi: 10.1007/BF00331193

Cantera, R., and Trujillo-Cenoz, O. (1996). Glial cells in insect ganglia. *Microsc. Res. Tech.* 35, 285–93.

Carlson, S. D., and Saint Marie, R. L. (1990). Structure and function of insect glia. *Annu. Rev. Entomol.* 35, 597–621. doi: 10.1146/annurev.en.35.010190.003121

Cheng, Y., and Hardin, P. E. (1998). Drosophila photoreceptors contain an autonomous circadian oscillator that can function without period mRNA cycling. *J. Neurosci.* 18, 741–750.

Damulewicz, M., Loboda, A., Bukowska-Strakova, K., Jozkowicz, A., Dulak, J., and Pyza, E. (2015). Clock and clock-controlled genes are differently expressed in the retina, lamina and in selected cells of the visual system of *Drosophila melanogaster*. *Front. Cell. Neurosci.* 9:353. doi: 10.3389/fncel.2015.00353

Damulewicz, M., Rosato, E., and Pyza, E. (2013). Circadian Regulation of the Na+/K+-atpase alpha subunit in the visual system is mediated by the pacemaker and by retina photoreceptors in *Drosophila melanogaster*. *PLoS ONE* 8:e73690. doi: 10.1371/journal.pone.0073690

Dircksen, H., Zahnow, C., Gaus, G., Keller, R., Rao, K. R., and Riehm, J. (1987). The ultrastructure of nerve endings containing pigment-dispersing hormone (PDH) in crustacean sinus glands: identification by an antiserum against a synthetic PDH. *Cell Tissue Res.* 250, 377–387. doi: 10.1007/BF00219082

Edery, I., Rutila, J. E., and Rosbash, M. (1994). Phase shifting of the circadian clock by induction of the Drosophila period protein. *Science* 263, 237–240. doi: 10.1126/science.8284676

Edwards, T. N., and Meinertzhagen, I. A. (2009). Photoreceptor neurons find new synaptic targets when misdirected by overexpressing runt in Drosophila. *J. Neurosci.* 29, 828–841. doi: 10.1523/JNEUROSCI.1022-08.2009

Edwards, T. N., and Meinertzhagen, I. A. (2010). The functional organisation of glia in the adult brain of Drosophila and other insects. *Prog. Neurobiol.* 90, 471–497. doi: 10.1016/j.pneurobio.2010.01.001

Edwards, T. N., Nuschke, A. C., Nern, A., and Meinertzhagen, I. A. (2012). Organization and metamorphosis of glia in the Drosophila visual system. *J. Comp. Neurol.* 520, 2067–2085. doi: 10.1002/cne.23071

Emery, P., Stanewsky, R., Helfrich-Förster, C., Emery-Le, M., Hall, J. C., and Rosbash, M. (2000). Drosophila CRY is a deep brain circadian photoreceptor. *Neuron* 26, 493–504. doi: 10.1016/S0896-6273(00)81181-2

Ewer, J., Frisch, B., Hamblen-Coyle, M. J., Rosbash, M., and Hall, J. C. (1992). Expression of the period clock gene within different cell types in the brain of Drosophila adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. *J. Neurosci.* 12, 3321–3349.

Fernández, M. P., Berni, J., and Ceriani, M. F. (2008). Circadian remodeling of neuronal circuits involved in rhythmic behavior. *PLoS Biol.* 6:e69. doi: 10.1371/journal.pbio.0060069

Giangrande, A. (1996). Development and organization of glial cells in *Drosophila melanogaster*. *Int. J. Dev. Biol.* 40, 917–927.

Giebułtowicz, J. M. (2000). Molecular mechanism and cellular distribution of insect circadian clocks. *Annu. Rev. Entomol.* 45, 769–793. doi: 10.1146/annurev.ento.45.1.769

Gorostiza, E. A., Depetris-Chauvin, A., Frenkel, L., Pírez, N., and Ceriani, M. F. (2014). Circadian pacemaker neurons change synaptic contacts across the day. *Curr. Biol.* 24, 2161–2167. doi: 10.1016/j.cub.2014.07.063

Górska-Andrzejak, J. (2013). Glia-related circadian plasticity in the visual system of Diptera. *Front. Physiol.* 4:36. doi: 10.3389/fphys.2013.00036

Górska-Andrzejak, J., Makuch, R., Stefan, J., Görlich, A., Semik, D., and Pyza, E. (2013). Circadian expression of the presynaptic active zone protein bruchpilot in the lamina of *Drosophila melanogaster*. *Dev. Neurobiol.* 73, 14–26. doi: 10.1002/dneu.22032

Górska-Andrzejak, J., Salvaterra, P. M., Meinertzhagen, I. A., Krzeptowski, W., Görlich, A., and Pyza, E. (2009). Cyclical expression of Na+/K+-ATPase in the visual system of *Drosophila melanogaster*. *J. Insect Physiol.* 55, 459–468. doi: 10.1016/j.jinsphys.2009.02.003

Halter, D. A., Urban, J., Rickert, C., Ner, S. S., Ito, K., Travers, A. A., et al. (1995). The homeobox gene repo is required for the differentiation and maintenance of glia function in the embryonic nervous system of *Drosophila melanogaster*. *Development* 121, 317–332.

Hardin, P. E. (2011). Molecular genetic analysis of circadian timekeeping in *Drosophila*. *Adv. Genet.* 74, 141–173. doi: 10.1016/B978-0-12-387690-4.00005-2

Helfrich-Förster, C. (1997). Development of pigment-dispersing hormone-immunoreactive neurons in the nervous system of *Drosophila melanogaster*. *J. Comp. Neurol.* 380, 335–354.

Helfrich-Förster, C. (1998). Robust circadian rhythmicity of *Drosophila melanogaster* requires the presence of lateral neurons: a brain-behavioral study of disconnected mutants. *J. Comp. Physiol.* 182, 435–453. doi: 10.1007/s003590050192

Helfrich-Förster, C. (2005). PDF has found its receptor. *Neuron* 48, 161–163. doi: 10.1016/j.neuron.2005.10.004

Helfrich-Förster, C., Shafer, O. T., Wülbeck, C., Grieshaber, E., Rieger, D., and Taghert, P. (2007). Development and morphology of the clock-gene-expressing lateral neurons of *Drosophila melanogaster*. *J. Comp. Neurol.* 500, 47–70. doi: 10.1002/cne.21146

Hermann-Luibl, C., and Helfrich-Förster, C. (2015). Clock network in Drosophila. *Curr. Opin. Insect Sci.* 7, 65–70. doi: 10.1016/j.cois.2014.11.003

Hyun, S., Lee, Y., Hong, S.-T., Bang, S., Paik, D., Kang, J., et al. (2005). Drosophila GPCR han is a receptor for the circadian clock neuropeptide PDF. *Neuron* 48, 267–278. doi: 10.1016/j.neuron.2005.08.025

Im, S. H., Li, W., and Taghert, P. H. (2011). PDFR and CRY signaling converge in a subset of clock neurons to modulate the amplitude and phase of circadian behavior in Drosophila. *PLoS ONE* 6:e18974. doi: 10.1371/journal.pone.0018974

Im, S. H., and Taghert, P. H. (2010). PDF receptor expression reveals direct interactions between circadian oscillators in Drosophila. *J. Comp. Neurol.* 518, 1925–1945. doi: 10.1002/cne.22311

Ito, K., Urban, J., and Technau, G. M. (1995). Distribution, classification, and development of Drosophila glial cells in the late embryonic and early larval ventral nerve cord. *Roux's Arch. Dev. Biol.* 204, 284–307.

Kerr, K. S., Fuentes-Medel, Y., Brewer, C., Barria, R., Ashley, J., Abruzzi, K. C., et al. (2014). Glial Wingless/Wnt regulates glutamate receptor clustering and synaptic physiology at the Drosophila neuromuscular junction. *J. Neurosci.* 34, 2910–2920. doi: 10.1523/JNEUROSCI.3714-13.2014

Klämbt, C., Hummel, T., Menne, T., Sadlowski, E., Scholz, H., and Stollewerk, A. (1996). Development and function of embryonic central nervous system glial cells in Drosophila. *Dev. Genet.* 18, 40–49.

Kremer, M. C., Jung, C., Batelli, S., Rubin, G. M., and Gaul, U. (2017). The glia of the adult Drosophila nervous system. *Glia* 65, 606–638. doi: 10.1002/glia.23115

Landskron, J., Chen, K. F., Wolf, E., and Stanewsky, R. (2009). A role for the PERIOD:PERIOD homodimer in the Drosophila circadian clock. *PLoS Biol.* 7:e3. doi: 10.1371/journal.pbio.1000003

Lear, B. C., Merrill, C. E., Lin, J.-M., Schroeder, A., Zhang, L., and Allada, R. (2005). A G protein-coupled receptor, groom-of-PDF, is required for PDF neuron action in circadian behavior. *Neuron* 48, 221–227. doi: 10.1016/j.neuron.2005.09.008

Lear, B. C., Zhang, L., and Allada, R. (2009). The neuropeptide PDF acts directly on evening pacemaker neurons to regulate multiple features of circadian behavior. *PLoS Biol.* 7:e1000154. doi: 10.1371/journal.pbio.1000154

Lin, Y., Stormo, G. D., and Taghert, P. H. (2004). The neuropeptide pigment-dispersing factor coordinates pacemaker interactions in the Drosophila circadian system. *J. Neurosci.* 24, 7951–7957. doi: 10.1523/JNEUROSCI.2370-04.2004

Matsuno, M., Horiuchi, J., Yuasa, Y., Ofusa, K., Miyashita, T., Masuda, T., et al. (2015). Long-term memory formation in Drosophila requires training-dependent glial transcription. *J. Neurosci.* 35, 5557–5565. doi: 10.1523/JNEUROSCI.3865-14.2015

Mertens, I., Vandingenen, A., Johnson, E. C., Shafer, O. T., Li, W., Trigg, J. S., et al. (2005). PDF receptor signaling in Drosophila contributes to both circadian and geotactic behaviors. *Neuron* 48, 213–219. doi: 10.1016/j.neuron.2005.09.009

Ng, F. S., and Jackson, F. R. (2015). The ROP vesicle release factor is required in adult Drosophila glia for normal circadian behavior. *Front. Cell. Neurosci.* 9:256. doi: 10.3389/fncel.2015.00256

Ng, F. S., Sengupta, S., Huang, Y., Yu, A. M., You, S., Roberts, M. A., et al. (2016). TRAP-seq Profiling and RNAi-based genetic screens identify conserved glial genes required for adult Drosophila behavior. *Front. Mol. Neurosci.* 9:146. doi: 10.3389/fnmol.2016.00146

Ng, F. S., Tangredi, M. M., and Jackson, F. R. (2011). Glial cells physiologically modulate clock neurons and circadian behavior in a calcium-dependent manner. *Curr. Biol.* 21, 625–634. doi: 10.1016/j.cub.2011.03.027

Nitabach, M. N., and Taghert, P. H. (2008). Organization of the Drosophila circadian control circuit. *Curr. Biol.* 18, R84–R93. doi: 10.1016/j.cub.2007.11.061

Nitabach, M. N., Wu, Y., Sheeba, V., Lemon, W. C., Strumbos, J., Zelensky, P. K., et al. (2006). Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. *J. Neurosci.* 26, 479–489. doi: 10.1523/JNEUROSCI.3915-05.2006

Park, J. H., Helfrich-Förster, C., Lee, G., Liu, L., Rosbash, M., and Hall, J. C. (2000). Differential regulation of circadian pacemaker output by separate clock genes in Drosophila. *Proc. Natl. Acad. Sci. U.S.A.* 97, 3608–3613. doi: 10.1073/pnas.97.7.3608

Peschel, N., Veleri, S., and Stanewsky, R. (2006). Veela defines a molecular link between Cryptochrome and Timeless in the light-input pathway to Drosophila's circadian clock. *Proc. Natl. Acad. Sci. U.S.A.* 103, 17313–17318. doi: 10.1073/pnas.0606675103

Pyza, E., and Górska-Andrzejak, J. (2004). Involvement of glial cells in rhythmic size changes in neurons of the housefly's visual system. *J. Neurobiol.* 59, 205–215. doi: 10.1002/neu.10307

Pyza, E., and Górska-Andrzejak, J. (2008). External and internal inputs affecting plasticity of dendrites and axons of the fly's neurons. *Acta Neurobiol. Exp.* (*Wars*). 68, 322–333.

Renn, S. C., Park, J. H., Rosbash, M., Hall, J. C., and Taghert, P. H. (1999). A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. *Cell* 99, 791–802. doi: 10.1016/S0092-8674(00)81676-1

Rosato, E., and Kyriacou, C. P. (2008). Sleep, arousal, and rhythms in flies. *Proc. Natl. Acad. Sci. U.S.A.* 105, 19567–19568. doi: 10.1073/pnas.0811124106

Shafer, O. T., Kim, D. J., Dunbar-Yaffe, R., Nikolaev, V. O., Lohse, M. J., and Taghert, P. H. (2008). Widespread receptivity to neuropeptide PDF throughout the neuronal circadian clock network of Drosophila revealed by real-time cyclic AMP imaging. *Neuron* 58, 223–237. doi: 10.1016/j.neuron.2008.02.018

Sheeba, V., Fogle, K. J., and Holmes, T. C. (2010). Persistence of morning anticipation behavior and high amplitude morning startle response following functional loss of small ventral lateral neurons in Drosophila. *PLoS ONE* 5:e11628. doi: 10.1371/journal.pone.0011628

Sheeba, V., Sharma, V. K., Gu, H., Chou, Y.-T., O'Dowd, D. K., and Holmes, T. C. (2008). Pigment dispersing factor-dependent and -independent circadian locomotor behavioral rhythms. *J. Neurosci.* 28, 217–227. doi: 10.1016/j.cois.2014.05.002

Siwicki, K. K., Eastman, C., Petersen, G., Rosbash, M., and Hall, J. C. (1988). Antibodies to the period gene product of Drosophila reveal diverse tissue distribution and rhythmic changes in the visual system. *Neuron* 1, 141–150. doi: 10.1016/0896-6273(88)90198-5

Stanewsky, R., Frisch, B., Brandes, C., Hamblen-Coyle, M. J., Rosbash, M., and Hall, J. C. (1997). Temporal and spatial expression patterns of transgenes containing increasing amounts of the Drosophila clock gene period and a lacZ reporter: mapping elements of the PER protein involved in circadian cycling. *J. Neurosci.* 17, 676–696.

Stark, W. S., and Carlson, S. D. (1986). Ultrastructure of capitate projections in the optic neuropil of Diptera. *Cell Tissue Res.* 246, 481–486. doi: 10.1007/BF00215187

Suh, J., and Jackson, F. R. (2007). Drosophila ebony activity is required in glia for the circadian regulation of locomotor activity. *Neuron* 55, 435–447. doi: 10.1016/j.neuron.2007.06.038

Takemura, S.-Y., Lu, Z., and Meinertzhagen, I. A. (2008). Synaptic circuits of the *Drosophila* optic lobe: the input terminals to the medulla. *J. Comp. Neurol.* 509, 493–513. doi: 10.1002/cne.21757

Tataroglu, O., and Emery, P. (2015). The molecular ticks of the Drosophila circadian clock. *Curr. Opin. Insect Sci.* 7, 51–57. doi: 10.1016/j.cois.2015.01.002

Weiss, R., Bartok, O., Mezan, S., Malka, Y., and Kadener, S. (2014). Synergistic interactions between the molecular and neuronal circadian networks drive robust behavioral circadian rhythms in *Drosophila melanogaster*. *PLoS Genet.* 10:e1004252. doi: 10.1371/journal.pgen.1004252

Xiong, W. C., Okano, H., Patel, N. H., Blendy, J. A., and Montell, C. (1994). repo encodes a glial-specific homeo domain protein required in the Drosophila nervous system. *Genes Dev.* 8, 981–994. doi: 10.1101/gad.8.8.981

Xu, K., DiAngelo, J. R., Hughes, M. E., Hogenesch, J. B., and Sehgal, A. (2011). The circadian clock interacts with metabolic physiology to influence reproductive fitness. *Cell Metab.* 13, 639–654. doi: 10.1016/j.cmet.2011.05.001

Yoshii, T., Hermann-Luibl, C., and Helfrich-Förster, C. (2016). Circadian light-input pathways in Drosophila. *Commun. Integr. Biol.* 9:e1102805. doi: 10.1080/19420889.2015.1102805

Yoshii, T., Todo, T., Wülbeck, C., Stanewsky, R., and Helfrich-Förster, C. (2008). Cryptochrome is present in the compound eyes and a subset of Drosophila's clock neurons. *J. Comp. Neurol.* 508, 952–966. doi: 10.1002/cne.21702

Yoshii, T., Wulbeck, C., Sehadova, H., Veleri, S., Bichler, D., Stanewsky, R., et al. (2009). The neuropeptide pigment-dispersing factor adjusts period and phase of Drosophila's clock. *J. Neurosci.* 29, 2597–2610. doi: 10.1523/JNEUROSCI.5439-08.2009

Zerr, D. M., Hall, J. C., Rosbash, M., and Siwicki, K. K. (1990). Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of Drosophila. *J. Neurosci.* 10, 2749–2762.

Zwarts, L., Van Eijs, F., and Callaerts, P. (2015). Glia in Drosophila behavior. *J. Comp. Physiol.* 201, 879–893. doi: 10.1007/s00359-014-0952-9

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 11 April 2018
doi: 10.3389/fphys.2018.00361

Different Levels of Expression of the Clock Protein PER and the Glial Marker REPO in Ensheathing and Astrocyte-Like Glia of the Distal Medulla of *Drosophila* Optic Lobe

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: *16 November 2017*
Accepted: *23 March 2018*
Published: *11 April 2018*

Citation:
Krzeptowski W, Walkowicz L, Płonczyńska A and Górska-Andrzejak J (2018) Different Levels of Expression of the Clock Protein PER and the Glial Marker REPO in Ensheathing and Astrocyte-Like Glia of the Distal Medulla of Drosophila Optic Lobe. Front. Physiol. 9:361. doi: 10.3389/fphys.2018.00361

Circadian plasticity of the visual system of *Drosophila melanogaster* depends on functioning of both the neuronal and glial oscillators. The clock function of the former is already quite well-recognized. The latter, however, is much less known and documented. In this study we focus on the glial oscillators that reside in the distal part of the second visual neuropil, medulla (dMnGl), in vicinity of the PIGMENT-DISPERSING FACTOR (PDF) releasing terminals of the circadian clock ventral Lateral Neurons (LNvs). We reveal the heterogeneity of the dMnGl, which express the clock protein PERIOD (PER) and the pan-glial marker REVERSED POLARITY (REPO) at higher (P1) or lower (P2) levels. We show that the cells with stronger expression of PER display also stronger expression of REPO, and that the number of REPO-P1 cells is bigger during the day than during the night. Using a combination of genetic markers and immunofluorescent labeling with anti PER and REPO Abs, we have established that the P1 and P2 cells can be associated with two different types of the dMnGl, the ensheathing (EnGl), and the astrocyte-like glia (ALGl). Surprisingly, the EnGl belong to the P1 cells, whereas the ALGl, previously reported to play the main role in the circadian rhythms, display the characteristics of the P2 cells (express very low level of PER and low level of REPO). Next to the EnGl and ALGl we have also observed another type of cells in the distal medulla that express PER and REPO, although at very low levels. Based on their morphology we have identified them as the T1 interneurons. Our study reveals the complexity of the distal medulla circadian network, which appears to consist of different types of glial and neuronal peripheral clocks, displaying molecular oscillations of higher (EnGl) and lower (ALGl and T1) amplitudes.

Keywords: glial oscillators, PER, REPO, astrocyte-like glia, ensheathing glia, circadian clocks, *Drosophila* visual system

INTRODUCTION

Glial cells of *Drosophila* are as diverse as their counterparts in vertebrates, with which they share many morphological and functional features (for review see Freeman and Doherty, 2006). The surface, cortex, and neuropil glia that are further divided into subtypes having distinct structures and associated with specific sets of neurons, reflect the diversity of glial functions (Edwards and Meinertzhagen, 2010; Edwards et al., 2012).

Glial cells that express the so called clock genes are considered to be the peripheral clocks (oscillators) in the fruit fly circadian (∼24 h) timing system, comparable to photoreceptors and other sensory neurons (reviewed in Jackson et al., 2015; Zwarts et al., 2015; Chi-Castañeda and Ortega, 2016). The fundamental feature of the clock function that enables regulating day and night cycles of various physiological functions is the rhythmic expression of the clock genes. *Drosophila* glial cells have been known for over a decade to rhythmically express the core genes of the circadian clock, such as *period* (*per*) and *timeless* (*tim*) (Zerr et al., 1990; Ewer et al., 1992; Ng et al., 2011). Recently, the expression of PER, the core repressor in the clock mechanism that blocks CLK/CYC-activated circadian transcription of target genes (Hardin, 2011), have been shown to oscillate in several glial subtypes, in a manner suggestive of the circadian clock functioning in these cells (Long and Giebultowicz, 2018). Studies of mammalian glial cells have likewise demonstrated the presence of functional clocks in astrocytes and microglia, which express e.g., *Per1* and *Per2* in a rhythmic manner (Prolo et al., 2005; Marpegan et al., 2011; Hayashi et al., 2013; Fonken et al., 2015; Brancaccio et al., 2017; Chi-Castañeda and Ortega, 2018). Therefore, the glial cells in both mammals and in *Drosophila* appear to be equipped with the same core components of the clock mechanism as the central clocks.

Apart from the studies on clock genes rhythmic expression in *Drosophila* glia, there are also reports suggesting that the glia participate in circadian regulation of behavioral rhythms, such as rhythmic locomotor behavior (also via physiological regulation of the neuronal circuitry driving these rhythms), and that gliotransmitters are involved in the circadian rhythmicity (Suh and Jackson, 2007; Ng et al., 2011; Ng and Jackson, 2015). Glial cells of *Drosophila* are also known to contribute to the circadian structural plasticity that so far has been reported in the clock and other brain structures of flies and mammals (Górska-Andrzejak, 2013; Bosler et al., 2015; Herrero et al., 2017). The so called epithelial glial cells (EGl) of the first optic neuropil (lamina) of Diptera display daily changes of their volume (Pyza and Górska-Andrzejak, 2004) and the level of expression of the catalytic α-subunit of sodium-potassium pump, the Na^+/K^+-ATPase (Górska-Andrzejak et al., 2009). The EGl also affect the rhythm of morphological changes of the L1 and L2 monopolar cells—the postsynaptic partners of the compound eye photoreceptors (Pyza and Górska-Andrzejak, 2004). They also modulate the circadian changes of abundance of the presynaptic protein Bruchpilot in photoreceptor terminals (Górska-Andrzejak et al., 2013). The glial clocks (and glia-to-neuron communication) also actively contribute to circadian remodeling of axonal projections of the clock neurons, the small ventral Lateral Neurons (s-LNvs) that control the rest-activity cycles in *Drosophila* (Fernández et al., 2008; Herrero et al., 2017). The acute disruption of glial cells internal clock abolishes the circadian changes of s-LNv projections (Fernández et al., 2008). In view of the evidence mentioned above, we follow Jackson (2011) and use the term "glial clocks" for the glia expressing the clock genes.

In this study, we focus on PER-expressing glial cells, which are located in the distal part of the second visual neuropil, the medulla (hereafter referred to as the distal medulla neuropil glia, dMnGl), in vicinity of the PIGMENT-DISPERSING FACTOR (PDF) releasing terminals of the circadian clock large Ventral Lateral Neurons (l-Nvs). According to many published categorizations of *Drosophila* glial cells (Awasaki et al., 2008; Doherty et al., 2009; Edwards and Meinertzhagen, 2010; Hartline, 2011; Edwards et al., 2012), the dMnGl contain two morphologically distinct types of glia, the astrocyte-like glia (ALGl) and the ensheathing glia (EnGl). Their cell bodies reside at the border between the neuropil and the cortex of the medulla in the estimated ratio of 1:1 (Kremer et al., 2017), whereas their processes span the medulla neuropil in the way reflecting its columnar organization (Kremer et al., 2017; Richier et al., 2017).

The ALGl extend complex processes of high structural density deep into neuropil (infiltrating the medulla layers of synaptic connections M1-M8). They are closely associated with neuronal synapses, express transporters for neurotransmitter clearance and contain multiple neurotransmitter recycling pathways (Stork et al., 2012; Richier et al., 2017). ALGl reveal enriched expression of genes that are involved in energy metabolism, redox reactions, and vesicle-mediated transport and secretion (Ng et al., 2016). RNAi-mediated knockdown of some of these genes has been shown to trigger changes in the level and/or circadian pattern of activity of flies, or to induce paralysis (Ng et al., 2016).

The EnGl processes, on the other hand, are closely associated with neuronal arborizations. They invade the synaptic neuropil of distal medulla as sparsely branched columnar structures showing characteristic branching pattern in the neuropil layers M3 and M6, where the photoreceptors R7 and R8 terminate, as well as in the serpentine layer, M7 (Kremer et al., 2017; Richier et al., 2017). Like their mammalian counterparts, the microglia, EnGl have been reported to phagocytose neuronal debris after axonal injury and also during normal synaptic growth (Doherty et al., 2009; Stork et al., 2012).

Like most of the *Drosophila* glia (with the exception of the midline glia), the ALGl and EnGl, express the REVERSED POLARITY (REPO), a homeodomain protein required for correct differentiation of glia in the embryonic nervous system, including the visual system (Campbell et al., 1994; Xiong et al., 1994; Halter et al., 1995). This transcriptional activator regulates expression of other genes (Yuasa et al., 2003). It was shown, that REPO controls synaptic growth at the *Drosophila* larval neuromuscular junction (NMJ), through the regulation of *wingless* (*wg*) expression (Kerr et al., 2014).

The main goal of our microscopic study was to provide a detailed characterization of the expression of the core clock protein PER and the glial marker REPO in the dMnGl, focusing on differences between the two distinct subtypes that jointly

populate this neuropil, the astrocyte-like, and ensheathing glia. They reside in a very strategic region of projections of the most important neuronal pacemakers (the LNvs) for generating behavioral rhythms (Helfrich-Förster et al., 2007). We reveal their heterogeneity with respect to the levels of PER and REPO and daily fluctuations in the abundance of the latter. We believe that our study may establish the foundation for further work on the type-dependent specificity of the glial clocks, as well as on their significance in functioning and structural plasticity of the distal medulla circadian network.

MATERIALS AND METHODS

Fly Strains

The following strains of *Drosophila melanogaster* were used: Canton-S (CS), *EAAT1*-Gal4 (*w*;* P{Eaat1-GAL4.R}2), *alrm*-Gal4, *tim*-Gal4, UAS-mCD8-GFP ($y^1 w^*$; P{UAS-mCD8.mGFP.LG}10b), UAS-S65T-GFP (*w**; P{UAS-GFP.S65T}Myo31DF^{T2}), Pdf^0 (*w+*; Pdf^0). Flies were reared on standard yeast-cornmeal-agar medium, under day/night cycles (LD 12:12; 12 h of light and 12 h of darkness; ZT0-denotes the beginning of the day; ZT12-denotes the beginning of the night), at 25 ± 1°C.

For studies on daily expression of glial marker REPO, males that emerged from pupae were kept for 6 days either in LD (one group) or DL (reversed cycle, second group). Following such entrainment, the flies kept in LD were decapitated 1 h (ZT1) and 4 h (ZT4) after the lights on, whereas the flies kept in DL, were dissected 1 h (ZT13) and 4 h (ZT16) after the lights off. For other experiments, the flies were always decapitated at the beginning of the day, at ZT1.

Immunolabeling

Following flies immobilization with CO_2, heads were dissected and fixed in 4% PFA (paraformaldehyde, Sigma-Aldrich) in 0.1M Phosphate Buffer (PB) for 2 h. They were washed in Phosphate Buffer Saline (PBS), cryoprotected (4°C, overnight) in 25% solution of sucrose in 0. 01 M PBS and mounted in Tissue-Tek medium (Cryomatrix, Thermo Scientific) for crysectioning. Cryosections of 20 μm thickness were cut and immunolabeled with primary antibodies as follows: mouse anti-REPO Ab (1:40, Developmental Studies Hybridoma Bank), mouse anti-PDF Ab (1:500, Developmental Studies Hybridoma Bank) rabbit anti-GFP Ab (1:1000, Novus Biologicals), mouse anti-GFP Ab (1:1000, Novus Biologicals), rabbit anti-PER Ab (1:1000, a kind gift of R. Stanewsky), and rabbit anti-*Drosophila melanogaster* MESENCEPHALIC ASTROCYTE-DERIVED NEUROTROPHIC FACTOR or DmMANF Ab (1:500, GenScript, prepared using PolyExpress Premium Antigen-Specific Affinity Purified pAb and tested for specificity on homogenates of *DmMANF*-deficient flies). Goat anti-rabbit and goat anti-mouse Abs conjugated with AlexaFluor488 (1:1000, Molecular Probes Invitrogen) or with Cy3 (1:500, Jackson ImmunoResearch Laboratories) were used as secondary antibodies. After final washing, slides were closed in either Vectashield, or DAPI-containing Vectashield, medium (Vector). In experiments on daily expression of glial marker REPO, heads that were collected at four different ZTs, were processed and immunolabeled in parallel, under the same conditions.

Image Acquisition and Analysis

Fluorescently labeled cryosections were examined using a confocal microscope, Zeiss LSM 510 META. For quantitative comparisons of PER and REPO-specific intensities, the same acquisition parameters were applied to images of the same experimental set. Morphometric analyses were performed using ImageJ software (NIH, Bethesda). The level of signal intensity was manually quantified as a Mean Gray Value (further referred as MGV): the sum of gray values in the selected area divided by the number of pixels within that area. For study on daily expression of glial marker REPO, fluorescence intensities of 20–30 REPO-positive nuclei of the dMnGl (in a single image of the optic lobe) was quantified and 10 hemispheres (from 10 individuals) were collected for each ZT group.

Statistics

Data were analyzed for normality using Shapiro-Wilk W-test. Datasets from two experimental groups were tested for significant differences using Mann-Whitney Test. One-way ANOVA or its non-parametric counterpart-Kruskal-Wallis Test, were used for multiple comparisons. N represents either the number of cells, or the number of optic lobes assayed. Data are expressed as means ± *SD*. *p*-values <0.05 were considered to be statistically significant ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

RESULTS

Two Populations of the dMnGl (P1 and P2) Express PER at Different Levels

The dMnGl (**Figure 1A**) display high level of PER-specific immunoreactivity, which is the result of strong PER expression (**Figure 1B**; Górska-Andrzejak et al., 2018). Closer inspection of the distal medulla has revealed, however, that next to the cells expressing PER at high level (hereafter referred to as P1), there are also cells that express PER at much lower level (hereafter referred to as P2) (**Figures 1C,D**). They (P2) were initially noticed in Pdf^0 mutant (**Figures 1C,E**), owing to the relatively high level of PER-specific signal in the glia of this mutant. In Pdf^0 the immunofluorescence in P2 was well visible even though it was 70% weaker than in P1 (Mann-Whitney Test, $Z = 3.84$ [$N_{P1} = 11$ and $N_{P2} = 10$], $p < 0.0001$) (**Figure 1F**). In CS glia, where the level of PER was generally lower than in Pdf^0, the P2 cells were barely discernible from the background noise (**Figure 1D**).

Two Populations of the dMnGl (P1 and P2) Express REPO at Different Levels

In the next step, we wanted to determine whether P1 and P2 displayed also differences in the level of REPO, the pan-glial marker. This conjecture was brought about by subtle, yet noticeable differences in REPO-specific immunofluorescence observed in different types of the optic lobe glia (**Figure 2A**), which might reflect some differences in the amplitudes of certain glial functions.

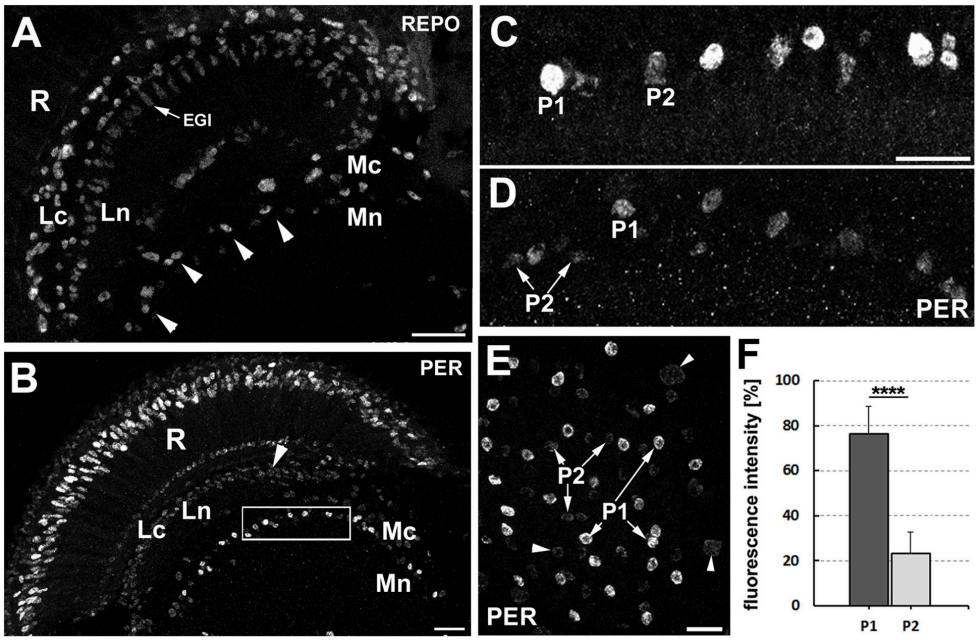

FIGURE 1 | (A) Location of the distal medulla neuropil glia (dMnGl) among other glial cells that are marked by REPO-specific immunofluorescence in the optic lobes (frontal section). The nuclei of the dMnGl (arrowheads) reside at the border between the cortex (Mc) and the neuropil (Mn) of the medulla. R, retina; Lc, lamina cortex; Ln, lamina neuropil; EGl, epithelial glial cells. Scale bar: 20 μm. **(B)** Frontal section of *Pdf* ^{0} optic lobe immunolabeled with anti-PER antibody. R, retina; Lc, lamina cortex; Ln, lamina neuropil; Mc, medulla cortex; Mn, medulla neuropil. The nuclei of the dMnGl (frame) reveal high level of PER-specific immunofluorescence in comparison with the nuclei of glia in Lc (arrowhead). Scale bar: 20 μm. **(C,D)** Small fragments of the Mc/Mn interface of *Pdf* ^{0} **(C)** and CS **(D)** shown in higher magnification reveal the unexpected presence of the dMnGl displaying low level of fluorescence (P2). The P2 cells are well visible in *Pdf* ^{0} **(C)**. They are positioned next to cells of high fluorescence (P1) in almost alternating order. Such arrangement can be also observed, although less clearly, in CS **(D)**. The images shown in **(C,D)** were collected at the same image acquisition parameters. Scale bar for **(C,D)**: 10 μm. **(E)** The frontal surface of *Pdf* ^{0} medulla. The fluorescence in P2 is comparable to the fluorescence in glial cells of the medulla cortex (arrowheads). Scale bar: 10 μm. **(F)** The average level of fluorescence (±*SD*) in P1 and P2 cells of *Pdf* ^{0} (****$p < 0.0001$).

The examination of the distal medulla of CS brains immunolabeled with anti-REPO Ab revealed again the presence of two types of cells that in this case differ in the intensity of REPO-specific fluorescence (**Figure 2B**). The 52% difference in the intensity of their fluorescence was statistically significant (Mann-Whitney Test, $U = 6$ [$N_{type1} = 10$ and $N_{type2} = 9$], $p < 0.01$). In *Pdf* ^{0} mutant we also observed such cells, displaying 49.5% difference in the REPO-specific signal (Mann-Whitney Test, $Z = 3.93$, [$N_{type1} = 11$ and $N_{type2} = 11$], $p < 0.0001$) (**Figure 2C**). Double immunolabeling (**Figure 2D**) showed that the cells identified earlier in PER labeling as P1express REPO also at high level, whereas the P2 cells express REPO at low level.

The Number of REPO-P1 Cells Is Bigger During the Day Than During the Night

Next, we wanted to know whether the level of REPO in the dMnGl changes during the day, which would imply daily oscillations in the REPO-controlled glial functions. It turned out that the average level of REPO-specific immunofluorescence measured in all the dMnGl at different times (ZTs) of the day and night (**Figure 3A**) did not show statistically significant differences (Kruskal-Wallis Test: $H[df = 3, N = 40] = 2.8$, $p > 0.05$). On the other hand, based on the Mean Gray Value (MGV) individual cells were distributed in two groups that can be identified as P1 and P2 (**Figure 3B**). Closer inspection revealed that the percentage of P1 (MGV > 40) and P2 (MGV < 40) vary throughout the day (as shown in **Figure 3C**). The number of P1 cells is bigger during the day than at night as shown for the population of 250 cells (from 10 specimens).

ALGl Express PER and REPO at Low Levels

Then, we wanted to find out whether the ALGl and EnGl can be associated with the two categories of cells: P1 and P2. In order to do this we determined the relative levels of PER and REPO expression in these two types of glia. Initially, we performed anti-PER and anti-REPO immunolabeling on the tissue of *alrm*-Gal4/UAS-S65T-GFP transgenic flies. The *alrm*-Gal4 driver is known to be specific for the ALGl cells and so S65T-GFP can be observed in their cytoplasm and nuclei (**Figure 4A**). The immunolabeling did not reveal any presence of PER in the GFP positive cells (ALGl), suggesting that these cells either do not express PER at all, or express it at non-detectable levels (**Figure 4B**). We found, however, that the ALGl express REPO at the level characteristic for the P2 cells (**Figures 4C,D,D'**). The ALGl cells do not belong, therefore, to the P1 population of either PER- or REPO-positive cells. They can be classified as P2 with

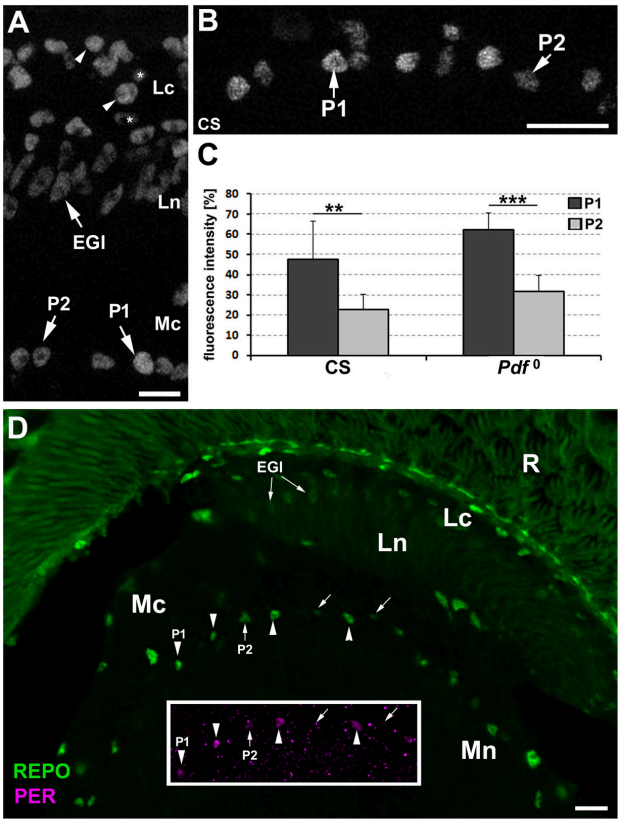

FIGURE 2 | (A) The cross section of the lamina and the distal part of the medulla of CS immunolabeled with anti-REPO antibody. The staining reveals differences in the intensity of REPO-specific fluorescence in various types of glia. In the lamina cortex (Lc) some glial cells show higher level of fluorescence (arrowheads) than others (asterisks). Fluorescence of the epithelial glia (EGl) in the lamina neuropil (Ln) is more uniform and rather low. The dMnGl show both higher (P1 cell) and lower (P2 cell) levels of REPO-specific fluorescence. Mc-medulla cortex. Scale bar: 5 μm. **(B)** The P1 and P2 cells of the REPO-positive dMnGl are arranged in almost alternating order. Scale bar: 10 μm. **(C)** The average level of REPO-specific fluorescence (± *SD*) of the P1 and P2 dMnGl in CS and *Pdf* ^{0} (**$p < 0.01$, ***$p < 0.0001$). **(D)** The frontal section of the lamina and the distal part of the medulla of CS labeled with anti-REPO and anti-PER (insert) antibodies. The nuclei of REPO-P1 cells (in the main picture, arrowheads) show strong PER-specific fluorescence (insert, arrowheads). The REPO- P2 cells (in the main picture, arrows) reveal weak or no PER-specific fluorescence (insert, arrows). R, retina; Lc, lamina cortex; Ln, lamina neuropil; EGl, the epithelial glia of the lamina; Mc, medulla cortex; Mn, medulla neuropil. Scale bar: 20 μm.

respect to REPO expression. Whether these cells do express small amounts of PER could not be determined based on this staining. Interestingly, the REPO-P1 cells display a very weak expression of GFP reporter of *alrm*-Gal4 driver (**Figures 4D,D'**).

The issues outlined above were subsequently resolved using the GFP reporter of a driver for the *Drosophila Excitatory Amino Acid Transporter 1*, *EAAT1*-Gal4, which also marks the glia that populate the distal medulla neuropil (Edwards et al., 2012). The obtained immunolabeling of *EAAT1*-Gal4/UAS-S65T-GFP optic lobes confirmed that the levels of expression of PER and REPO in the GFP-positive cells (the ALGl nuclei) are lower than in the P1 cells (**Figures 5A,B,D,D'**). PER is detectable, but its level is exceptionally low (inserts in **Figure 5B**). On this view, the GFP-positive cells (ALGl) should be regarded as P2 with respect to both REPO and PER expressions.

We have noticed, however, that the P2 population of cells does not consist entirely of glia. At least some of these GFP-positive cells send projections to the first optic neuropil-the lamina (**Figure 5D**), which means that they do not belong to the ALGl. Their terminals in the lamina (**Figures 5E,F**), as revealed by the driver based expression of membranous mCD8-GFP reporter (*EAAT1*-Gal4/UAS-mCD8-GFP), have the morphology of baskets enveloping the lamina synaptic units, called cartridges (insert in **Figure 5F**). Since such a morphology is typical for T1 interneurons, we assume that *EAAT1*-Gal4 drives expression also to T1 cells, and that they belong to the P2 group.

EnGl Express High Levels of PER and REPO

To confirm that it is the EnGl that express PER and REPO at high levels we performed anti-PER and anti-REPO immunolabeling on the tissue of *NP6520*-Gal4/UAS-mCD8-GFP transgenic flies. *NP6520*-Gal4 is the marker for ensheathing glia (Awasaki et al., 2008; Richier et al., 2017). The immunolabeling revealed strong expression of PER and REPO in GFP-positive cells (EnGl) at the border of cortex and neuropil of the medulla (**Figure 6**). The EnGl of the distal medulla express also *Drosophila melanogaster* MESENCEPHALIC ASTROCYTE-DERIVED NEUROTROPHIC FACTOR (DmMANF), as revealed by the immunolabeling using anti-DmMANF Ab (**Figure 6C**). It is present not only in the perinuclear space of endoplasmic reticulum (ER) of EnGl, but also in their long processes that penetrate the neuropil of the medulla (**Figures 6C–D"**). In fact, DmMANF was mostly observed in the processes of EnGl in the medulla neuropil, as the majority of DmMANF-positive processes that were most clearly visible belonged to EnGl (were also GFP-positive; **Figures 6D–D"**).

Both P1 and P2 Cells Express TIM

tim-gal4 line is often used as a driver line for clock cells (Ozkaya and Rosato, 2012) due to its strong expression in pacemakers. Since our other study (Górska-Andrzejak et al., 2018) revealed that the dMnGl express GFP reporter of *tim*-Gal4 at much higher level than the rest of the optic lobe (**Figure 7A**), we expected TIM to be expressed at high level in the P1 cells (**Figure 7**). Our results showed, however, that not only the P1 but also the P2 and some of the REPO-negative cells express TIM (**Figure 7C**). It is worth emphasizing that we did not see any negative correspondence of REPO level with the GFP reporter of tim, in contrast to what we observed for *alrm*-GFP and *EAAT1*-GFP lines (**Figures 4**, **5**).

DISCUSSION

The dMnGl Express Different Levels of PER and REPO

Our detailed microscopic investigation revealed that the dMnGl, which are known to contain two morphologically distinct types of glia, the ALGl, and EnGl (Awasaki et al., 2008; Doherty

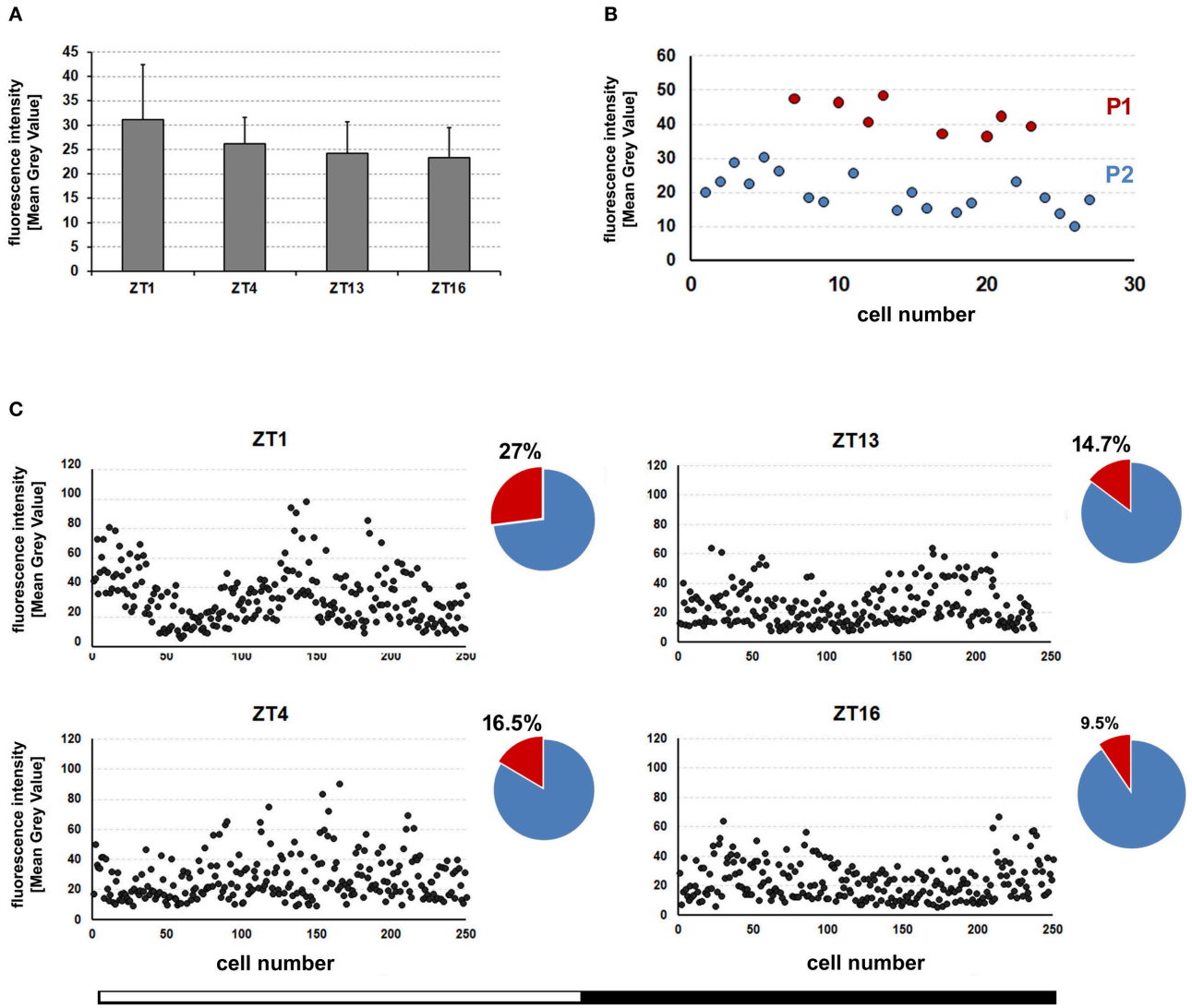

FIGURE 3 | (A) The average (±*SD*) level of REPO-specific fluorescence in the nuclei of the dMnGl of CS flies collected at different times (ZT1, ZT4, ZT13, and ZT16) of the day. For each time point the fluorescence was measured in the dMnGl of 10 hemispheres ($N = 10$). **(B)** The consecutive measurements of REPO fluorescence in the dMnGl ($N = 27$) of an exemplary optic lobe, revealing distinct populations of cells with higher (P1) and lower (P2) levels of fluorescence at ZT1. **(C)** The measurements of REPO fluorescence in the dMnGl of flies collected at designated ZTs. Each chart shows measurements for 250 nuclei from 10 optic lobes. The accompanying pie charts illustrate percentages of nuclei with the intensity of fluorescence below (blue) and above (red) the MGV of 40.

et al., 2009; Edwards and Meinertzhagen, 2010; Hartline, 2011; Edwards et al., 2012), differ with respect to the level of expression of the clock protein PER and the glial marker REPO. Surprisingly, the ALGl express low levels of both PER and REPO (P2), whereas the EnGl display relatively high levels of these two proteins (P1). The amplitude of the circadian (PER-controlled) and glial (REPO-controlled) functions of ALGl and EnGl may therefore, significantly differ.

Obviously, the mere findings that different cells express different levels of PER do not necessarily imply that their molecular clocks are substantially different, especially that the clock proteins may be also engaged in non-clock functions (Beaver et al., 2003; Sakai et al., 2004; Houl et al., 2006; Chen et al., 2012). For example, the fruit fly's *per* (and none of the other clock genes) is crucial also for the formation of long-term memory (LTM). *per* expressing cells that regulate the LTM formation might, therefore, be distinct from the clock neurons (Sakai et al., 2004; Chen et al., 2012). The fact that such difference between ALGl and EnGl is not visible in the case of TIM expression (based on the level of GFP reporter of *tim*-Gal4 driver; **Figure 7**) could also indicate an additional function of PER (next to the clock function) in either both or only one of these two types of dMnGl glia.

Nevertheless, it is also known that the abundance of PER, the prime repressor in the mechanism of the circadian clock (Landskron et al., 2009; Hardin, 2011), sets the pace of the clock

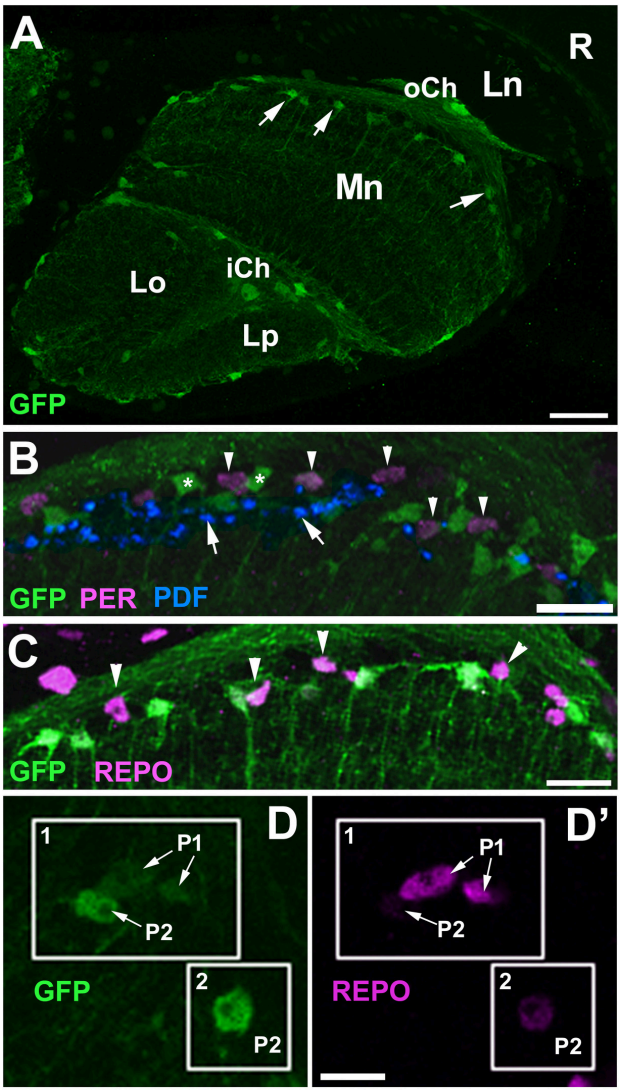

FIGURE 4 | Colocalization of PER and REPO to the astrocyte-like glia (ALGl) of *alrm*-Gal4/UAS-S65T-GFP flies collected at ZT1. **(A)** Localization of ALGl (arrows) in the distal part of the medulla neuropil (Mn) in horizontal section. R, retina; Ln, lamina neuropil; oCh, outer chiasm; iCh, inner chiasm; Lo, lobula; Lp, lobula plate. Scale bar: 20 μm. **(B)** GFP-expressing ALGl (asterisks) in the region of PDF-positive terminals (arrows) of l-LNv neurons do not express PER. PER-positive nuclei (arrowheads) are located in between the cell bodies of ALGl. Scale bar: 10 μm. **(C)** GFP-negative nuclei of non-ALGl (arrowheads) express REPO at high level. Scale bar: 10 μm. (**D**,**D'**) ALGl and non-ALGl cells of the distal medulla in higher magnification. ALGl cells (box 1 and 2) that express GFP at high level **(D)**, show weak expression of REPO **(D')**, whereas non-ALGl cells (box 1) that express GFP at barely detectable level **(D)**, show strong REPO expression **(D')**. Scale bar: 5 μm.

and the phase of the circadian rhythm (Baylies et al., 1987). Levels of *per* RNA are correlated with the period length. Therefore, flies with the lowest levels of PER have slow-running biological clocks (Baylies et al., 1987) and the phase of the rhythm can be altered by temporary increase of PER concentration (Edery et al., 1994). Hence, in the cells that express PER at different levels, such as P1 and P2, the pace of the clocks may differ. Additionally, as we saw in our other study (Górska-Andrzejak et al., 2018), the dMnGl expressed the highest level of GFP of all the optic lobe glia, when examined in *tim*-Gal4/UAS-S65T-GFP transgenic flies. Consequently, at least some of the dMnGl must have high levels of both TIM and PER, and so may belong to the robust molecular oscillators (with high-amplitude cycling of both PER and TIM) among the glia. The fact that the percentage of dMnGl with the highest level of REPO displays daily fluctuations supports this notion, especially because the timing of its maximum (at ZT1) is consistent with an increased PDF immunoreactivity seen early in the morning (Park et al., 2000).

The ALGl Reveal Low Levels of PER and REPO

As we were unable to colocalize the *alrm*-Gal4 and *EAAT1*-Gal4-driven GFP with high levels of PER and REPO we conclude that the astrocytic glia belong to the P2 population of dMnGl. No staining of PER in the ALGl was also observed by Long and Giebultowicz (2018) in several areas of the central brain at two different time points (ZT22 and ZT10), which typically correspond with high and low PER expression. It is thus unlikely that low level of PER at ZT1 can be caused by some untypical daily pattern of PER expression. Our study of dMnGl conducted at several time points (Górska-Andrzejak et al., 2018) did not reveal any irregularities in the daily pattern of PER that would indicate untypical expression in the ALGl. On this view, we are quite confident that the ALGl should be classified as the P2 cells.

The low level of PER in the ALGl of *Drosophila* is surprising since these glia were reported to be crucial in behavioral rhythmicity (Suh and Jackson, 2007; Ng et al., 2011). Surprising as it may be, the well-documented role of ALGl in behavioral rhythmicity indicates that also the cells, like ALGl, that express PER at low (or not detectable) levels may play important roles in the circadian network of *Drosophila*. It is worth mentioning, that the majority of ALGl express the glia-specific protein, EBONY (Jackson et al., 2015), which cycles in the fly's head under control of PER/TIM-based intracellular oscillator (Ueda et al., 2002; Suh and Jackson, 2007), and which is vital in regulation of behavioral rhythmicity (Suh and Jackson, 2007). The *ebony* gene mutation was shown to alter the circadian activity rhythms (Newby and Jackson, 1991) and its phenotype can be rescued by induction of *ebony* expression under *repo*-Gal4 driver (Suh and Jackson, 2007).

Perhaps the glia that express EBONY (or other proteins similar to EBONY) need not express PER at such a high level to play the function of the peripheral clocks. Incidentally, another group of the neuropil glia that express EBONY are the epithelial glial cells (EGl; Górska-Andrzejak et al., 2009) of the lamina (**Figure 1A**), which like ALGl of the distal medulla express relatively low levels of PER (**Figure 1B**) and REPO (**Figures 2A,D**). Being regarded as the astrocyte-type glia of the lamina, in spite of their columnar rather than astrocytic morphology (Edwards and Meinertzhagen, 2010), they are another example of the ALGl that display low levels of PER and REPO.

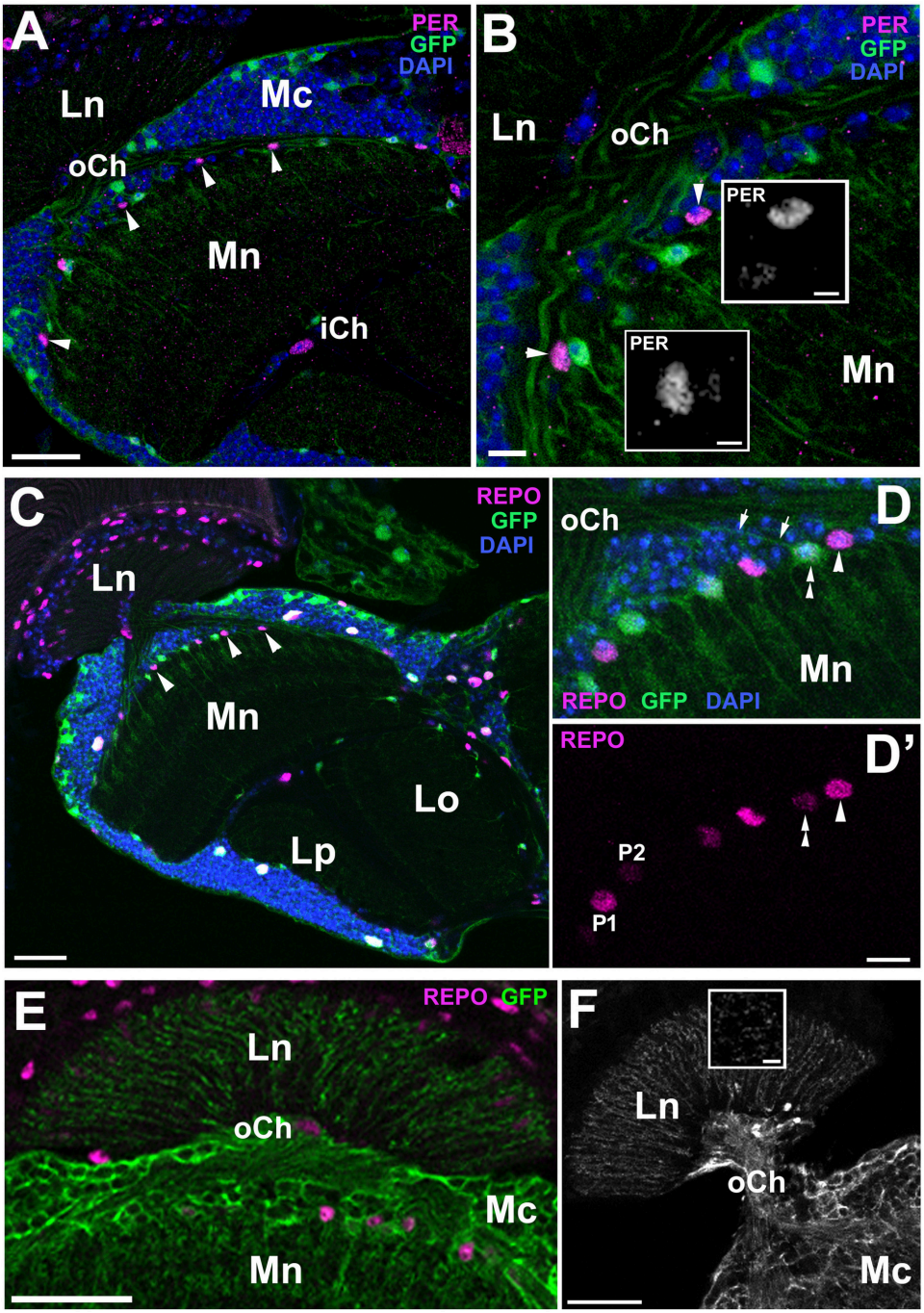

FIGURE 5 | Frontal sections of the optic lobe of *EAAT1*-Gal4/UAS-S65T-GFP and *EAAT1*-Gal4/UAS-mCD8-GFP transgenic flies collected at ZT1 and immunolabeled with anti-PER and anti-REPO antibodies. **(A)** PER-positive nuclei of the dMnGl (arrowheads) are clearly visible in the distal part of the medulla neuropil (Mn) of *EAAT1*-Gal4/UAS-S65T-GFP flies. Ln, lamina neuropil; oCh, outer chiasm; Mc, medulla cortex; iCh, inner chiasm. Scale bar: 20 μm. **(B)** Higher magnification of the distal medulla showing that GFP-positive cells express PER (inserts) at barely detectable level. The neighboring GFP-negative cells (arrowheads) express PER at high level. Scale bar: 5 μm and 2 μm (in inserts). **(C)** REPO-positive nuclei of the dMnGl (arrowheads) are well visible in the distal part of the medulla neuropil (Mn) of *EAAT1*-Gal4/UAS-S65T-GFP flies. Ln, lamina neuropil; Lo, lobula; Lp, lobula plate. Scale bar: 20 μm. **(D,D')** Higher magnification of the distal medulla neuropil (Mn) of *EAAT1*-Gal4/UAS-S65T-GFP flies viewed in a projection of 10 μm thick image stack. GFP-negative cells (arrowhead in **D**) express REPO at higher level (arrowhead in **D'**), whereas GFP-positive cells (double arrowhead in **D**) express lower level of REPO (double arrowhead in **D'**). Some of GFP-positive cells send projections to the lamina (arrows in **D**). DAPI-positive nuclei visualize the medulla cortex. oCh, outer chiasm. Scale bar: 5 μm. **(E,F)** Morphology of GFP-positive processes in the lamina of *EAAT1*-Gal4/UAS-mCD8-GFP flies in frontal **(E)** and horizontal **(F)** sections. The basket-like terminals, which surround each of the lamina synaptic units, so called cartridges, are characteristic for the processes of T1 interneurons. Insert in **(F)** shows the cross section of T1 processes in the lamina. Ln, lamina neuropil; oCh, outer chiasm; Mc, medulla cortex; Mn, medulla neuropil. Scale bars: 20 μm for **(E,F)**, 5 μm for insert.

It Is the EnGl That Express Higher Levels of PER and REPO

Our study have revealed that the EnGl express PER at high level. Therefore, they may be regarded as robust oscillators in comparison with other types of glia, just like the l-LNv neurons in comparison with other clock neurons (Helfrich-Förster, 1998; Helfrich-Förster et al., 2007). The l-LNvs display the molecular rhythms of PER and TIM that are phase-advanced and of higher amplitude with respect to other clock neurons. Therefore, it is presumed that their input may be particularly robust and they are vital for light-mediated modulation of arousal and sleep (Sheeba et al., 2008, 2010).The question remains whether the EnGl support l-LNvs in this modulation. In this context, the fact that the percentage of REPO-P1 cells was found to be the biggest at the beginning of the day (**Figure 3**) also seems to be relevant.

It is an interesting coincidence that the age-related decline in PER occurred in various glial subtypes, but was not observed in the case of EnGl (Long and Giebultowicz, 2018; study on the age-dependent changes in the expression of PER in the *Drosophila* glia). This confirms their robustness as glial oscillators. The age related decline in PER does not occur in the neuronal pacemakers of the central clock, but it has been found in the photoreceptors of the retina (Long and Giebultowicz, 2018).

The higher level of PER (and REPO) in EnGl may indicate the need for stronger circadian regulation of the functions carried out by EnGl, e.g., the ensheathment of R-cells axon bundles. Being the robust circadian oscillators, the EnGl could modulate their capacity to process information in a circadian manner. As they wrap and insulate neuronal axons (Cameron et al., 2016), they increase the conduction velocity of electrical impulses (in both vertebrate and invertebrate nervous systems), and consequently, enhance the neuronal capacity to process information at a faster pace.

Studies on development of the nervous system in *Drosophila* indicate that this type of glia are able to undergo complex morphological changes to accommodate neuronal axons (Banerjee and Bhat, 2008; Subramanian et al., 2017). In the adult brain, on the other hand, the ensheathing glia (but not astrocytes) express the engulfment receptor *Draper*, which enables glial membranes to contact degenerating axons and proceed with engulfment of axonal debris. Blocking of endocytosis specifically in the EnGl cells inhibits the process of severed axons clearance (Doherty et al., 2009).

Interestingly, as we showed the EnGl of *Drosophila* express the DmMANF, a novel evolutionary conserved (in ∼50% identical to human MANF) neurotrophic factor (Petrova et al., 2003) that not only protects neurons against apoptosis and supports their survival (like other neurotrophic factors), but also plays a conserved immune modulatory function (Neves et al., 2016). It has been shown that the silencing of DmMANF in glial cells induces the appearance of so called MANF immunoreactive cells (MiCs) during metamorphosis. These are the migratory cells that closely resemble macrophages/hemocytes and vertebrate microglia. They express RELISH and DRAPER suggestive of their immune response activation (Stratoulias and Heino, 2015b).

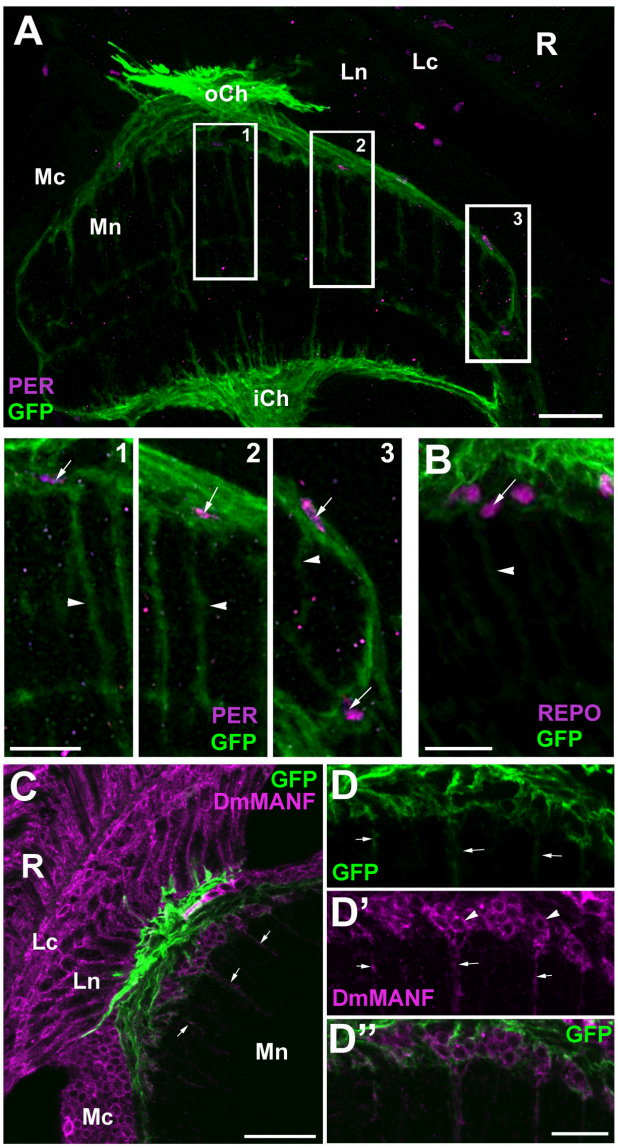

FIGURE 6 | (A) Horizontal section of the medulla of *NP6520*-Gal4/UAS-mCD8-GFP flies immunolabeled with anti-PER antibody. The nuclei of GFP-expressing EnGl (frames 1–3) are PER-positive, which is well visible in higher magnification (panels 1–3). The processes of EnGl (arrowheads in panels 1–3) span the medula neuropil (Mn). R, retina; Lc, Lamina cortex; Ln, lamina neuropil; oCh, outer chiasm; Mc, medulla cortex; iCh, inner chiasm. Scale bars: 20 μm for **(A)** and 10 μm for panel 1, 2, and 3. **(B)** The nuclei of GFP-positive EnGl show high level of REPO-specific immunofluorescence (arrow). Arrowhead-the EnGl processes. Scale bar: 10 μm. **(C)** The distal part of the medulla in horizontal section of *NP6520*-Gal4/UAS-mCD8-GFP flies immunolabeled with anti-DmMANF antibody. The processes of EnGl are marked with arrows. R, retina; Lc, lamina cortex; Ln, lamina neuropil; Mc, medulla cortex; Mn, medulla neuropil. Scale bar: 20 μm. **(D-D")** Higher magnification of EnGl marked in **(C)**. The GFP-positive processes of EnGl (**D**, arrows) are marked with DmMANF **(D',D")**. DmMANF is also visible in the perinuclear space of cell bodies in the medulla cortex (D', arrowheads). Scale bar: 10 μm.

Thus, the ensheathing glia appear to play the similar role as the highly rhythmic microglia in the mammalian brain (for review see Salter and Stevens, 2017). For example, the hippocampal

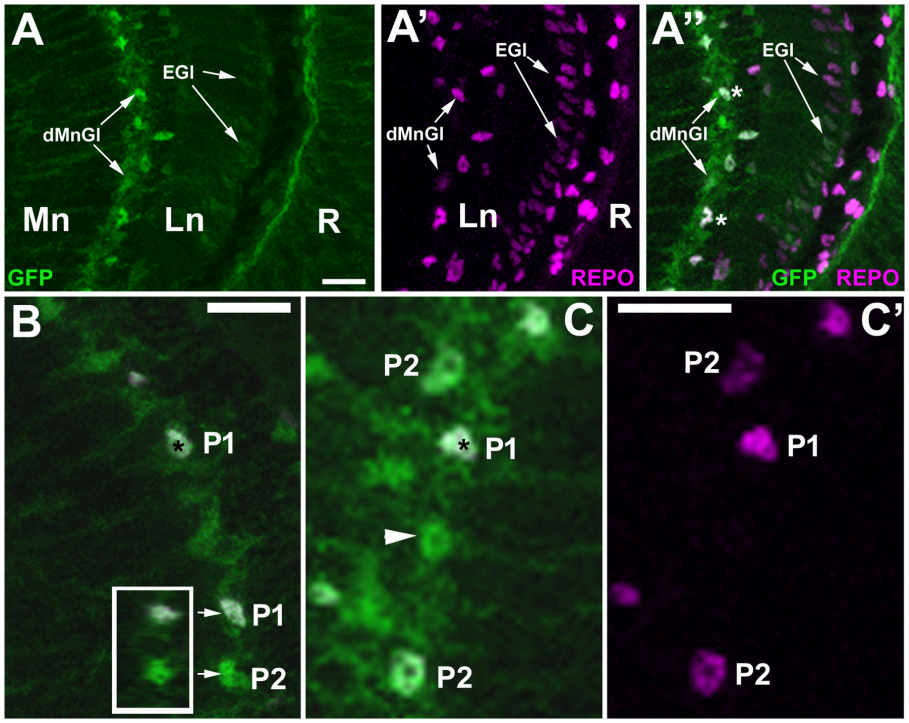

FIGURE 7 | Anti-REPO immunolabeling of GFP-labeled *tim*-expressing cells in the visual system of *tim*-Gal4/UAS-S65T-GFP flies collected at ZT1. **(A–A")** The strong fluorescence of GFP-reporter is observed in the dMnGl, whereas the weak fluorescence is visible in the other glial cells, e.g., the epithelial glial cells (EGl) of the lamina neuropil (Ln). R, retina; Mn, medulla neuropil. **(B)** Exemplary GFP-positive cells revealing higher (P1) and lower (P2) levels of REPO-specific fluorescence. Insert: the orthogonal view (*z*-stack projection) of P1 and P2 (arrows) showing that their nuclei are complete (so the difference in the intensity of REPO-specific immunofluorescence results entirely from the differences in the level of REPO expression). **(C,C')** TIM-positive cells (green) in the distal medulla show either high (P1), low (P2), or no expression of REPO (arrowhead). The nuclei of cells revealing high level of expression of GFP and REPO are marked with asterisks. Scale bars in all panels: 10 μm.

microglia have been shown to rhythmically expresses core clock genes: *Per1, Per2, Rev-erb, Bmal1*, as well as several pro-inflammatory cytokines. They also display profound differences in immune stimulation throughout the day (Hayashi et al., 2013; Fonken et al., 2015). The strong molecular rhythm of PER oscillations in the *Drosophila* EnGl definitely adds to its similarity to microglia.

In the *Drosophila* embryonic nervous system DmMANF can be found only in glia (Palgi et al., 2009), while it is present in both the glia and the neurons of the adult brain (Stratoulias and Heino, 2015a). In glia it is present in both cell somata and processes. In neurons, on the other hand, it can be found only in the somata (Stratoulias and Heino, 2015a). In our recent study (Walkowicz et al., 2017) we found that DmMANF is strongly expressed in the epithelial glial cells (EGl) of the first visual neuropil (the lamina). Here we show, that it is also clearly present in the cell bodies and, most importantly, the processes of EnGl (**Figures 6C,D"**).

The processes of EnGl in the distal medulla are enlarged and form a broad meshwork (Kremer et al., 2017). Since they are closely associated with R7 and R8 photoreceptor terminals, just like the processes of the lamina ensheathing glia (also called marginal glia) with the terminals of R1-R6 photoreceptors (Edwards et al., 2012; Kremer et al., 2017), one might put forth a hypothesis that the EnGl may be involved in the regulation of daily morphological changes in the photoreceptor terminals in the distal medulla. In the *Drosophila* medulla various types of axons innervate the neuropil unit (column), but they must target distinct layers while using the same tract. Even the subtle structural changes provided by the EnGl should thus enable modulation of the information flow during the day and night, affecting functions of the visual system. In the mammalian retina renewal of the photoreceptor outer segments is regulated in a circadian manner and this process is based on engulfing and phagocytosis of photoreceptors by the neighboring pigmented epithelium (reviewed in Kevany and Palczewski, 2010).

We found that the EnGl express REPO at higher level than the ALGl. It seems important in view of the recent findings (Matsuno et al., 2015) that the expression of REPO (regulated by neuronal protein KLINGON, KLG), just like *per* expression (Sakai et al., 2004; Chen et al., 2012), is vital for the formation of the long term memory (LTM). Experiments showed that *repo* mutants are defective for LTM and conditional knockdown of *repo* under control of ALGl-specific driver line results in reduction of 24 h memory, while it is not affected by similar inhibition in EnGl (Matsuno et al., 2015). It appears, therefore, that only the astrocyte glia is involved in this process. One should

take into consideration, however, that this may be also caused by the fact that the EnGl, even without REPO, should still be able to partake in LTM formation, owing to the high level of PER, exceeding that in the ALGl.

The capability of changing morphology and governing the phagocytosis, as well as for the high expression of PER and daily fluctuations in REPO abundance suggest that both morphology and functioning of the EnGl of distal medulla has the capacity to undergo robust daily/circadian changes. The occurrence of daily alterations in glial morphology is known from the study on the epithelial glia of the lamina in the housefly (Pyza and Górska-Andrzejak, 2004). They are associated with oscillations of the axon diameter and the size of dendritic tree in L2 monopolar cells during the day and night (Pyza and Meinertzhagen, 1999; Weber et al., 2009). Moreover, the contribution of glial clocks to the circadian structural plasticity of the pacemaker neurons (the small LNvs) have just been reported by Herrero et al. (2017). The impaired glial clocks abolish circadian structural remodeling without affecting other clock-controlled outputs (Herrero et al., 2017).

The T1 Interneurons Express PER and REPO at Very Low Levels

Interestingly, the so called T1 interneurons (Hamanaka and Meinertzhagen, 2010) first identified by Fischbach and Dittrich (1989) occurred to display low levels of PER and REPO. Because of the very presence of PER, we cannot rule out the possibility that the T1 interneurons are part of the medulla circadian network. Interestingly, however, these cells are perceived as neuronal anomaly (Takemura et al., 2008) as they fail to make presynaptic sites at chemical synapses in both the lamina and the medulla neuropils (that they innervate). Since their outputs have not been found in detailed EM study, the cells appear to be entirely postsynaptic (Hamanaka and Meinertzhagen, 2010). What we have found about T1 makes this cell even more peculiar (but also more interesting) as it expresses a very low level of the glial marker, REPO (**Figure 5**), in addition to expression of EAAT1 transporter (also often expressed by glia) that mediates the high affinity uptake of glutamate or aspartate into cells. Unveiling its precise function, however, requires further study.

CONCLUSIONS

Our results revealed that the two types of dMnGl, the astrocyte like glia (ALGl) and the ensheathing glia (EnGl), differ among each other also with respect to the level of expression of the clock protein PER and the glial marker REPO. Since the ALGl glia controlling circadian behavior show little or no PER expression we conclude that the high levels of PER may not be necessary for the circadian functions in the peripheral oscillators, such as the ALGl. This makes the functioning of the circadian mechanism in the ALGl and other peripheral oscillators even more intriguing. On the other hand, the high level of PER and REPO in the EnGl, as well as the presence of daily changes in the number of cells that express REPO at relatively high levels, may indicate that the EnGl are highly rhythmic, just like their vertebrate counterparts, the microglia. They may constitute the particularly important and/or influential glial component of the *Drosophila* circadian network and deserve more consideration in future study on the role of glia in the circadian rhythms.

Our study reveal the heterogeneity of glial oscillators and the complexity of the distal medulla circadian network, thus establishing an essential basis for the study of its functioning. We believe that further study should be focused on the type-dependent properties of glia in the circadian network and clarification of the EnGl circadian function.

AUTHOR CONTRIBUTIONS

WK and LW: performed experiments, collected and analyzed data, and prepared the manuscript. AP: performed some experiments, collected, and analyzed data. JG-A: designed the study, performed experiments, collected and analyzed data, and prepared the manuscript.

FUNDING

The study was supported by the grant K/ZDS/007356 and the Laboratory of Confocal Microscopy in the Institute of Zoology and Biomedical Research, as well as the National Science Centre in Poland (grant number 2014/15/B/NZ3/04754).

ACKNOWLEDGMENTS

The authors express their gratitude for the fly strains that the Department of Cell Biology and Imaging received: to C. Förster (University of Würzburg) for Pdf^0 strain, F. Rouyer (Paris Saclay Institute of Neuroscience) for *tim*-Gal4 strain, C. Klämbt (University of Münster) for *alrm*-Gal4 strain, and Bloomington Drosophila Stock Center (USA) for *EAAT1*-Gal4 (8849), UAS-mcD8-GFP (5137), UAS-S65T-GFP (1521) lines. The authors also wish to express their gratitude to M. Andrzejak for his assistance in preparation of this manuscript.

REFERENCES

Awasaki, T., Lai, S.-L., Ito, K., and Lee, T. (2008). Organization and postembryonic development of glial cells in the adult central brain of Drosophila. *J. Neurosci.* 28, 13742–13753. doi: 10.1523/JNEUROSCI.4844-08.2008

Banerjee, S., and Bhat, M. A. (2008). Glial ensheathment of peripheral axons in Drosophila. *J. Neurosci. Res.* 86, 1189–1198. doi: 10.1002/jnr.21574

Baylies, M. K., Bargiello, T. A., Jackson, F. R., and Young, M. W. (1987). Changes in abundance or structure of the per gene product can alter periodicity of the *Drosophila* clock. *Nature* 326, 390–392. doi: 10.1038/326390a0

Beaver, L. M., Rush, B. L., Gvakharia, B. O., and Giebultowicz, J. M. (2003). Noncircadian regulation and function of clock genes period and timeless in oogenesis of *Drosophila melanogaster*. *J. Biol. Rhythms* 18, 463–472. doi: 10.1177/0748730403259108

Bosler, O., Girardet, C., Franc, J.-L., Becquet, D., and François-Bellan, A.-M. (2015). Structural plasticity of the circadian timing system. An overview from flies to mammals. *Front. Neuroendocrinol.* 38, 50–64. doi: 10.1016/j.yfrne.2015.02.001

Brancaccio, M., Patton, A. P., Chesham, J. E., Maywood, E. S., and Hastings, M. H. (2017). Astrocytes control circadian timekeeping in the suprachiasmatic nucleus via glutamatergic signaling. *Neuron* 93, 1420.e5–1435.e5. doi: 10.1016/j.neuron.2017.02.030

Cameron, S., Chen, Y., and Rao, Y. (2016). Borderless regulates glial extension and axon ensheathment. *Dev. Biol.* 414, 170–180. doi: 10.1016/j.ydbio.2016.04.020

Campbell, G., Göring, H., Lin, T., Spana, E., Andersson, S., Doe, C. Q., et al. (1994). RK2, a glial-specific homeodomain protein required for embryonic nerve cord condensation and viability in Drosophila. *Development* 120, 2957–2966.

Chen, C.-C., Wu, J.-K., Lin, H.-W., Pai, T.-P., Fu, T.-F., Wu, C.-L., et al. (2012). Visualizing long-term memory formation in two neurons of the *Drosophila* brain. *Science* 335, 678–685. doi: 10.1126/science.1212735

Chi-Castañeda, D., and Ortega, A. (2016). Clock genes in glia cells: a rhythmic history. *ASN Neuro.* 8, 1–13. doi: 10.1177/1759091416670766

Chi-Castañeda, D., and Ortega, A. (2018). Glial cells in the genesis and regulation of circadian rhythms. *Front. Physiol.* 9:88. doi: 10.3389/fphys.2018.00088

Doherty, J., Logan, M. A., Tasdemir, O. E., and Freeman, M. R. (2009). Ensheathing glia function as phagocytes in the adult *Drosophila* brain. *J. Neurosci.* 29, 4768–4781. doi: 10.1523/JNEUROSCI.5951-08.2009

Edery, I., Rutila, J. E., and Rosbash, M. (1994). Phase shifting of the circadian clock by induction of the *Drosophila* period protein. *Science* 263, 237–240. doi: 10.1126/science.8284676

Edwards, T. N., and Meinertzhagen, I. A. (2010). The functional organisation of glia in the adult brain of *Drosophila* and other insects. *Prog. Neurobiol.* 90, 471–497. doi: 10.1016/j.pneurobio.2010.01.001

Edwards, T. N., Nuschke, A. C., Nern, A., and Meinertzhagen, I. A. (2012). Organization and metamorphosis of glia in the *Drosophila* visual system. *J. Comp. Neurol.* 520, 2067–2085. doi: 10.1002/cne.23071

Ewer, J., Frisch, B., Hamblen-Coyle, M. J., Rosbash, M., and Hall, J. C. (1992). Expression of the period clock gene within different cell types in the brain of *Drosophila* adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. *J. Neurosci.* 12, 3321–3349.

Fernández, M. P., Berni, J., and Ceriani, M. F. (2008). Circadian remodeling of neuronal circuits involved in rhythmic behavior. *PLoS Biol.* 6:e69. doi: 10.1371/journal.pbio.0060069

Fischbach, K.-F., and Dittrich, A. P. M. (1989). The optic lobe of *Drosophila melanogaster*. I. A Golgi analysis of wild-type structure. *Cell Tissue Res.* 258, 441–475. doi: 10.1007/BF00218858

Fonken, L. K., Frank, M. G., Kitt, M. M., Barrientos, R. M., Watkins, L. R., and Maier, S. F. (2015). Microglia inflammatory responses are controlled by an intrinsic circadian clock. *Brain Behav. Immun.* 45, 171–179. doi: 10.1016/j.bbi.2014.11.009

Freeman, M. R., and Doherty, J. (2006). Glial cell biology in *Drosophila* and vertebrates. *Trends Neurosci.* 29, 82–90. doi: 10.1016/j.tins.2005.12.002

Górska-Andrzejak, J. (2013). Glia-related circadian plasticity in the visual system of Diptera. *Front. Physiol.* 4:36. doi: 10.3389/fphys.2013.00036

Górska-Andrzejak, J., Chwastek, E. M., Walkowicz, L., and Witek, K. (2018). On variations in the level of PER in glial clocks of *Drosophila* optic lobe and its negative regulation by PDF signaling. *Front. Physiol.* 9:230. doi: 10.3389/fphys.2018.00230

Górska-Andrzejak, J., Makuch, R., Stefan, J., Görlich, A., Semik, D., and Pyza, E. (2013). Circadian expression of the presynaptic active zone protein bruchpilot in the lamina of *Drosophila melanogaster*. *Dev. Neurobiol.* 73, 14–26. doi: 10.1002/dneu.22032

Górska-Andrzejak, J., Salvaterra, P. M., Meinertzhagen, I. A., Krzeptowski, W., Görlich, A., and Pyza, E. (2009). Cyclical expression of Na+/K+-ATPase in the visual system of *Drosophila melanogaster*. *J. Insect Physiol.* 55, 459–468. doi: 10.1016/j.jinsphys.2009.02.003

Halter, D. A., Urban, J., Rickert, C., Ner, S. S., Ito, K., Travers, A. A., et al. (1995). The homeobox gene repo is required for the differentiation and maintenance of glia function in the embryonic nervous system of *Drosophila melanogaster*. *Development* 121, 317–332.

Hamanaka, Y., and Meinertzhagen, I. A. (2010). Immunocytochemical localization of synaptic proteins to photoreceptor synapses of *Drosophila melanogaster*. *J. Comp. Neurol.* 518, 1133–1155. doi: 10.1002/cne.22268

Hardin, P. E. (2011). Molecular genetic analysis of circadian timekeeping in Drosophila. *Adv. Genet.* 74, 141–173. doi: 10.1016/B978-0-12-387690-4.00005-2

Hartline, D. K. (2011). The evolutionary origins of glia. *Glia* 59, 1215–1236. doi: 10.1002/glia.21149

Hayashi, Y., Koyanagi, S., Kusunose, N., Okada, R., Wu, Z., Tozaki-Saitoh, H., et al. (2013). The intrinsic microglial molecular clock controls synaptic strength via the circadian expression of cathepsin S. *Sci. Rep.* 3:2744. doi: 10.1038/srep02744

Helfrich-Förster, C. (1998). Robust circadian rhythmicity of *Drosophila melanogaster* requires the presence of lateral neurons: a brain-behavioral study of disconnected mutants. *J. Comp. Physiol. A* 182, 435–453. doi: 10.1007/s003590050192

Helfrich-Förster, C., Shafer, O. T., Wülbeck, C., Grieshaber, E., Rieger, D., and Taghert, P. (2007). Development and morphology of the clock-gene-expressing lateral neurons of *Drosophila melanogaster*. *J. Comp. Neurol.* 500, 47–70. doi: 10.1002/cne.21146

Herrero, A., Duhart, J. M., and Ceriani, M. F. (2017). Neuronal and glial clocks underlying structural remodeling of pacemaker neurons in Drosophila. *Front. Physiol.* 8:918. doi: 10.3389/fphys.2017.00918

Houl, J. H., Yu, W., Dudek, S. M., and Hardin, P. E. (2006). *Drosophila* CLOCK is constitutively expressed in circadian oscillator and non-oscillator cells. *J. Biol. Rhythms* 21, 93–103. doi: 10.1177/0748730405283697

Jackson, F. R. (2011). Glial cell modulation of circadian rhythms. *Glia* 59, 1341–1350. doi: 10.1002/glia.21097

Jackson, F. R., Ng, F. S., Sengupta, S., You, S., and Huang, Y. (2015). Glial cell regulation of rhythmic behavior. *Methods Enzymol.* 552, 45–73. doi: 10.1016/bs.mie.2014.10.016

Kerr, K. S., Fuentes-Medel, Y., Brewer, C., Barria, R., Ashley, J., Abruzzi, K. C., et al. (2014). Glial wingless/wnt regulates glutamate receptor clustering and synaptic physiology at the *Drosophila* neuromuscular junction. *J. Neurosci.* 34, 2910–2920. doi: 10.1523/JNEUROSCI.3714-13.2014

Kevany, B. M., and Palczewski, K. (2010). Phagocytosis of retinal rod and cone photoreceptors. *Physiology* 25, 8–15. doi: 10.1152/physiol.00038.2009

Kremer, M. C., Jung, C., Batelli, S., Rubin, G. M., and Gaul, U. (2017). The glia of the adult *Drosophila* nervous system. *Glia* 65, 606–638. doi: 10.1002/glia.23115

Landskron, J., Chen, K. F., Wolf, E., and Stanewsky, R. (2009). A role for the PERIOD:PERIOD homodimer in the *Drosophila* circadian clock. *PLoS Biol.* 7:e1000003. doi: 10.1371/journal.pbio.1000003

Long, D. M., and Giebultowicz, J. M. (2018). Age-related changes in the expression of the circadian clock protein PERIOD in *Drosophila* glial cells. *Front. Physiol.* 8:1131. doi: 10.3389/fphys.2017.01131

Marpegan, L., Swanstrom, A. E., Chung, K., Simon, T., Haydon, P. G., Khan, S. K., et al. (2011). Circadian regulation of ATP release in astrocytes. *J. Neurosci.* 31, 8342–8350. doi: 10.1523/JNEUROSCI.6537-10.2011

Matsuno, M., Horiuchi, J., Yuasa, Y., Ofusa, K., Miyashita, T., Masuda, T., et al. (2015). Long-term memory formation in *Drosophila* requires training-dependent glial transcription. *J. Neurosci.* 35, 5557–5565. doi: 10.1523/JNEUROSCI.3865-14.2015

Neves, J., Zhu, J., Sousa-Victor, P., Konjikusic, M., Riley, R., Chew, S., et al. (2016). Immune modulation by MANF promotes tissue repair and regenerative success in the retina. *Science* 353:aaf3646. doi: 10.1126/science.aaf3646

Newby, L. M., and Jackson, F. R. (1991). *Drosophila ebony* mutants have altered circadian activity rhythms but normal eclosion rhythms. *J. Neurogenet.* 7, 85–101. doi: 10.3109/01677069109066213

Ng, F. S., and Jackson, F. R. (2015). The ROP vesicle release factor is required in adult *Drosophila* glia for normal circadian behavior. *Front. Cell. Neurosci.* 9:256. doi: 10.3389/fncel.2015.00256

Ng, F. S., Sengupta, S., Huang, Y., Yu, A. M., You, S., Roberts, M. A., et al. (2016). TRAP-seq profiling and RNAi-based genetic screens identify conserved glial genes required for adult *Drosophila* behavior. *Front. Mol. Neurosci.* 9:146. doi: 10.3389/fnmol.2016.00146

Ng, F. S. S., Tangredi, M. M. M., and Jackson, F. R. R. (2011). Glial cells physiologically modulate clock neurons and circadian behavior in a calcium-dependent manner. *Curr. Biol.* 21, 625–634. doi: 10.1016/j.cub.2011.03.027

Ozkaya, O., and Rosato, E. (2012). The circadian clock of the fly: a neurogenetics journey through time. *Adv. Genet.* 77, 79–123. doi: 10.1016/B978-0-12-387687-4.00004-0

Palgi, M., Lindström, R., Peränen, J., Piepponen, T. P., Saarma, M., and Heino, T. I. (2009). Evidence that DmMANF is an invertebrate neurotrophic factor supporting dopaminergic neurons. *Proc. Natl. Acad. Sci. U.S.A.* 106, 2429–2434. doi: 10.1073/pnas.0810996106

Park, J. H., Helfrich-Förster, C., Lee, G., Liu, L., Rosbash, M., and Hall, J. C. (2000). Differential regulation of circadian pacemaker output by separate clock genes in Drosophila. *Proc. Natl. Acad. Sci. U.S.A.* 97, 3608–3613. doi: 10.1073/pnas.97.7.3608

Petrova, P., Raibekas, A., Pevsner, J., Vigo, N., Anafi, M., Moore, M. K., et al. (2003). MANF: a new mesencephalic, astrocyte-derived neurotrophic factor with selectivity for dopaminergic neurons. *J. Mol. Neurosci.* 20, 173–188. doi: 10.1385/JMN:20:2:173

Prolo, L. M., Takahashi, J. S., and Herzog, E. D. (2005). Circadian rhythm generation and entrainment in astrocytes. *J. Neurosci.* 25, 404–408. doi: 10.1523/JNEUROSCI.4133-04.2005

Pyza, E., and Górska-Andrzejak, J. (2004). Involvement of glial cells in rhythmic size changes in neurons of the Housefly's visual system. *J. Neurobiol.* 59, 205–215. doi: 10.1002/neu.10307

Pyza, E., and Meinertzhagen, I. A. (1999). Daily rhythmic changes of cell size and shape in the first optic neuropil in *Drosophila melanogaster*. *J. Neurobiol.* 40, 77–88. doi: 10.1002/(SICI)1097-4695(199907)40:1<77::AID-NEU7>3.0.CO;2-0

Richier, B., Vijandi, C., de Mackensen, S., and Salecker, I. (2017). Lapsyn controls branch extension and positioning of astrocyte-like glia in the *Drosophila* optic lobe. *Nat. Commun.* 8:317. doi: 10.1038/s41467-017-00384-z

Sakai, T., Tamura, T., Kitamoto, T., and Kidokoro, Y. (2004). A clock gene, period, plays a key role in long-term memory formation in Drosophila. *Proc. Natl. Acad. Sci. U.S.A.* 101, 16058–16063. doi: 10.1073/pnas.0401472101

Salter, M. W., and Stevens, B. (2017). Microglia emerge as central players in brain disease. *Nat. Med.* 23, 1018–1027. doi: 10.1038/nm.4397

Sheeba, V., Fogle, K. J., and Holmes, T. C. (2010). Persistence of morning anticipation behavior and high amplitude morning startle response following functional loss of small ventral lateral neurons in Drosophila. *PLoS ONE* 5:e11628. doi: 10.1371/journal.pone.0011628

Sheeba, V., Sharma, V. K., Gu, H., Chou, Y.-T., O'Dowd, D. K., and Holmes, T. C. (2008). Pigment dispersing factor-dependent and -independent circadian locomotor behavioral rhythms. *J. Neurosci.* 28, 217–227. doi: 10.1523/JNEUROSCI.4087-07.2008

Stork, T., Bernardos, R., and Freeman, M. R. (2012). Analysis of glial cell development and function in Drosophila. *Cold Spring Harb. Protoc.* 2012, 1–17. doi: 10.1101/pdb.top067587

Stratoulias, V., and Heino, T. I. (2015a). Analysis of the conserved neurotrophic factor MANF in the *Drosophila* adult brain. *Gene Expr. Patterns* 18, 8–15. doi: 10.1016/j.gep.2015.04.002

Stratoulias, V., and Heino, T. I. (2015b). MANF silencing, immunity induction or autophagy trigger an unusual cell type in metamorphosing *Drosophila* brain. *Cell. Mol. Life Sci.* 72, 1989–2004. doi: 10.1007/s00018-014-1789-7

Subramanian, A., Siefert, M., Banerjee, S., Vishal, K., Bergmann, K. A., Curts, C. C. M., et al. (2017). Remodeling of peripheral nerve ensheathment during the larval-to-adult transition in Drosophila. *Dev. Neurobiol.* 77, 1144–1160. doi: 10.1002/dneu.22502

Suh, J., and Jackson, F. R. (2007). *Drosophila* ebony activity is required in glia for the circadian regulation of locomotor activity. *Neuron* 55, 435–447. doi: 10.1016/j.neuron.2007.06.038

Takemura, S.-Y., Lu, Z., and Meinertzhagen, I. A. (2008). Synaptic circuits of the *Drosophila* optic lobe: the input terminals to the medulla. *J. Comp. Neurol.* 509, 493–513. doi: 10.1002/cne.21757

Ueda, H. R., Matsumoto, A., Kawamura, M., Iino, M., Tanimura, T., and Hashimoto, S. (2002). Genome-wide transcriptional orchestration of circadian rhythms in Drosophila. *J. Biol. Chem.* 277, 14048–14052. doi: 10.1074/jbc.C100765200

Walkowicz, L., Kijak, E., Krzeptowski, W., Górska-Andrzejak, J., Stratoulias, V., Woznicka, O., et al. (2017). Downregulation of DmMANF in glial cells results in neurodegeneration and affects sleep and lifespan in *Drosophila melanogaster*. *Front. Neurosci.* 11:610. doi: 10.3389/fnins.2017.00610

Weber, P., Kula-Eversole, E. E., and Pyza, E. E. (2009). Circadian control of dendrite morphology in the visual system of *Drosophila melanogaster*. *PLoS ONE* 4:e4290. doi: 10.1371/journal.pone.0004290

Xiong, W. C., Okano, H., Patel, N. H., Blendy, J. A., and Montell, C. (1994). repo encodes a glial-specific homeo domain protein required in the Drosophila nervous system. *Genes Dev.* 8, 981–994. doi: 10.1101/gad.8.8.981

Yuasa, Y., Okabe, M., Yoshikawa, S., Tabuchi, K., Xiong, W.-C., Hiromi, Y., et al. (2003). *Drosophila* homeodomain protein REPO controls glial differentiation by cooperating with ETS and BTB transcription factors. *Development* 130, 2419–2428. doi: 10.1242/dev.00468

Zerr, D. M., Hall, J. C., Rosbash, M., Siwicki, K. K., Hall, J., and Rosbash, M. (1990). Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of Drosophila. *J. Neurosci.* 10, 2749–2762.

Zwarts, L., Van Eijs, F., and Callaerts, P. (2015). Glia in *Drosophila* behavior. *J. Comp. Physiol. A* 201, 879–893. doi: 10.1007/s00359-014-0952-9

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 09 January 2018
doi: 10.3389/fphys.2017.01131

Age-Related Changes in the Expression of the Circadian Clock Protein PERIOD in *Drosophila* Glial Cells

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Circadian clocks consist of molecular negative feedback loops that coordinate physiological, neurological, and behavioral variables into "circa" 24-h rhythms. Rhythms in behavioral and other circadian outputs tend to weaken during aging, as evident in progressive disruptions of sleep-wake cycles in aging organisms. However, less is known about the molecular changes in the expression of clock genes and proteins that may lead to the weakening of circadian outputs. Western blot studies have demonstrated that the expression of the core clock protein PERIOD (PER) declines in the heads of aged *Drosophila melanogaster* flies. This age-related decline in PER does not occur in the central pacemaker neurons but has been demonstrated so far in retinal photoreceptors. Besides photoreceptors, clock proteins are also expressed in fly glia, which play important roles in neuronal homeostasis and are further categorized into subtypes based on morphology and function. While previous studies of mammalian glial cells have demonstrated the presence of functional clocks in astrocytes and microglia, it is not known which glial cell types in *Drosophila* express clock proteins and how their expression may change in aged individuals. Here, we conducted immunocytochemistry experiments to identify which glial subtypes express PER protein suggestive of functional circadian clocks. Glial cell subtypes that showed night-time accumulation and day-time absence in PER consistent with oscillations reported in the pacemaker neurons were selected to compare the level of PER protein between young and old flies. Our data demonstrate that some glial subtypes show rhythmic PER expression and the relative PER levels become dampened with advanced age. Identification of glial cell types that display age-related dampening of PER levels may help to understand the cellular changes that contribute to the loss of homeostasis in the aging brain.

Keywords: glia, circadian biology, aging, *Drosophila*, glial clocks

OPEN ACCESS

Edited by:
Jolanta Górska-Andrzejak, Jagiellonian University, Poland

Reviewed by:
Colleen J. Doherty, North Carolina State University, United States
Gustavo Bueno Rivas, University of Florida, United States
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Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: *30 September 2017*
Accepted: *21 December 2017*
Published: *09 January 2018*

Citation:
Long DM and Giebultowicz JM (2018) Age-Related Changes in the Expression of the Circadian Clock Protein PERIOD in Drosophila Glial Cells. Front. Physiol. 8:1131. doi: 10.3389/fphys.2017.01131

INTRODUCTION

Many molecular, cellular, and physiological processes in most organisms are coordinated with the predictable changes of the 24-h solar day. The circadian clock provides the mechanism of time keeping that is based on a negative feedback loop of transcriptional activators and repressors that generate endogenous molecular oscillations of circa 24 h (Hardin, 2011). A core repressor in the clock mechanism is encoded by the gene *period* (*per*) and the translocation of the PER

protein into the cell nuclei followed by its degradation is the fundamental feature of clock function (Hardin, 2011). In both mammals and *Drosophila*, the circadian system consists of central and peripheral clocks (Hardin and Panda, 2013). Central pacemaker neurons in mammals are located in the suprachiasmatic nuclei (SCN). The central pacemaker neurons driving behavioral rest/activity rhythms consist of a network of about 150 neurons in the *Drosophila* brain. In addition to these central pacemaker neurons, mammals have intrinsic peripheral clocks in cells of fat tissues, kidneys, liver, and most other organs. Many of these peripheral tissues that express autonomous oscillators coordinate local tissue-specific processes. Similarly to mammals, peripheral clocks are widespread in fly tissues and function independently of the central pacemaker coordinating tissue-specific physiological processes (Giebultowicz, 2001). Within the nervous system, peripheral clocks are present in retinal photoreceptor cells and in other sensory neurons. In addition to neurons, some glial cells rhythmically express circadian clock genes in both mammals (Prolo et al., 2005; Marpegan et al., 2011; Hayashi et al., 2013) and in *Drosophila* (Ng et al., 2011). Early studies showed that PER protein is expressed in non-neuronal cells (Zerr et al., 1990; Ewer et al., 1992) and suggested that PER expression in these presumed glia is sufficient for manifestation of behavioral rhythmicity (Ewer et al., 1992). However, which glial cell subtypes express the PER-based oscillator and what their roles may be in the timekeeping processes remain poorly understood.

As organisms age, circadian rhythms tend to dampen as demonstrated in behavioral and molecular experiments both in mammals (Reddy and O'Neill, 2010) and *Drosophila* (Giebultowicz and Long, 2015). This phenomenon is implicated in declining cellular homeostasis and in various pathologies of aging, including altered inflammatory responses (Fonken et al., 2016), neurodegenerative diseases (Musiek et al., 2013) and impaired memory formation (Kondratova et al., 2010). In addition, physiological aging and late life diseases are accelerated by chronic disruption of clock functions in mammals (Kondratov et al., 2006; Antoch et al., 2008; Yu and Weaver, 2011; Hastings and Goedert, 2013). Similar to mammals, mutations in core clock genes accelerate aging phenotypes in *Drosophila* (Krishnan et al., 2009). Disruptions of the circadian clock in flies predispose them to neurodegeneration, although it is not known which clocks are involved (Krishnan et al., 2009, 2012). It was shown that *Drosophila per*^{01} mutants have increased levels of oxidative damage and neurodegeneration compared to age-matched controls (Krishnan et al., 2009). However, it is not known to what extent *per* mRNA is expressed in the glia, and consequently, whether loss of *per* in these cells could contribute to neurodegeneration and aging in general.

Glial cells play important roles in such processes as neuronal guidance during development, neuronal homeostasis, clearance of damaged tissues, and neurotransmitter recycling (Freeman and Doherty, 2006; Edwards and Meinertzhagen, 2010; Stork et al., 2012). Recent studies implicate mammalian astrocytes in neuroprotection via involvement in toxin clearance from the brain during sleep (Xie et al., 2013) and removal of damaged mitochondria from neurons in the process of transmitophagy (Davis et al., 2014). Glial cells were first classified based upon their location within the brain as surface, cortex, and neuropil glia. Recent classifications in mammals include astrocytes, microglia, oligodendrocytes, and Schwann cells. The *Drosophila* adult central nervous system (CNS) has five glial subtypes divided into three main categories, namely surface, cortex or neuropil glia.

Surface glia consist of two distinct subtypes, the perineurial and subperineurial glia. Perineurial glia are narrow, oblong cells that make up the outermost covering of the adult CNS (Awasaki et al., 2008). During development, these cells increase their cell division to maintain complete coverage of the adult *Drosophila* nervous system (Avet-Rochex et al., 2012). The function of this glial cell subtype in the adult fly brain remains largely unknown (Edwards and Meinertzhagen, 2010), but a recent study suggests that perineurial glia may be important in the transport of trehalose (the main energy supplying carbohydrate in insects) across the blood-brain barrier (Volkenhoff et al., 2015). Subperineurial glia are large, flat polyploidal cells (Unhavaithaya and Orr-Weaver, 2012) that reside just underneath the perineurial glia layer. Unlike perineurial glia, subperineurial glia undergo endoreplication during larval development to increase their cell size to maintain coverage of the brain through metamorphosis (Unhavaithaya and Orr-Weaver, 2012). These cells contain several tight septate junctions, form the blood-brain barrier of *Drosophila*, and separate the CNS from pathogens, xenobiotics, and the high electrolyte content of the hemolymph ultimately protecting neuronal function (Limmer et al., 2014; Weiler et al., 2017). Consistent with these functions, the transcriptome of surface glia is enriched for gene categories associated with drug metabolism, cell adhesion, and various transporters (DeSalvo et al., 2014).

Cortex glia make contact with the subperineurial glia through adherens junctions and envelope neuronal cell bodies that reside in the cortex providing metabolic support to them (Edwards and Meinertzhagen, 2010). One cortex glial cell can cover many neuronal bodies, which gives these cells a mesh-like appearance (Awasaki et al., 2008).

Located below the cortex are two types of neuropil glia, astrocytes and ensheathing glia. Ensheathing glia have a fibrous lamellar morphology (Awasaki et al., 2008) and act as phagocytes of the brain, similar to mammalian microglia. These glia respond to axonal injury through the *Draper* receptor signaling pathway (Doherty et al., 2009). Astrocyte glial cell bodies are located at the cortex/neuropil border and have projections that are closely associated with neuronal synapses and contain multiple neurotransmitter recycling pathways (Stork et al., 2012). A recent study of the transcriptome of fly astrocytes showed enriched expression of genes involved in metabolism, redox reactions, neurotransmitter synthesis and transport (Ng et al., 2016). RNAi-mediated knockdown of some of these genes revealed alterations in behavior including changes in activity level, activity onset, and mechanical stress induced paralysis (Ng et al., 2016).

It has been established that some glial subtypes express circadian clock genes in a rhythmic manner. In mammals, both astrocytes (Prolo et al., 2005; Marpegan et al., 2011) and microglia (Hayashi et al., 2013; Fonken et al., 2015) rhythmically express Per1 and Per2 proteins. Cultured astrocytes from Per1::luciferase

transgenic rats and knock-in mice are capable of maintaining modest rhythms in circadian clock gene expression that can be entrained by physiologically relevant temperature changes (Prolo et al., 2005). Rhythmic expression of several clock genes was also shown in cortical microglia by qRT-PCR (Hayashi et al., 2013; Fonken et al., 2015). Expression of the circadian clock gene *per* in glia have been also suggested in flies (Ewer et al., 1992) and this was confirmed more recently although, it is not clear which glial subtypes express PER protein (Ng et al., 2011).

Although impairments of the circadian system are believed to be involved in accelerated aging, little is known about how the circadian clock in different tissues is altered across the lifespan. In *Drosophila*, PER expression remains robust in central pacemaker neurons (Luo et al., 2012) but is significantly reduced in retinal photoreceptors (Luo et al., 2012; Rakshit et al., 2012). While glia have many important roles in maintaining nervous system homeostasis, it is not known which glial subtypes express the core clock protein PER and whether PER levels remain similar across lifespan or decline with age. To address these questions, we took advantage of the fact that glial subtypes of *Drosophila* have unique expression patterns and can be labeled separately by GFP via cell-type specific drivers. We performed 2-timepoint immunocytochemical experiments to identify *Drosophila* glia subtypes that express PER protein and determined that the PER level in these cells is reduced in old fly brains compared to young.

METHODS

Fly Rearing and Genetics

Drosophila melanogaster were maintained on diet containing yeast (35 g/l), cornmeal (50 g/l), and molasses (5%). Temperature was maintained at 25 ± 1°C with a 12:12 h light/dark cycle with fluorescent light of luminous energy of 8 ± 2 μmol $m^{-2}s^{-1}$. We used mated males in all experiments to minimize differences in lifespan, which may vary with sex and mating status. Males were aged in groups of 50 in polypropylene cages (Genesee Scientific, San Diego, CA) inverted over 35 mm petri dish (BD Falcon, San Jose, CA) containing 15 mL of diet. Diet dishes were replaced every 2–3 days. Young (day 5) and old (day 55) males expressing nuclear GFP in specific glial cell subtypes were obtained by crossing *w;*UAS-GFP with nuclear localization signal (Bloomington Drosophila Stock Center stock 4775) males with females carrying GAL4 drivers expressing in the following glia types: perineurial glia, *NP6293*-GAL4 (Awasaki et al., 2008); subperineurial glia *moody*-GAL4 (Schwabe et al., 2005); cortex glia *NP577*-GAL4 and *NP2222*-GAL4; ensheathing glia *NP6520*-GAL4 (Awasaki et al., 2008) and *mz0709*-GAL4 (Ito et al., 1995); astrocytes *alrm*-GAL4 (Doherty et al., 2009). UAS-GFP with the nuclear localization signal was chosen to clearly discern nuclear overlap between GFP and PER protein; however, some GFP was also visible in the cytoplasm of glial cells.

Immunocytochemistry (ICC)

Flies for brain dissection were collected at Zeitgeber time (ZT) 22 and ZT10 which correspond to high and low levels of PER protein in wild-type flies, respectively (Long et al., 2014). Whole brain mounts were made using established protocol (Long et al., 2014). Brains were incubated for 48 h in primary antibodies 1:500 chicken monoclonal anti-GFP (Aves Laboratories) and 1:10,000 pre-absorbed rabbit anti-PER, rinsed 6 times in phosphate buffered saline with 0.5% Triton-X (Fisher Scientific, Pittsburgh, PA) and incubated overnight in secondary antibodies Alexa Fluor 488 anti-chicken (1:500) and Alexa Fluor 555 anti-rabbit (1:1,000) (Life Technologies). After the final rinse, brains were mounted on microscope slides in Vectashield mounting media with DAPI (Vector Laboratories, Burlingame, California). Images were taken with a Zeiss LSM 780 NLO scanning confocal microscope (Zeiss) with all laser parameters set for optimal signal in young fly brains at ZT22 for each genotype and then held constant while imaging young ZT10 as well as old ZT22 and ZT10 flies of the same genotype. Young and old per^{01} mutant flies were dissected and stained along with each genotype using the same protocol. Lack of PER staining signal in per^{01} mutants was used as a negative control.

Image Analysis and PER Quantification

In order to quantify the relative fluorescence of PER signal at ZT10 and ZT22 in each glia subtype, images were reviewed and maximum intensity projections were created using ZEN 2012 software (Zeiss). Due to their location on the outer surface of the adult *Drosophila* brain, in order to capture a sufficient number of surface glial cells for measurement, multiple images of non-overlapping 6 μm stacks were captured in several regions of each brain. The area of focus for perineurial glia was the dorsal brain while the surface of the optic lobes was used for subperineurial glial cells. For all other glial subtypes, a single 11 μm thick stack from each brain was used for PER signal quantification. PER levels were evaluated by measuring the fluorescence intensity in an average of 15 GFP-positive cell nuclei located in the region of interest specified below. After converting the mean level of fluorescence to the Mean Gray Value the intensity was quantified using Fiji ImageJ software (Schindelin et al., 2012). For each stack, measurements of non-specific background fluorescence were taken from the adjacent areas of similar size as glial cell nuclei (avoiding non-specific red speckles). The background values were averaged and subtracted from the averaged PER measurements obtained from that stack. Five to seven brains were used to measure PER at given time point and age. Statistical significance for average intensity of PER staining between young and old brains at ZT22 was calculated by unpaired *t*-test with Welch's correction using GraphPad Prism 6 (GraphPad Prism v6.0;GraphPad Software Inc. San Diego, CA). The *p*- and *t*-values and the degrees of freedom (df) from these measurements are provided in the results section and in the figure captions.

RESULTS

We investigated which glia subtypes express the circadian clock protein PER in a manner similar to that of the pacemaker neurons and whether the relative amounts of PER signal change with age. To label specific glial cells, we employed the GAL4/UAS system (Brand and Perriman, 1993) using GAL4 lines to drive GFP expression in subtypes of glia with specific location and function in combination with immunocytochemistry to measure

PER levels. It has been reported that PER expression in lateral and dorsal pacemaker neurons are equally strong in young and old *Drosophila* brains (Luo et al., 2012); therefore, the presence of PER staining in these neurons was used as a positive control. These cells have rhythmic PER expression with high levels at ZT22 and lack PER at ZT10 in wild type flies (Long et al., 2014); therefore, we selected these time points to examine glial cells.

PER Is Expressed in Both Layers of the Surface Glia

Surface glia consist of two distinct glial subtypes namely perineurial and subperineurial glia. Perineurial glial cells were labeled by crossing UAS-GFP to *NP6293*-GAL4 driver line, which specifically marks this layer of glia (Awasaki et al., 2008). All GFP-positive nuclei of cells on the dorsal surface of the brain showed PER signal at ZT22 but not at ZT10, suggesting rhythmic expression in the perineurial glia in young brains (**Figures 1A,B**). PER expression persisted in the brains of old flies but the average signal at ZT22 was significantly reduced (**Figure 1C**, p-value < 0.0001, t-value $= 8.928$, df $= 9.171$). Subperineurial glial cells were visualized via *moody*-GAL4 driver line (Schwabe et al., 2005) combined with UAS-GFP. We observed that GFP-labeled cells surrounded the entire brain in both young and old flies. PER signal was quantified in GFP positive subperineurial glia surrounding the optic lobe (**Figures 2A,B**). Although some GFP leaked and was observed in the cytoplasm of the subperineurial glia predominant signal came from their large nuclei. Subperineurial glia showed nuclear PER signal at ZT22 (**Figure 2A**, arrowheads) but not at ZT10 in brains of young flies suggesting rhythmic expression of this protein. PER was discernible from the background in old brains at ZT22 but the average PER signal was significantly reduced compared to young flies (**Figure 2C**, p-value ≤ 0.0031, t-value $= 3.719$, df $=11.56$).

PER Is Weakly Expressed in Cortex Glia at ZT22

Cortex glia surround neuronal cell bodies that reside underneath the surface glia. To visualize cortex glia cells, we used two drivers, *NP2222*-GAL4 or *NP577*-GAL4 (Awasaki et al., 2008) combined with UAS-GFP. GFP-positive cells were abundant in both young and old flies having a mesh-like appearance as described previously (Awasaki et al., 2008). Given their large population and prolific distribution in the cortex, we focused on a small subset of GFP-positive cortex glia in the vicinity of the dorsal lateral pacemaker neurons (**Figures 3A,B**). PER signal was detected in both cortex glia lines at ZT22 but not at ZT10 similar to the circadian expression of PER in the lateral neurons (**Figure 3A**). The relative level of PER signal in cortex glia in brains of *NP2222*-GAL4>GFP flies was much lower than in the lateral neurons located nearby, but was present at ZT22 and not at ZT10 (**Figure 3A**). Analysis of PER signal in cortex glia in this region showed that the average PER levels were significantly reduced in old fly brains compared to young (**Figure 3C**, p-value ≤ 0.0099, t-value $= 3.784$, df $= 5.743$).

Variable PER Expression in the Neuropil Glia

Neuropil glia consists of two morphologically distinct subtypes, the ensheathing glia and astrocytes. Ensheathing glia were visualized via *mz0709*-GAL4 (Ito et al., 1995) or *NP6520*-GAL4 (Awasaki et al., 2008) drivers combined with UAS-*GFP*. PER was detected in GFP-positive cells in both lines; however, *mz0709*-GAL4 has been reported to drive expression also in the subperineurial glia (Dutta et al., 2016); therefore, *NP6520*-GAL4>GFP flies were used for PER signal measurement. GFP-positive cells were observed at the border between cortex and several neuropil compartments in the central brain (**Figures 4A,B**). At ZT10, these ensheathing glial cells were negative for PER signal in both young and old brains (not shown); however, many of these cells were PER positive at ZT22 in both ages (**Figure 4A**). The intensity of PER signal was variable from brain to brain and cell to cell and there was no significant difference in average PER signal between young and old brains (**Figure 4C**, p-value $= 0.8452$, t-value $= 0.2017$, df $=7.961$).

Another group of glial cells marked with GFP via *NP6520*-GAL4 driver was observed in the medulla segment of the optic lobe (**Figures 5A,B**). Based on their position and large oblong nuclei, these cells appear to represent the giant glial cells of the medulla (Tix et al., 1997). Cell nuclei were PER negative at ZT10, but PER was detected at ZT22 albeit with somewhat variable intensity from cell to cell (**Figure 5A**). Nevertheless, the average intensity of PER signal was significantly reduced in these cells in the brains of old files compared to young (**Figure 5C**, p-value ≤ 0.0100, t-value $= 3.056$, df $= 12$) indicating that PER levels in these glial cells are reduced as the function of age similar to other glia types discussed above.

A second group of prominent neuropil glia are astrocytes which were visualized via *alrm*-GAL4 driver (Doherty et al., 2009) combined with UAS-GFP. Interestingly, it appears that *alrm*-GAL4 marked as GFP-positive the same giant glial cells in the medulla that were also labeled via ensheathing glia *NP6520*-GAL4 driver. PER was again detected in these cells at ZT22 (**Figures 6A,B**) with the average signal lower in old flies (not shown). This suggests that these cells share features of both ensheathing and astrocyte glial cells.

In mammals, astrocytes located among the central clock neurons in the SCN show robust oscillations in Per-reporter. Therefore, we investigated whether astrocytes located in the central brain neuropil are PER-positive in flies. These cells were marked with GFP via *alrm*-GAL4 driver and due to the leakiness of nuclear GFP also show some projections that extended into the neuropil (**Figure 6C**). GFP-positive astrocytes were examined in several areas of the central brain but PER protein staining was not detected in any of these cells at either ZT22 or ZT10 while nearby ventral lateral pacemaker neurons were PER-positive at ZT22 as expected (**Figures 6B,C**).

DISCUSSION

In this study, we show that most glial cell subtypes of the adult *Drosophila* CNS express PER in a manner suggesting

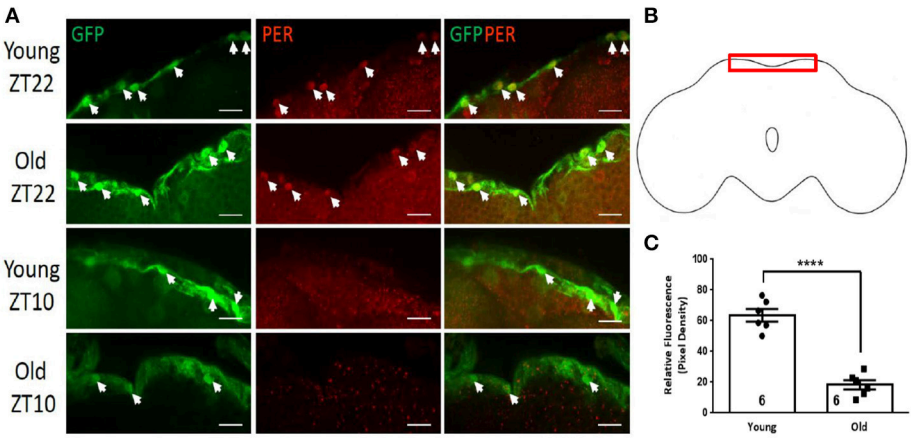

FIGURE 1 | PER expression in perineurial glia of the dorsal brain. GFP-positive perineurial glia covering brain surface visualized in *NP6293*-GAL4>GFP flies. **(A)** Representative brains showing GFP, PER, and combined labeling of the cell nuclei (arrowheads) in young (5 days) and old (55 days) brains at ZT22. At ZT10, PER is absent in perineurial glia from both young old brains. Scale bars equal 10 μm. **(B)** Brain outline indicating location of cells shown in **(A)** in the dorsal surface region. **(C)** Graph showing the average relative fluorescence of PER in perineurial glia. PER levels at ZT22 were significantly lower in old brains (****$p \leq 0.0001$, $t = 8.928$, df $= 9.171$). Number of brains analyzed are shown within each bar; error bars indicate standard error of the mean (SEM).

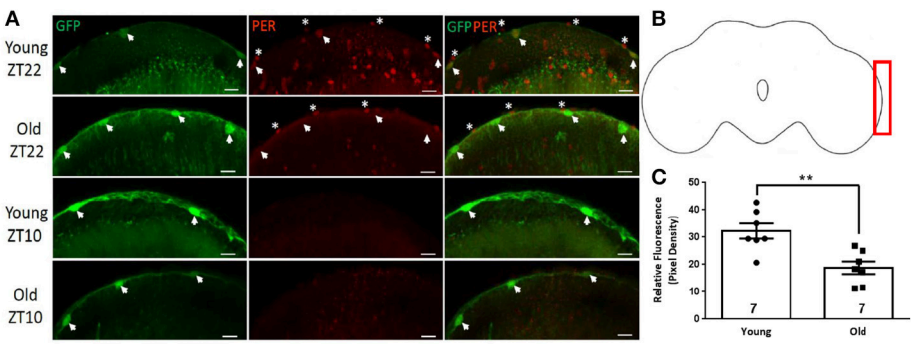

FIGURE 2 | Subperineurial glial cells express PER. Close up of the optic lobe surface in brains of *moody*-GAL4>GFP flies. **(A)** Large GFP-positive nuclei of subperineurial glia (arrowheads) are PER-positive in the brains of young (5 day) flies at ZT22, but PER protein is almost absent in glia of old brains at ZT22, although PER is detected in the outer layer of perineurial glia not labeled by GFP (asterisks). Other PER-positive cells in young brains at ZT22 not marked by GFP are likely different types of glia. Subperineurial glia in young and old brains are PER-negative at ZT10. Scale bars equal 10 μm. **(B)** Brain outline indicating location of cells shown in **(A)** at the surface of the optic lobe. **(C)** Graph showing the average relative fluorescence of PER in subperineurial glia at ZT22. PER level is significantly lower in old brains (**$p = 0.0031$, $t = 3.719$, df $= 11.56$). Number of brains analyzed are shown within each bar; error bars indicate SEM.

that circadian clock may function in these cells. These glial subtypes include perineurial glia, subperineurial glia, cortex glia, ensheathing glia of the central brain and the giant glia located in the medulla. While rhythmic PER expression in the medulla was reported previously (Suh and Jackson, 2007; Gorska-Andrzejak et al., 2009), our data suggest clock function in several other types of glial cells. However, the astrocyte glia appear to be an exception as we did not detect PER protein in these cells at either time point examined.

Our finding that perineurial and subperineurial glia express PER protein is consistent with a recent study of the surface glia transcriptome of adult *Drosophila* compared to the transcriptomes of all neurons, all glia, and to total brain lysates (DeSalvo et al., 2014). While not the focus of the study, their data do list *per* mRNA and other circadian clock genes as expressed in the surface glia (DeSalvo et al., 2014). Moreover, the core clock gene *Clk* was identified as one of the top 50 genes enriched in surface glia when compared to all glia (DeSalvo et al., 2014). The perineurial and subperineurial glia have distinct non-overlapping roles in the formation and maintenance of the blood-brain barrier that are not well understood (Awasaki et al., 2008; DeSalvo et al., 2014); the presence of the circadian clock in these cells may help to understand their functioning in the future.

Cortex glia constitute about 20% of the glia in the adult *Drosophila* brain (Kremer et al., 2017), but this subtype is relatively understudied in flies. Based on our results, cortex glia express PER protein similarly to the pacemaker neurons albeit at a much lower level even in young flies. However, we cannot exclude that cortex glia in other brain regions could show higher PER levels. Cortex glia are presumed to provide trophic

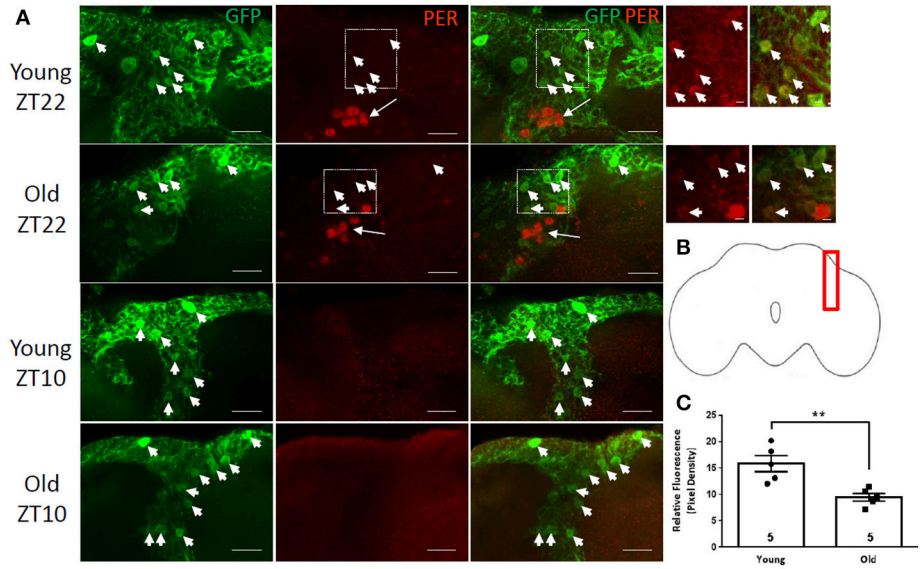

FIGURE 3 | PER is weakly express in GFP-positive cortex glia. Images of the region containing PER-positive dorsal lateral neurons (arrows) and GFP-positive cortex glia (arrowheads) in brains of *NP2222*-GAL4>GFP flies. **(A)** In comparison to neurons, very weak PER staining is observed in GFP-positive cortex glia at ZT22 in young flies and is further reduced in old flies. No PER is detected in young or old brains at ZT10. Scale bars equal 10 μm. Right: enlarged images of the outlined regions show weak but discernible PER signal in cortex glia in young and old brains at ZT22. Scale bars equal 2 μm. **(B)** Brain outline indicating location of cells shown in **(A)** in the lateral region of the brain. **(C)** Graph showing the average relative PER fluorescence in cortex glia at ZT22. PER level is significantly lower but still detectable in old brains (**$p = 0.0099$, $t = 3.784$, df $= 5.743$). Number of brains analyzed are shown within each bar; error bars indicate SEM.

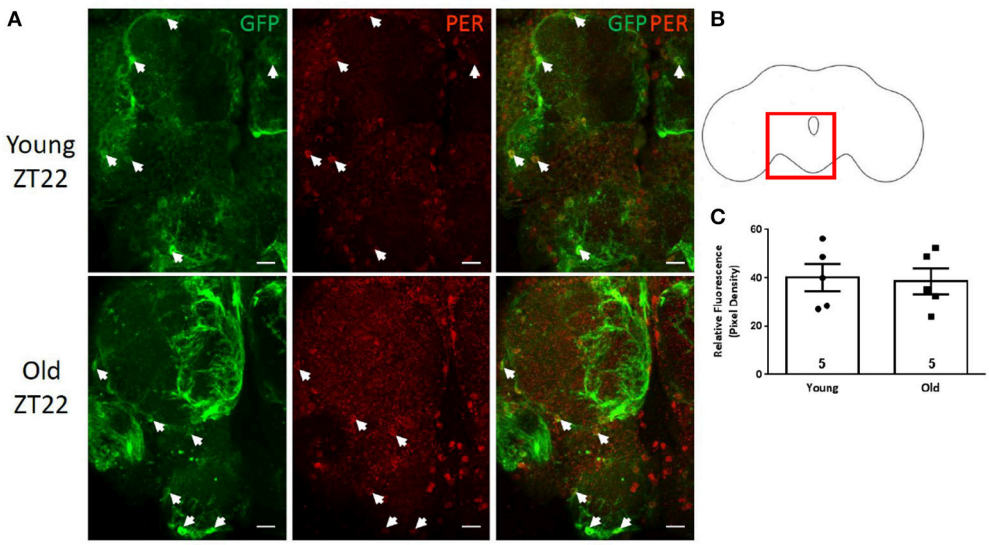

FIGURE 4 | PER is variably expressed in the neuropil ensheathing glia. Ensheathing glia are labeled with GFP (arrowheads) in *NP6520*-GAL4>GFP brains. **(A)** PER signal is detected in a subset of GFP-positive cells at ZT22 in the central brain of 5 and 55 day old flies. PER levels vary from cell to cell. No PER was detected in young or old brains at ZT10 (data not shown). Scale bars equal 10 μm. **(B)** Brain outline indicating location of cells shown in **(A)** in the region of the ventral central brain. **(C)** Graph showing the average relative PER fluorescence in ensheathing glia. PER levels are not significantly different between young and old flies ($p = 0.8452$, $t = 0.2017$, df $= 7.961$). Number of brains analyzed are shown within each bar; error bars indicate SEM.

support to the neuronal cell bodies they envelop (Edwards and Meinertzhagen, 2010). A recent study supports this idea by demonstrating that genes involved with β-oxidation are expressed in cortex glia suggesting that these cells may generate and transport ketone bodies (Schulz et al., 2015). Cortex glia are known to produce Ca^{+2} oscillations and disruptions of

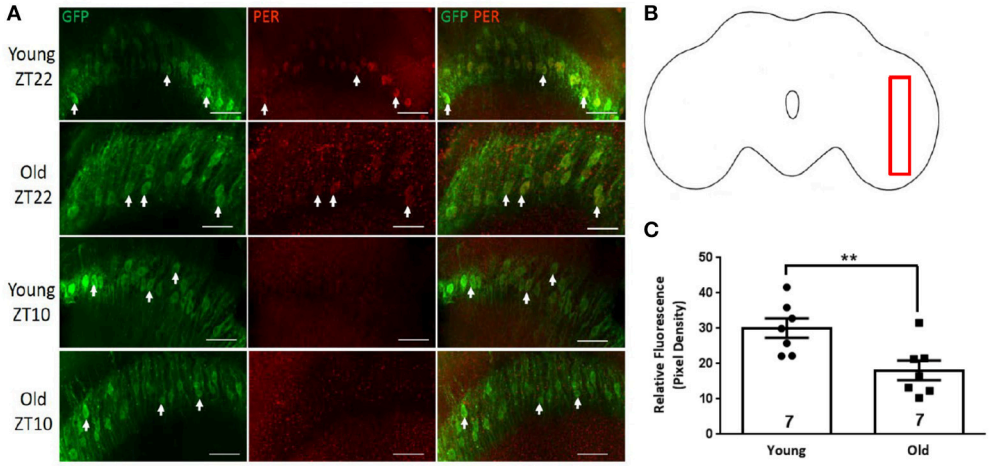

FIGURE 5 | PER expression in glia of the medulla. A prominent group of GFP-positive giant glial cells in the medulla of *NP6520*-GAL4>GFP brains. **(A)** PER protein was detected in young brains and at a lower level in old brains at ZT22 but was absent in both at ZT10. Scale bars equal 20 μm. **(B)** Brain outline indicating location of cells shown in **(A)** in the region of the medulla. **(C)** Graph showing average relative PER fluorescence of in giant glia at ZT22. PER level is significantly lower in old brains ($^{**}p = 0.0100$, $t = 3.056$, df = 12). Number of brains analyzed are shown within each bar; error bars indicate SEM. Arrowheads indicate GFP labeled glial cell nuclei.

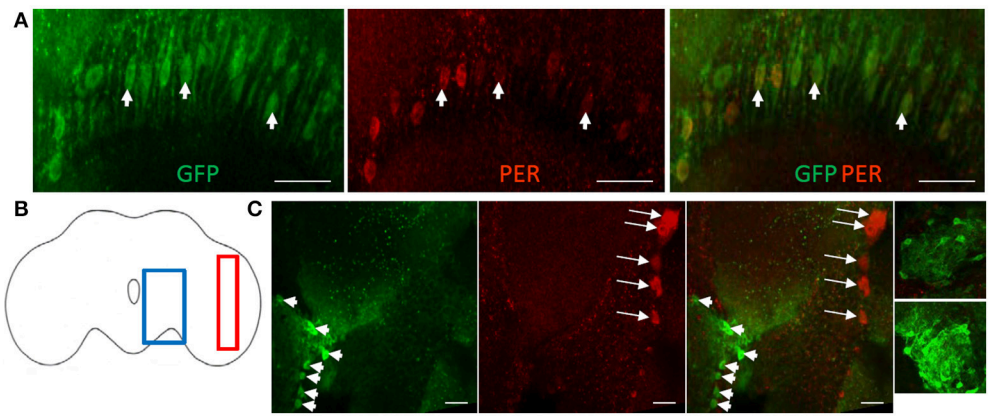

FIGURE 6 | Astrocyte-like cells labeled with GFP via *alrm*-GAL4 driver. **(A)** This driver appears to be active in the same PER-positive giant glial cells of the medulla that were marked with ensheathing glia driver (see **Figure 5**). Scale bars equal 20 μm. **(B)** Brain outline indicating approximate location of cells shown in **(A)** in red and cells shown in **(C)** in blue. **(C)** Astrocytes labeled with GFP via *alrm*-GAL4 and located in the central brain (arrowheads) are PER-negative while ventral lateral neurons located nearby (arrows) show PER signal at ZT22. Scale bars equal 10 μm. Two small images on the right show additional PER-negative astrocytes in the central brain.

these oscillations by mutations in the glial-specific Na^+/Ca^{2+}, K^+ exchanger encoded by *zydeco* significantly decrease seizure threshold in flies (Melom and Littleton, 2013). While cortex glia are important for neuronal health and function, the role of potentially low amplitude PER oscillations in these cells has yet to be addressed.

Ensheathing glial cells are closely associated with neuronal arborizations and synaptic regions. We determined that these cells express PER in the central brain in *Drosophila*. It has been reported that the equivalent mammalian cells, the microglia express Per1 and Per2 in a circadian manner (Hayashi et al., 2013; Fonken et al., 2015). The ensheathing glia of *Drosophila* uniquely express key components of the glial phagocytic machinery such as the engulfment receptor *Draper* (Doherty et al., 2009). Interestingly, a recent study comparing circadian transcriptome in heads of young and old flies indicated that *drpr* mRNA show a rhythmic profile in young flies but the rhythm is dampened in old (Kuintzle et al., 2017).

We determined that the astrocyte glia in the central brain in the vicinity of the pacemaker neurons do not express PER. These results are consistent with a previous report that the astrocytes of the central brain are PER/TIM negative (Suh and Jackson, 2007). The absence of PER in fly astrocytes is somewhat unexpected given that mammalian astrocyte cultures from Per1::luciferase transgenic rats and knock-in mice are capable of maintaining modest rhythms in circadian clock genes expression (Prolo et al., 2005; Marpegan et al., 2011). In fact, several recent studies have demonstrated that mammalian astrocytes

are important for controlling circadian timekeeping. Astrocyte-specific loss of core clock gene BMAL1 (the mammalian ortholog of *cycle*) via two independent methods was shown to alter circadian locomotor activity whereas expression of clock-associated kinase CK1ε in astrocytes was sufficient to lengthen the period of PER oscillations (Tso et al., 2017). Other recent studies also demonstrated astrocytes roles in circadian timekeeping through glia-neuron communication involving different signaling molecules (Barca-Mayo et al., 2017; Brancaccio et al., 2017). Consistent with the lack of PER in the fly central brain astrocytes, a study of the astrocyte transcriptome did not identify any of the core circadian clock genes (Ng et al., 2016). While astrocytes in both flies and mammals associate with neuronal synapses and have similar star-shaped morphologies and molecular markers, it is conceivable that circadian clock function could have been acquired later in evolution aided by the substantial proliferation of glia in mammals (Kremer et al., 2017).

Little is known about clock-controlled output processes in *Drosophila* glia. In the lamina of the fly visual system, glial cells show rhythmic changes in volume coordinated with the volume changes in the photoreceptor-contacting interneurons (Gorska-Andrzejak, 2013). These rhythmic changes in structure coincide with the rhythmic expression of the α-subunit of the sodium pump, Na^+/K^+-ATPase, which is in high abundance in glia and its rhythmic expression is *per*-dependent, as per^{01} mutants lack rhythmic expression of this subunit (Gorska-Andrzejak et al., 2009). A recent study implicates glial cell oscillators in the control of *Gclc*, a rhythmically expressed component of the rate-limiting enzyme in glutathione synthesis (Chow et al., 2016). This study reported that pan glial knockdown of the circadian clock gene *cycle* via *loco*-GAL4>*cycRNAi* was sufficient to significantly decrease rhythmicity of *Gclc* expression (Chow et al., 2016).

The ubiquitous and rhythmic expression of PER in glia reported here opens more questions regarding the functional significance of glial clocks. The role of glia in locomotor activity rhythms is not clear. Pan-glial knockdown of the circadian clock genes *per* or *cryptochrome* via RNAi failed to alter the free-running behavior of adult *Drosophila* (Ng et al., 2011). Yet, other studies have shown that *Drosophila* glial cell functions are required for behavioral rhythmicity (Jackson, 2011; Ng et al., 2011, 2016; Jackson et al., 2015). Finally, pan-glia knockdown of several astrocyte enriched genes can cause significant changes in activity level, sensitivity to mechanical stress, and/or alterations in circadian locomotor activity (Ng et al., 2016).

One of the important findings of our study is that, with the exception of the central brain neuropil glia, all PER expressing glial subtypes display significant age-related decline in PER protein levels. These data are consistent with the age-related decrease in PER protein reported by Western blot in whole heads (Luo et al., 2012; Rakshit et al., 2012; Kuintzle et al., 2017). Previous immunofluorescent studies detected PER decline in the retinal photoreceptors of old flies (Luo et al., 2012; Rakshit et al., 2012). Our data now show that similar decline occurs in the majority of glial cells. Although the reasons for age-related decrease in PER protein shown here are not known, it could be related to reported reduction of TIM protein in the heads of old flies (Luo et al., 2012; Rakshit et al., 2012). TIM protein is known to be required for PER stability (Hardin, 2011). It is also well known that PER is an essential repressor of CLK/CYC-activated circadian transcription of target genes (Hardin, 2011); therefore, our data suggest that the repressive arm of the circadian clock weakens in glia during aging due to decline of nuclear localized PER protein. Consistent with this hypothesis, a recent RNA-seq study showed that *per* mRNA expression is higher in the heads of old flies compared to young while protein levels are decreased in old (Kuintzle et al., 2017).

Our detailed analysis of PER expression suggests that circadian clock may function in several glial subtypes. These data should facilitate future functional analysis of glial circadian clocks and their roles in homeostasis of the nervous system. The age-related decline in PER protein expression in various glial subtypes may provide new ways of investigating the physiological processes that decline with age.

AUTHOR CONTRIBUTIONS

This study was conceived, analyzed, and written by DL and JG. All experiments were performed by DL.

ACKNOWLEDGMENTS

This work was supported by the National Institute of Aging of NIH under award number R01AG045830 and R21AG052950 to JG. The authors also wish to acknowledge the Confocal Microscopy Facility of the Center for Genome Research and Biocomputing at Oregon State University. This publication was made possible in part by award number 1337774 from the National Science Foundation, MRI: Acquisition of Confocal and Two-Photon Excitation Microscope. Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study. We would like to thank Dr. R Jackson for all NP-GAL4 lines, *mz0709*-GAL4, and *alrm*-GAL4, Dr. R. Stanewsky for rabbit anti-PER primary antibody, and Eileen Chow for reading the manuscript.

REFERENCES

Antoch, M. P., Gorbacheva, V. Y., Vykhovanets, O., Toshkov, I. A., Kondratov, R. V., Kondratova, A. A., et al. (2008). Disruption of the circadian clock due to the clock mutation has discrete effects on aging and carcinogenesis. *Cell Cycle* 7, 1197–1204. doi: 10.4161/cc.7.9.5886

Avet-Rochex, A., Kaul, A. K., Gatt, A. P., Mcneill, H., and Bateman, J. M. (2012). Concerted control of gliogenesis by InR/TOR and FGF signalling in the Drosophila post-embryonic brain. *Development* 139, 2763–2772. doi: 10.1242/dev.074179

Awasaki, T., Lai, S. L., Ito, K., and Lee, T. (2008). Organization and postembryonic development of glial cells in the adult central brain of Drosophila. *J. Neurosci.* 28, 13742–13753. doi: 10.1523/JNEUROSCI.4844-08.2008

Barca-Mayo, O., Pons-Espinal, M., Follert, P., Armirotti, A., Berdondini, L., and De Pietri Tonelli, D. (2017). Astrocyte deletion of Bmal1 alters daily locomotor

activity and cognitive functions via GABA signalling. *Nat. Commun.* 8:14336. doi: 10.1038/ncomms14336

Brancaccio, M., Patton, A. P., Chesham, J. E., Maywood, E. S., and Hastings, M. H. (2017). Astrocytes control circadian timekeeping in the suprachiasmatic nucleus via glutamatergic signaling. *Neuron* 93, 1420–1435.e5. doi: 10.1016/j.neuron.2017.02.030

Brand, A. H., and Perriman, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118, 401–415.

Chow, E. S., Long, D. M., and Giebultowicz, J. M. (2016). Circadian rhythm in mRNA expression of the glutathione synthesis gene Gclc is controlled by peripheral glial clocks in *Drosophila melanogaster*. *Physiol. Entomol.* 41, 369–377. doi: 10.1111/phen.12164

Davis, C. H., Kim, K. Y., Bushong, E. A., Mills, E. A., Boassa, D., Shih, T., et al. (2014). Transcellular degradation of axonal mitochondria. *Proc. Natl. Acad. Sci. U.S.A.* 111, 9633–9638. doi: 10.1073/pnas.1404651111

DeSalvo, M. K., Hindle, S. J., Rusan, Z. M., Orng, S., Eddison, M., Halliwill, K., et al. (2014). The Drosophila surface glia transcriptome: evolutionary conserved blood-brain barrier processes. *Front. Neurosci.* 8:346. doi: 10.3389/fnins.2014.00346

Doherty, J., Logan, M. A., Tasdemir, O. E., and Freeman, M. R. (2009). Ensheathing glia function as phagocytes in the adult Drosophila brain. *J. Neurosci.* 29, 4768–4781. doi: 10.1523/JNEUROSCI.5951-08.2009

Dutta, S., Rieche, F., Eckl, N., Duch, C., and Kretzschmar, D. (2016). Glial expression of Swiss cheese (SWS), the Drosophila orthologue of neuropathy target esterase (NTE), is required for neuronal ensheathment and function. *Dis. Model. Mech.* 9, 283–294. doi: 10.1242/dmm.022236

Edwards, T. N., and Meinertzhagen, I. A. (2010). The functional organisation of glia in the adult brain of Drosophila and other insects. *Prog. Neurobiol.* 90, 471–497. doi: 10.1016/j.pneurobio.2010.01.001

Ewer, J., Frish, B., Hamblen-Coyle, M. J., Rosbash, M., and Hall, J. C. (1992). Expression of the period clock gene within different cell types in the brain of Drosophila adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. *J. Neurosci.* 12, 3321–3349.

Fonken, L. K., Frank, M. G., Kitt, M. M., Barrientos, R. M., Watkins, L. R., and Maier, S. F. (2015). Microglia inflammatory responses are controlled by an intrinsic circadian clock. *Brain Behav. Immun.* 45, 171–179. doi: 10.1016/j.bbi.2014.11.009

Fonken, L. K., Kitt, M. M., Gaudet, A. D., Barrientos, R. M., Watkins, L. R., and Maier, S. F. (2016). Diminished circadian rhythms in hippocampal microglia may contribute to age-related neuroinflammatory sensitization. *Neurobiol. Aging* 47, 102–112. doi: 10.1016/j.neurobiolaging.2016.07.019

Freeman, M. R., and Doherty, J. (2006). Glial cell biology in Drosophila and vertebrates. *Trends Neurosci.* 29, 82–90. doi: 10.1016/j.tins.2005.12.002

Giebultowicz, J. M. (2001). Peripheral clocks and their role in circadian timing: insights from insects. *Philos. Trans. R. Soc. B* 356, 1791–1799. doi: 10.1098/rstb.2001.0960

Giebultowicz, J. M., and Long, D. M. (2015). Ageing and Circadian rhythms. *Curr. Opin. Insect Sci.* 7, 82–86. doi: 10.1016/j.cois.2015.03.001

Gorska-Andrzejak, J. (2013). Glia-related circadian plasticity in the visual system of Diptera. *Front. Physiol.* 4:36. doi: 10.3389/fphys.2013.00036

Gorska-Andrzejak, J., Salvaterra, P. M., Meinertzhagen, I. A., Krzeptowski, W., Gorlich, A., and Pyza, E. (2009). Cyclical expression of Na^+/K^+-ATPase in the visual system of *Drosophila melanogaster*. *J. Insect Physiol.* 55, 459–468. doi: 10.1016/j.jinsphys.2009.02.003

Hardin, P. E. (2011). Molecular genetic analysis of circadian timekeeping in Drosophila. *Adv. Genet.* 74, 141–173. doi: 10.1016/B978-0-12-387690-4.00005-2

Hardin, P. E., and Panda, S. (2013). Circadian timekeeping and output mechanisms in animals. *Curr. Opin. Neurobiol.* 23, 724–731. doi: 10.1016/j.conb.2013.02.018

Hastings, M. H., and Goedert, M. (2013). Circadian clocks and neurodegenerative diseases: time to aggregate? *Curr. Opin. Neurobiol.* 23, 880–887. doi: 10.1016/j.conb.2013.05.004

Hayashi, Y., Koyanagi, S., Kusunose, N., Okada, R., Wu, Z., Tozaki-Saitoh, H., et al. (2013). The intrinsic microglial molecular clock controls synaptic strength via the circadian expression of cathepsin S. *Sci. Rep.* 3:2744. doi: 10.1038/srep02744

Ito, K., Urban, J., and Technau, G. M. (1995). Distribution, classification, and development of Drosophila glial cells in the late embryonic and early larval ventral nerve cord. *Roux. Arch. Dev. Biol.* 204, 284–307. doi: 10.1007/BF02179499

Jackson, F. R. (2011). Glial cell modulation of circadian rhythms. *Glia* 59, 1341–1350. doi: 10.1002/glia.21097

Jackson, F. R., Ng, F. S., Sengupta, S., You, S., and Huang, Y. (2015). Glial cell regulation of rhythmic behavior. *Meth. Enzymol.* 552, 45–73. doi: 10.1016/bs.mie.2014.10.016

Kondratov, R. V., Kondratova, A. A., Gorbacheva, V. Y., Vykhovanets, O. V., and Antoch, M. P. (2006). Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock. *Genes Dev.* 20, 1868–1873. doi: 10.1101/gad.1432206

Kondratova, A. A., Dubrovsky, Y. V., Antoch, M. P., and Kondratov, R. V. (2010). Circadian clock proteins control adaptation to novel environment and memory formation. *Aging* 2, 285–297. doi: 10.18632/aging.100142

Kremer, M. C., Jung, C., Batelli, S., Rubin, G. M., and Gaul, U. (2017). The glia of the adult Drosophila nervous system. *Glia* 65, 606–638. doi: 10.1002/glia.23115

Krishnan, N., Kretzschmar, D., Rakshit, K., Chow, E., and Giebultowicz, J. (2009). The circadian clock gene period extends healthspan in aging *Drosophila melanogaster*. *Aging* 1, 937–948. doi: 10.18632/aging.100103

Krishnan, N., Rakshit, K., Chow, E. S., Wentzell, J. S., Kretzschmar, D., and Giebultowicz, J. M. (2012). Loss of circadian clock accelerates aging in neurodegeneration-prone mutants. *Neurobiol. Dis.* 45, 1129–1135. doi: 10.1016/j.nbd.2011.12.034

Kuintzle, R. C., Chow, E. S., Westby, T. N., Gvakharia, B. O., Giebultowicz, J. M., and Hendrix, D. A. (2017). Circadian deep sequencing reveals stress-response genes that adopt robust rhythmic expression during aging. *Nat. Commun.* 8:14529. doi: 10.1038/ncomms14529

Limmer, S., Weiler, A., Volkenhoff, A., Babatz, F., and Klambt, C. (2014). The Drosophila blood-brain barrier: development and function of a glial endothelium. *Front. Neurosci.* 8:365. doi: 10.3389/fnins.2014.00365

Long, D. M., Blake, M. R., Dutta, S., Holbrook, S. D., Kotwica-Rolinska, J., Kretzschmar, D., et al. (2014). Relationships between the circadian system and Alzheimer's disease-like symptoms in Drosophila. *PLoS ONE* 9:e106068. doi: 10.1371/journal.pone.0106068

Luo, W., Chen, W. F., Yue, Z., Chen, D., Sowcik, M., Sehgal, A., et al. (2012). Old flies have a robust central oscillator but weaker behavioral rhythms that can be improved by genetic and environmental manipulations. *Aging Cell* 11, 428–438. doi: 10.1111/j.1474-9726.2012.00800.x

Marpegan, L., Swanstrom, A. E., Chung, K., Simon, T., Haydon, P. G., Khan, S. K., et al. (2011). Circadian regulation of ATP release in astrocytes. *J. Neurosci.* 31, 8342–8350. doi: 10.1523/JNEUROSCI.6537-10.2011

Melom, J. E., and Littleton, J. T. (2013). Mutation of a NCKX eliminates glial microdomain calcium oscillations and enhances seizure susceptibility. *J. Neurosci.* 33, 1169–1178. doi: 10.1523/JNEUROSCI.3920-12.2013

Musiek, E. S., Lim, M. M., Yang, G., Bauer, A. Q., Qi, L., Lee, Y., et al. (2013). Circadian clock proteins regulate neuronal redox homeostasis and neurodegeneration. *J. Clin. Invest.* 123, 5389–5400. doi: 10.1172/JCI70317

Ng, F. S., Sengupta, S., Huang, Y., Yu, A. M., You, S., Roberts, M. A., et al. (2016). TRAP-seq profiling and RNAi-based genetic screens identify conserved glial genes required for adult Drosophila behavior. *Front. Mol. Neurosci.* 9:146. doi: 10.3389/fnmol.2016.00146

Ng, F. S., Tangredi, M. M., and Jackson, F. R. (2011). Glial cells physiologically modulate clock neurons and circadian behavior in a calcium-dependent manner. *Curr. Biol.* 21, 625–634. doi: 10.1016/j.cub.2011.03.027

Prolo, L. M., Takahashi, J. S., and Herzog, E. D. (2005). Circadian rhythm generation and entrainment in astrocytes. *J. Neurosci.* 25, 404–408. doi: 10.1523/JNEUROSCI.4133-04.2005

Rakshit, K., Krishnan, N., Guzik, E. M., Pyza, E., and Giebultowicz, J. M. (2012). Effects of aging on the molecular circadian oscillations in Drosophila. *Chronobiol. Int.* 29, 1–10. doi: 10.3109/07420528.2011.635237

Reddy, A. B., and O'Neill, J. S. (2010). Healthy clocks, healthy body, healthy mind. *Trends Cell Biol.* 20, 36–44. doi: 10.1016/j.tcb.2009.10.005

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019

Schulz, J. G., Laranjeira, A., Van Huffel, L., Gartner, A., Vilain, S., Bastianen, J., et al. (2015). Glial beta-oxidation regulates Drosophila energy metabolism. *Sci. Rep.* 5:7805. doi: 10.1038/srep07805

Schwabe, T., Bainton, R. J., Fetter, R. D., Heberlein, U., and Gaul, U. (2005). GPCR signaling is required for blood-brain barrier formation in drosophila. *Cell* 123, 133–144. doi: 10.1016/j.cell.2005.08.037

Stork, T., Bernardos, R., and Freeman, M. R. (2012). Analysis of glial cell development and function in Drosophila. *Cold Spring Harb. Protoc.* 2012, 1–17. doi: 10.1101/pdb.top067587

Suh, J., and Jackson, F. R. (2007). Drosophila ebony activity is required in glia for circadian regulation of locomotor activity. *Neuron* 55, 435–447. doi: 10.1016/j.neuron.2007.06.038

Tix, S., Eule, E., Fischbach, K. F., and Benzer, S. (1997). Glia in the chiasms and medulla of the *Drosophila melanogaster* optic lobes. *Cell Tissue Res.* 289, 397–409. doi: 10.1007/s004410050886

Tso, C. F., Simon, T., Greenlaw, A. C., Puri, T., Mieda, M., and Herzog, E. D. (2017). Astrocytes regulate daily rhythms in the suprachiasmatic nucleus and behavior. *Curr. Biol.* 27, 1055–1061. doi: 10.1016/j.cub.2017.02.037

Unhavaithaya, Y., and Orr-Weaver, T. L. (2012). Polyploidization of glia in neural development links tissue growth to blood-brain barrier integrity. *Genes Dev.* 26, 31–36. doi: 10.1101/gad.177436.111

Volkenhoff, A., Weiler, A., Letzel, M., Stehling, M., Klambt, C., and Schirmeier, S. (2015). Glial glycolysis is essential for neuronal survival in Drosophila. *Cell Metab.* 22, 437–447. doi: 10.1016/j.cmet.2015.07.006

Weiler, A., Volkenhoff, A., Hertenstein, H., and Schirmeier, S. (2017). Metabolite transport across the mammalian and insect brain diffusion barriers. *Neurobiol. Dis.* 107, 15–31. doi: 10.1016/j.nbd.2017.02.008

Xie, L., Kang, H., Xu, Q., Chen, M. J., Liao, Y., Thiyagarajan, M., et al. (2013). Sleep drives metabolite clearance from the adult brain. *Science* 342, 373–377. doi: 10.1126/science.1241224

Yu, E. A., and Weaver, D. R. (2011). Disrupting the circadian clock: gene-specific effects on aging, cancer, and other phenotypes. *Aging* 3, 479–493. doi: 10.18632/aging.100323

Zerr, D. M., Hall, J. C., Rosbash, M., and Siwicki, K. K. (1990). Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of Drosophila. *J. Neurosci.* 10, 2749–2762.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 14 November 2017
doi: 10.3389/fphys.2017.00918

Neuronal and Glial Clocks Underlying Structural Remodeling of Pacemaker Neurons in *Drosophila*

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A number of years ago we reported that ventral Lateral Neurons (LNvs), which are essential in the control of rest-activity cycles in *Drosophila*, undergo circadian remodeling of their axonal projections. This structural plasticity gives rise to changes in the degree of connectivity, which could provide a means of transmitting time of day information. Thus far, work from different laboratories has shown that circadian remodeling of adult projections relies on activity-dependent and -independent mechanisms. In terms of clock- dependent mechanisms, several neuronal types undergoing circadian remodeling hinted to a differential effect of clock genes; while *per* mutants exhibited poorly developed axonal terminals giving rise to low complexity arbors, *tim* mutants displayed a characteristic hyper branching phenotype, suggesting these genes could be playing additional roles to those ascribed to core clock function. To shed light onto this possibility we altered clock gene levels through RNAi- mediated downregulation and expression of a dominant negative form exclusively in the adult LNvs. These experiments confirmed that the LNv clock is necessary to drive the remodeling process. We next explored the contribution of glia to the structural plasticity of the small LNvs through acute disruption of their internal clock. Interestingly, impaired glial clocks also abolished circadian structural remodeling, without affecting other clock-controlled outputs. Taken together our data shows that both neuronal and glial clocks are recruited to define the architecture of the LNv projections along the day, thus enabling a precise reconfiguration of the circadian network.

Keywords: circadian remodeling, structural plasticity, LNvs, cell autonomous clocks

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Edited by:
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Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: *18 September 2017*
Accepted: *30 October 2017*
Published: *14 November 2017*

Citation:
Herrero A, Duhart JM and Ceriani MF (2017) Neuronal and Glial Clocks Underlying Structural Remodeling of Pacemaker Neurons in Drosophila. Front. Physiol. 8:918. doi: 10.3389/fphys.2017.00918

INTRODUCTION

Plasticity—the ability to make adaptive changes—is an integral property of the nervous system. There are numerous examples of functional and structural plasticity in invertebrates and vertebrates (Holtmaat and Svoboda, 2009; Bozorgmehr et al., 2013), highlighting its relevance. Plasticity occurs at different scales in time and structure, ranging from milliseconds to hours and from dendritic spines and axonal boutons to entire axonal and dendritic arbors. Structural plasticity contributes to synaptic and circuit function, and it is affected during aging (Barnes, 2001) and disease (Bernardinelli et al., 2014); despite their relevance, the mechanisms underlying structural plasticity, especially large-scale terminal remodeling in the adult brain remains elusive. Over the years, examples of structural remodeling of neuronal terminals taking place along the day have accumulated (Pyza and Meinertzhagen, 1999; Mehnert et al., 2007; Becquet et al., 2008; Fernández et al., 2008; Appelbaum et al., 2010), and have been shown to depend on an intact circadian clock (Fernández et al., 2008).

The circadian clock is conserved throughout the animal kingdom. In *Drosophila,* 150 neurons in the adult brain support a circadian pacemaker. This molecular clock depends on the activity of the transcription factors CLOCK (CLK) and CYCLE (CYC), which drive circadian oscillations by promoting rhythmic transcription of several key genes, including *period* (*per*), *timeless* (*tim*), and *clockwork orange* (*cwo)*, which repress CLK-CYC-mediated transcription (Ozkaya and Rosato, 2012). The coordinated operation of the circadian network is necessary for the adapted responses to synchronizing environmental stimuli. Clock neurons are anatomically clustered in distinct groups: small and large ventral-lateral (s-LNvs, l-LNvs, and the fifth s-LNv), the dorsal-lateral (LNds), the lateral posterior (LPNs) and three subgroups of dorsal neurons (DNs1-3). Only the LNvs express a neuropeptide called PIGMENT DISPERSING FACTOR (PDF), which plays a major role in the synchronization of the circadian network. PDF is essential for normal circadian activity patterns in light: dark cycles (LD) and for persistent circadian rhythms under constant free running conditions (DD). In fact, PDF synchronizes the phase of the sLNvs and DN1s, while slows down the pace and increases the amplitude of the LNds and the PDF negative 5th sLNv (Lin et al., 2004; Lear et al., 2009; Yoshii et al., 2009; Im et al., 2011).

PDF immunoreactivity changes throughout the day in the sLNv axonal termini, indicating that its regulation is under clock control. In addition, the sLNv axonal terminals exhibit a higher degree of arborization during the day and a reduced complexity at night, accompanying the changes in PDF levels. This phenomenon, called circadian structural plasticity, is lost in per^{01} and tim^{01} mutants, indicating that it depends on a functional clock, although substantial structural differences are observed (Fernández et al., 2008).

Glial cells have a critical role in plasticity and synaptic transmission. Recent studies in *Drosophila* have implicated glial cells in the regulation of neuronal excitability, vision, circadian behavior, sleep, behavioral sensitivity to drugs, and olfaction (Borycz et al., 2002; Bainton et al., 2005; Stuart et al., 2007; Suh and Jackson, 2007; Ng et al., 2011; Seugnet et al., 2011; Melom and Littleton, 2013; Chaturvedi et al., 2014; Liu et al., 2014; Chen et al., 2015). Despite little is known about *Drosophila* circadian gliotransmitters, there is vast evidence of their relevance in circadian rhythmicity (Ng et al., 2011; Ng and Jackson, 2015). Several studies have reported rhythmic expression of clock proteins and other neural proteins (e.g., PER, TIM, Ebony) in glial cells of the adult brain (Suh and Jackson, 2007), although the importance of glial clocks in circadian outputs has not been extensively studied yet, prompting us to analyze its relevance in structural plasticity.

Given the implications to the circadian network of an active sLNv terminal remodeling, i.e., daily changes in connectivity (Gorostiza et al., 2014), we inquired whether affecting the molecular clock, particularly in the adult LNvs, would abolish circadian plasticity. To this end we deregulated specific clock genes and analyzed the impact of these genetic interventions on structural remodeling; interestingly, despite altering different clock genes similarly affected molecular oscillations, the terminals adopted a different configuration, suggesting that additional mechanisms are recruited. We next addressed the possibility that the clock in glial cells actively contributes to the structural plasticity of sLNv terminals, and uncovered that adult-specific impairment of their molecular clock also disrupts circadian remodeling. Thus, both, the molecular clock in LNvs and glia are necessary for sustaining this unusual form of plasticity.

MATERIALS AND METHODS

Fly Rearing and Strains

Flies were grown and maintained at 25°C in vials containing standard cornmeal medium under 12:12 h LD cycles. For adult specific induction either the GeneSwitch or TARGET systems were employed (McGuire et al., 2004). GeneSwitch expression was induced transferring 2 day-old flies to vials containing food supplemented with RU486 (mifepristone, Sigma, USA) in 80% ethanol to a final concentration of 200 μg/ml, or with the same amount of ethanol (vehicle) in control treatments. Adult-specific termosensitive Gal4 expression was induced transferring flies raised at 23°C during development to 30°C for 48 h. The *pdf*-GeneSwitch (*pdf*-GS) line was generated in our laboratory (Depetris-Chauvin et al., 2011); Stocks UAS-cyc^{DN} (#36317, Tanoue et al., 2004), UAS-$tim^{RNAi}I$ (#29583), UAS-per^{RNAi} (#40878 and #31285, I and II, respectively), *pdf*-Gal4 (#6900), *repo*-Gal4 (#7415) were obtained from the Bloomington Stock Center. The UAS-tim^{RNAi} *II* (#2886) stock was obtained from the Vienna RNAi Stock Center. *pdf*-dsRed was generously provided by J. Blau.

Adult Locomotor Activity

For locomotor activity experiments adult male flies were entrained for 3 days in 12:12 LD cycles at 25°C and then transferred to constant darkness (DD) at 25°C. Males were placed in glass tubes containing standard food and monitored for activity with infrared detectors and an automated data collection system (TriKinetics, Waltham, MA). Activity was monitored for 14 days (4 in LD and 9–10 in DD). Period, FFT and rhythmicity in DD were estimated using ClockLab software (Actimetrics, Evanston, IL) as previously described (Ceriani et al., 2002; Depetris-Chauvin et al., 2011).

Immunohistochemistry and Image Acquisition

Adult fly heads were fixed with 4% p-formaldehyde (pH 7.5) for 30–40 min at room temperature. Brains were dissected and rinsed four times in PT buffer (PBS with 0.1% Triton X-100) for 30 min. Samples were blocked in 7% normal goat serum (in PT) for 1 h, and incubated with primary antibodies at room temperature for 2 days. The primary antibodies employed were chicken anti-GFP 1:500 (Aves Labs, Inc, USA), rabbit anti-DsRed 1:500 (Clontech, USA) and homemade rat anti-*Drosophila*-PDF 1:500 (Depetris-Chauvin et al., 2011). Samples were washed 4 x 15 min in PT, and incubated with secondary antibody at 1:250 for 2 h at room temperature. Secondary antibodies were washed 4 x 15 min in PT and mounted in Vectashield antifade mounting medium (Vector Laboratories, USA). The secondary antibodies

used were Cy2-conjugated donkey anti-rabbit, Alexa Fluor 647-conjugated AffiniPure donkey anti-rat and Cy3-conjugated AffiniPure donkey anti-rabbit (Jackson ImmunoResearch, USA). Images were taken on a Zeiss LSM 710 confocal microscope.

Structural Plasticity Analysis and PDF Immunoreactivity

Images were taken with a 40× objective and an optical zoom of 2×. CD8GFP signal was adjusted to threshold levels generating a selection that delimits the area of sLNv axonal terminals. This selection was then applied to the PDF channel and mean intensity was measured. For the analysis of PDF immunoreactivity all pictures were taken employing the same confocal settings and quantification was performed using Image J software (downloaded from http://rsbweb.nih.gov/ij/). Structural plasticity was analyzed by Scholl analysis, as reported (Fernández et al., 2008). In all cases the analysis was performed blind.

Quantitative Real-Time PCR

Total RNA isolation from fly head extracts was performed using Trizol (Invitrogen, Carlsbad, CA). Superscript III was used for reverse transcription (ThermoFisher Scientific, USA) and FastStart Universal SYBR Green Master (Roche) was used for quantitative real-time PCR following manufacturer's instructions. The real-time assays were conducted in a Stratagene Mx3000P QPCR System (La Jolla, CA) using SYBR green as the detection system and ROX as the reference dye. The primers were designed using Primer3 (available online at http://frodo.wi.mit.edu/primer3/). mRNA levels were assessed from three independent RNA extractions and two technical replicates were performed on each sample. Only primer pairs with efficiency between 90 and 110% were used. The following primers were employed, to detect *rpl49* (fw 5′GAACAAGAAGGCCCATCG TA3′; rev 5′AGTAACAGGCTTRGGCTTGC3′); *per* (for 5′GAC CGAATCCCTGCTCAATAA3′; rev 5′GGACTTCTTGCTCTT CTCACC3′); *tim* (fw 5′GGTAAACGGATCGCACTTCTCG3′; rev 5′AAGAGACATTGTCGCTGTTTAAT3′); *dClk* (fw 5′CAG AGTCAGTTGCAGGATCAA3′; rev 5′GCAGATATGTGTAGC GGGATAG3′); *cyc* (fw 5′TGGACAATCACCCGAACATAC3′; rev 5′CTGAGGCAGGAAACCAATCA3′).

Data Analysis and Statistics

Statistical analyses were performed with the InfoStat package version 2009 (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). In all graphs, experimental groups with different letters indicate statistically significant differences, with a $p < 0.05$. Validation of RNAi lines was tested with a Student's *t*-test. Clock gene oscillation under different clock modulations was analyzed by One-way ANOVA within each clock gene, followed by Tukey's *post-hoc* test. Effects on structural plasticity were analyzed by Two-way ANOVA, followed by Tukey's *post-hoc* test. Effects on PDF levels were analyzed by Kruskal–Wallis One way ANOVA, followed by Conover's *post-hoc* test. Number of flies or brains in each experiment is referred as *n*, and the number of experiments is referred as *N*, and was used for statistical analysis.

RESULTS

Disrupting the Molecular Clock in the Adult LNvs Impairs Locomotor Rhythmicity

To address the possibility that the circadian remodeling of sLNv terminals exclusively depends on their own molecular clock we took advantage of the spatially restricted Gal4/UAS system to alter clock protein levels exclusively in the LNvs. To monitor the efficiency of the different strategies and potentially uncover a differential effect, we first analyzed locomotor activity patterns in flies in which different clock genes were constitutively deregulated through RNAi-mediated silencing (*per*, *tim*), or through the expression of a dominant-negative CYC version (CYC^{DN}, Tanoue et al., 2004).

Control (*pdf*-Gal4>+) flies showed a clear rhythmic pattern in the presence of synchronizing cues (LD cycles) as well as in DD (**Figure 1A**). In DD this rhythm has a period of around 24 h and flies consolidate their activity across the subjective day (**Table 1**). Downregulating *per* and *tim* mRNA levels in the LNvs (through *pdf*-Gal4) employing different RNAi lines triggered significant effects on the patterns of locomotor activity, mostly a ∼80% decrease in the percentage of rhythmicity (**Figure 1B**); surprisingly, period length was not different from the control (Supplementary Figure 1 and **Table 1**). We next measured steady state levels of *per* and *tim* mRNA by quantitative real-time PCR in total RNA extracts from wild type (*tim*-Gal4,*dcr2*>+) and flies expressing specific RNAis (per^{RNAi} and tim^{RNAi}) at the peak of their endogenous levels (CT14, CT stands for circadian time, and refers to the hours passed since the last day to night transition). Significant differences were observed, with a decrease to about 30% compared to wild type levels both in the case of *per* and *tim* mRNA (**Figure 1C**). To overcome potential unspecific effects of the knockdown strategy, we employed a second RNAi line that showed similar results (Supplementary Figure 1). Thus, both RNAis are efficient to downregulate clock protein levels and affect clock outputs (i.e., behavioral rhythmicity).

In addition, we took advantage of a well-characterized dominant negative variant of CYC, called CYC^{DN} (Tanoue et al., 2004) to block CLK/CYC activated transcription. As previously reported, expressing cyc^{DN} in the LNvs produced a drastic reduction of behavioral rhythmicity (**Figures 1A,B**; see **Table 1** for an in depth analysis of behavioral parameters) down to about 14%, suggesting this strategy is a very efficient one to impair clock function (Tanoue et al., 2004). Taken together, these experiments support the relevance of LNvs in the control of rhythmic rest-activity cycles since deregulating different core clock genes in a cluster-specific fashion significantly impacts the consolidation of rhythmic locomotor behavior.

Modulation of Different Clock Components Leads to Dampened Oscillations

Rhythmicity of clock-controlled outputs depends on the precise regulation of the cell autonomous molecular clock. Since affecting different clock protein levels in the LNvs altered behavioral patterns to a different degree we assessed the state of the molecular clock after long term deregulation of each specific clock protein. To this end we examined *per*, *tim*, *dClk,* and *cyc*

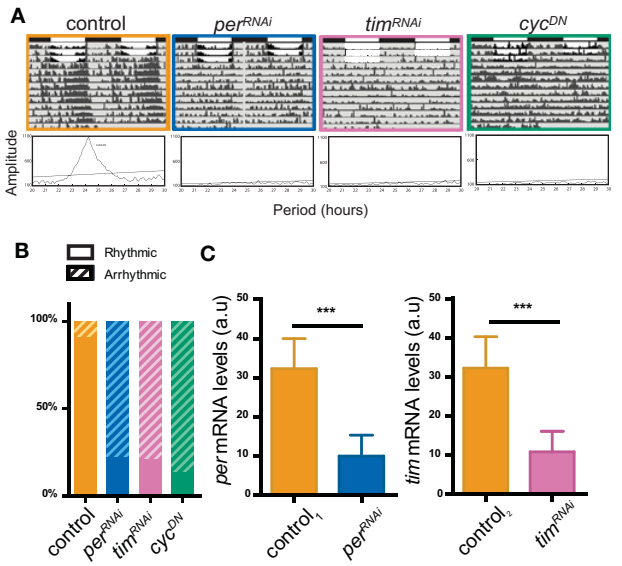

FIGURE 1 | Impaired LNv clocks result in disrupted locomotor activity patterns. **(A)** Representative double-plotted actograms and their respective periodograms of the different genotypes: UAS-*dcr2*, UAS-*per*^{RNAi}*I* (#40878), UAS-*tim*^{RNAi}*I* (#29583), and UAS-*cyc*^{DN} under the control of *pdf*-Gal4. Locomotor activity of individual flies was recorded for 4 days under 12:12 LD cycles and then transferred to DD (gray area) for 9 additional days. In the actograms, white bars represent day, black bars represent night. For every fly actogram, periodograms of the free-running rhythm in DD are shown (bottom). Rhythmic behavior of a typical male control is shown (orange). *per*^{RNAi} (in blue), *tim*^{RNAi} (in pink), and *cyc*^{DN} (in green) expressing animals are shown. All genetic manipulations gave rise to a largely arrhythmic locomotor behavior. **(B)** Percentage of rhythmicity. Data represents at least three independent experiments; over 44 flies were analyzed. **(C)** Validation of the effectiveness of the RNAis employed. Both UAS-*per*^{RNAi} and UAS-*tim*^{RNAi} were expressed under *tim*-Gal4. Levels are normalized to *rpl49*. Both *tim and per* mRNA levels are reduced compared to their respective control. Student's *t*-test showed a significant difference in levels of expression. Triple asterisks (***) indicate significant differences with $p < 0.001$. Three independent experiments were performed.

mRNA levels at two timepoints during the day, CT2 and CT14, upon deregulation of the different genes using the pan-circadian driver *tim*-Gal4.

Interestingly, we found that the amplitude of the peak/trough oscillation of *per* or *tim* transcript levels was greatly reduced in the conditions tested regardless of the clock protein targeted, that is, as a result of impairing repression or activation (Supplementary Figure 2 and **Figure 2**). As expected (Lerner et al., 2015), *dClk* mRNA levels were low compared to those of *per* and *tim* in controls, which could partially account for the difficulty in determining a precise change in peak/trough amplitude (only two-fold in this set of measurements for the controls). Accordingly, a clear effect on the amplitude of *dClk* oscillations was detectable in *tim*^{RNAi} and *cyc*^{DN}. In addition, despite *cyc* levels were originally reported not to cycle by northern blot (Rutila et al., 1998), qPCR analysis showed a shallow (three-fold) cycling in endogenous *cyc* levels in controls (higher at CT14 than CT2), that were not significantly affected in any of the combinations analyzed (Supplementary Figure 2).

In sum these results highlight that deregulation of different clock components results in a dampened molecular clock, regardless of the affected process (that is, impaired CLK/CYC mediated transcription or its repression), and confirm that any of these genetic interventions are useful to assess the relevance of the cell autonomous clock on a particular output, i.e., the control of the structural plasticity of the sLNv terminals.

Circadian Structural Plasticity Is Differentially Altered by Clock Genes

A number of years ago we reported morphological changes of the axonal terminals of the sLNvs across the day and showed that this phenomenon is under the control of the circadian clock since it is abolished in *per*^{01} and *tim*^{01} mutants (Fernández et al., 2008). However, despite circadian changes in the complexity of the sLNv axonal arbor was abrogated in both null mutants, the overall morphology of the terminals was quite distinct, suggesting PER and TIM could be playing additional "non-circadian" roles. Thus, we set out to examine the impact of altering different molecular clock components specifically in the LNvs on the architecture of the dorsal terminals exclusively in the adult brain to avoid potential developmental effects.

We took advantage of an inducible Gal4 version termed GeneSwitch with restricted expression to the LNv neurons (*pdf*-GS, Depetris-Chauvin et al., 2011), combined with a membrane-tethered version of GFP (CD8GFP) to describe the complexity of the axonal arborizations, along with *per*^{RNAi}, *tim*^{RNAi}, or *cyc*^{DN} to address the role of the LNv clock in structural plasticity. Flies transferred to RU486-containing food 2 days after eclosion were dissected at CT2 and CT14 on DD3 (**Figure 3A**). As previously reported, the overall structure of the dorsal terminals is more complex in the morning and less arborized at night time in controls (**Figures 3B,C**, shown in orange; Fernández et al., 2008; Gorostiza et al., 2014). Interestingly, affecting the negative elements of the molecular clock, *per* (shown in blue) and *tim* (in pink), the complexity was significantly reduced from that displayed by controls in the subjective morning (**Figures 3B,C**), and resembled the nighttime configuration of control terminals. On the other hand, expressing *cyc*^{DN} (in green) gave rise to maximally spread axonal termini throughout the day; in fact, the architecture of the termini (to the level described through confocal microscopy) was different from that of controls at any timepoint; specifically, the number of higher order neurites (ramifications of primary and secondary processes) was clearly increased compared to controls, suggesting that actively impairing CYC function in the adult triggers clear morphological defects, beyond those anticipated from affecting the endogenous molecular clock.

PDF immunoreactivity in the axonal terminals at the dorsal protocerebrum has been shown to oscillate in a circadian fashion both under LD and DD conditions (Park et al., 2000); remarkably, this cycling is blocked in mutants with impaired clock function (Park et al., 2000). Immunohistochemistry analysis on whole-mount adult brains dissected at times when PDF levels peak and reach a trough was examined. PDF levels were assessed at CT2 and CT14 upon adult- specific expression of either

TABLE 1 | Detailed circadian parameters for all the behavioral experiments performed.

Genotype	%R ± SEM	Tau ± SEM	FFT ± SEM	Power ± SEM	*N*	*n*
*pdf*GAL4> +	90.69 ± 3.44^{A}	24.26 ± 0.4	0.035 ± 0.009	725.37 ± 353.4	3	66
*pdf*GAL4>*per^{RNAi} I*	22.31 ± 9.78^{B}	23.94 ± 0.12	0.023 ± 0.008	247.42 ± 143.71	3	70
pdfGAL4>tim^{RNAi} I	20.72 ± 15.99^{B}	23.50 ± 0.24	0.013 ± 0.003	347.40 ± 126.48	3	61
*pdf*GAL4>*cyc^{DN}*	13.73 ± 3.34^{B}	23.67 ± 0.09	0.018 ± 0.004	175.60 ± 53.67	3	46
pdfGAL4>per^{RNAi} II	43.54 ± 13.91^{AB}	23.72 ± 0.09	0.024 ± 0.007	336.92 ± 189.72	3	73
pdfGAL4>tim^{RNAi} II	46.26 ± 16.75^{AB}	24.33 ± 0.44	0.022 ± 0.007	506.65 ± 155.42	3	78

The analysis included the assessment of period (Tau) and different measurements of rhythm strength, such as FFT, power, and percentage of rhythmicity (%R). Average ± S.E.M. of different clock deregulating genotypes along with the control is shown. Different letters indicate statistically significant differences with a $p < 0.05$ (One-way ANOVA with a Tukey post-hoc test). N indicates the number of experiments, which was used for statistical analysis. n indicates the number of animals tested. per^{RNAi} I (Bloomington Stock Center: #40878) and tim^{RNAi} I (Bloomington Stock Center: #29583) refers to the RNAis used in the main figures. The ones indicated in gray correspond to the RNAi lines shown in Supplementary Figure 1, as follows: per^{RNAi} II (Bloomington Stock Center: #31285) and tim^{RNAi} II (VDRC Stock Center: #2886).

per^{RNAi}, *tim^{RNAi}*, or *cyc^{DN}* along with controls. Control *pdf*-GS > CD8GFP flies (orange) in the presence of RU486 exhibited a significant difference in PDF immunoreactivity between these two time points (**Figure 3D**). In contrast, PDF immunoreactivity at the sLNv dorsal terminals in every experimental condition was significantly different to controls. Such difference was more pronounced when expressing *per^{RNAi}* (blue) or *tim^{RNAi}* (pink), in which the amplitude of the oscillation is markedly reduced, even when compared to *cyc^{DN}* (green).

In sum, these experiments demonstrate that circadian remodeling of the sLNv terminals is driven by the LNv molecular clock. Furthermore, affecting the positive and negative elements of the feedback loop triggered a distinctive "architecture" of the axonal termini. Under these conditions, not only structural plasticity but also PDF levels are altered, indicating that both outputs are dependent on the correct operation of the LNv molecular oscillator.

Glial Clocks Also Contributes to Circadian Remodeling of the sLNv Terminals

Having demonstrated that the LNv clock is necessary for the remodeling of the sLNv termini, we wondered whether additional clocks could contribute to this phenomenon. One evident candidate is the one in glial cells, which plays a role in rhythmic locomotor behavior (Ng et al., 2011); in addition, it is well established that astrocytes modulate the activities of many different neuronal synapses, further strengthening this possibility. Flies expressing *cyc^{DN}*, in our hands the most effective means to block CLK/CYC activated transcription (**Figures 1**, **2**), was employed to acutely interrupt the glial clock in the adult brain.

At the restrictive temperature (23°C, **Figure 4A**), where no expression of the dominant negative CYC is achieved, *repo*-Gal4;*tub*-Gal80^{TS} >*cyc^{DN}* flies exhibited the expected remodeling of the sLNv terminals, more elaborated during the subjective day than at night (**Figures 4B,C**). In contrast, disrupting the clock in glia led to the absence of circadian remodeling, resulting in a minimally spread arbor, reminiscent of controls at night (**Figures 4B,C**). Controls shifted to the permissive temperature still displayed circadian remodeling (Supplementary Figure 3). These observations bring further support to the notion that glial clocks play an active role regulating circadian clock outputs, and more specifically, are essential for circadian structural plasticity.

We also explored PDF immunoreactivity in the dorsal protocerebrum, given its established relevance to this form of plasticity (Depetris-Chauvin et al., 2014). Surprisingly, we found that PDF levels still change between the subjective day and night when the clock in glia is impaired; in fact, under these conditions PDF levels were not different from controls (**Figure 4D**), providing further evidence that these two clock outputs (PDF levels and structural remodeling) can be uncoupled (Depetris-Chauvin et al., 2011).

Together these results indicate that circadian structural plasticity of the sLNvs depends not only on its own molecular clock, but also that glial clocks actively contribute to this form of plasticity.

DISCUSSION

A number of years ago we discovered that in wild type flies there are conspicuous structural changes in neurons that are key to the control of circadian locomotor activity (the sLNvs), which undergo remodeling of their axonal arborizations on daily basis (Fernández et al., 2008). Those initial observations led to additional discoveries, namely, that circadian remodeling involves changes in the number of synapses and connectivity, and concomitantly, that sLNvs neurons contact different postsynaptic targets across the day (Gorostiza et al., 2014); thus, structural plasticity results in changes in the strength of the communication between circadian clusters that could contribute to seasonal adaptation (Gorostiza et al., 2014; Petsakou et al., 2015). Circadian structural remodeling has been observed in clock brain structures and in other brain regions receiving input from the circadian clock (Bosler et al., 2015); interestingly, it has been shown to coexist with circadian changes in neuronal excitability and synaptic efficacy within and outside clock structures, but the precise relationship between these two forms of plasticity remains poorly understood (Frank and Cantera, 2014).

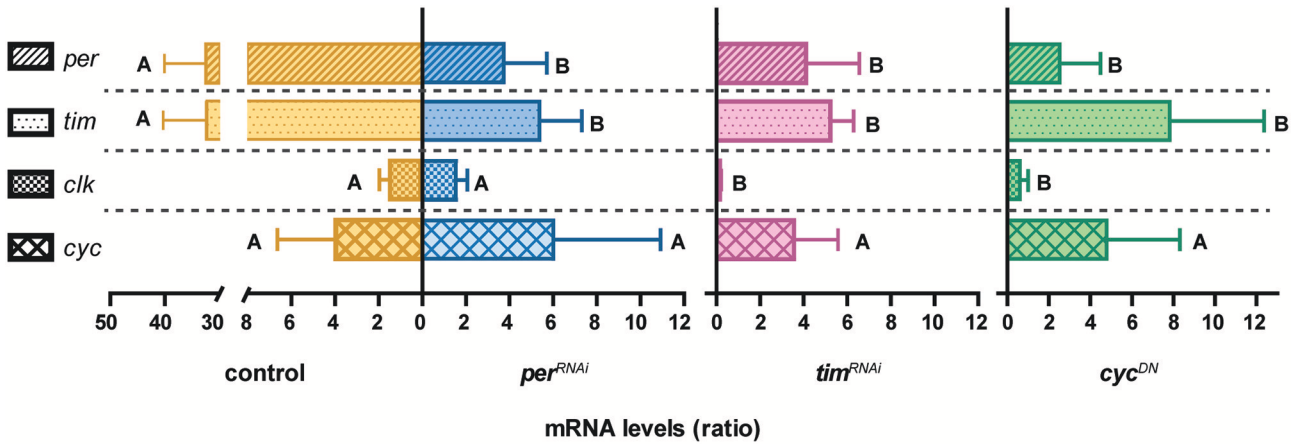

FIGURE 2 | Different clock components trigger dampened molecular oscillations. For each gene, the ratio describing higher/lower mRNA levels is plotted (that is, CT14/CT2 for *per*, *tim* and *cyc*; and CT2/CT14 for *clk*). Levels are normalized to the reference gene *rpl49*. Statistical analysis was performed comparing individual transcript levels (indicated by a dashed line). The genotypes analyzed are as follows: control (orange), per^{RNAi} (blue), tim^{RNAi} (pink), and cyc^{DN} (green), under *tim*-Gal4. Different letters indicate statistically significant differences with a $p < 0.05$ (One-way ANOVA with a Tukey *post-hoc* test). Three independent experiments were performed.

Cell Autonomous Mechanisms Underlie Continuous Remodeling of Neuronal Terminals

Rhythmic changes in neuronal morphology reported thus far in *Drosophila* include those in axonal caliber, branching complexity, synaptic vesicles and synapse numbers. One of the structures undergoing daily and circadian remodeling is the visual system that shows changes in the number of synaptic contacts as well as in the morphology of neurons and glial cells (Pyza and Meinertzhagen, 1999; Weber et al., 2009; Damulewicz et al., 2013; Gorska-Andrzejak et al., 2013); likewise, cyclical changes in neuronal morphology are exhibited by motor neurons in the adult (Mehnert et al., 2007; Mehnert and Cantera, 2008; Ruiz et al., 2010, 2013), as well as by interneurons in the central brain (Fernández et al., 2008).

In terms of the underlying molecular processes that trigger structural changes not much is known; activity dependent and independent mechanisms appear to be recruited to drive terminal remodeling in the central brain (Depetris-Chauvin et al., 2011; Sivachenko et al., 2013), associated to changes in the degree of fasciculation (Sivachenko et al., 2013), pruning (Depetris-Chauvin et al., 2014), and actin cytoskeleton remodeling (Petsakou et al., 2015), although the timing of these events has not been explored in any length.

As it is the case for many clock-controlled outputs, it was expected that at least part of the molecules responsible for orchestrating active structural remodeling show circadian modulation of gene expression, protein stability and/or activity. Consequently, circadian remodeling would be directly controlled by a cell-autonomous circadian clock, i.e., the one operating in those specific neurons, a possibility that had not been specifically examined yet. Thus, to examine whether the LNv molecular clock is necessary to drive structural remodeling we resorted to different genetic strategies to obliterate molecular oscillations. Surprisingly, while adult-specific downregulation of the repressors of the molecular clock (through the expression of per^{RNAi} and tim^{RNAi}) resulted in less complex arborization patterns, impairing CLK/CYC mediated transcriptional activation (through the expression of cyc^{DN}) correlated with maximally spread terminals, despite an overall similar effect on clock genes at the mRNA level. Closer inspection of the architecture of the arborizations suggests that additional phenomena are also affected; particularly in the case of CYC^{DN} expression, not only the terminals are complex throughout the day as controls exhibit in the early (subjective) morning, but also membrane integrity appears severely disrupted unlike neuronal terminals in controls. Additional experiments are required to understand this process in full; one obvious candidate that could mediate the altered morphology is the circadian modulation of actin dynamics (Petsakou et al., 2015) that directly impacts on structural integrity and plasticity of neurons and their synapses. These results underscore that the circadian clock not only drives circadian remodeling but it could additionally play an active role in maintaining neuronal shape (Mehnert and Cantera, 2011).

Distinct Long Term and Acute Effects Derived from Clock Disruption

While addressing the circadian nature of the remodeling phenomenon in different structures it became clear that loss-of-function mutations in *per* and *tim* not only abolish circadian remodeling but also trigger abnormal branching, which in turn would suggest that circadian plasticity plays a role in maintaining normal morphology (Mehnert and Cantera, 2011). Incidentally, *per* and *tim* null mutants showed quite distinct phenotypes in the architecture of the neuronal terminals (Mehnert et al., 2007; Fernández et al., 2008), suggesting that both proteins could play additional roles than those limited to the circadian clock. To delve further into this possibility,

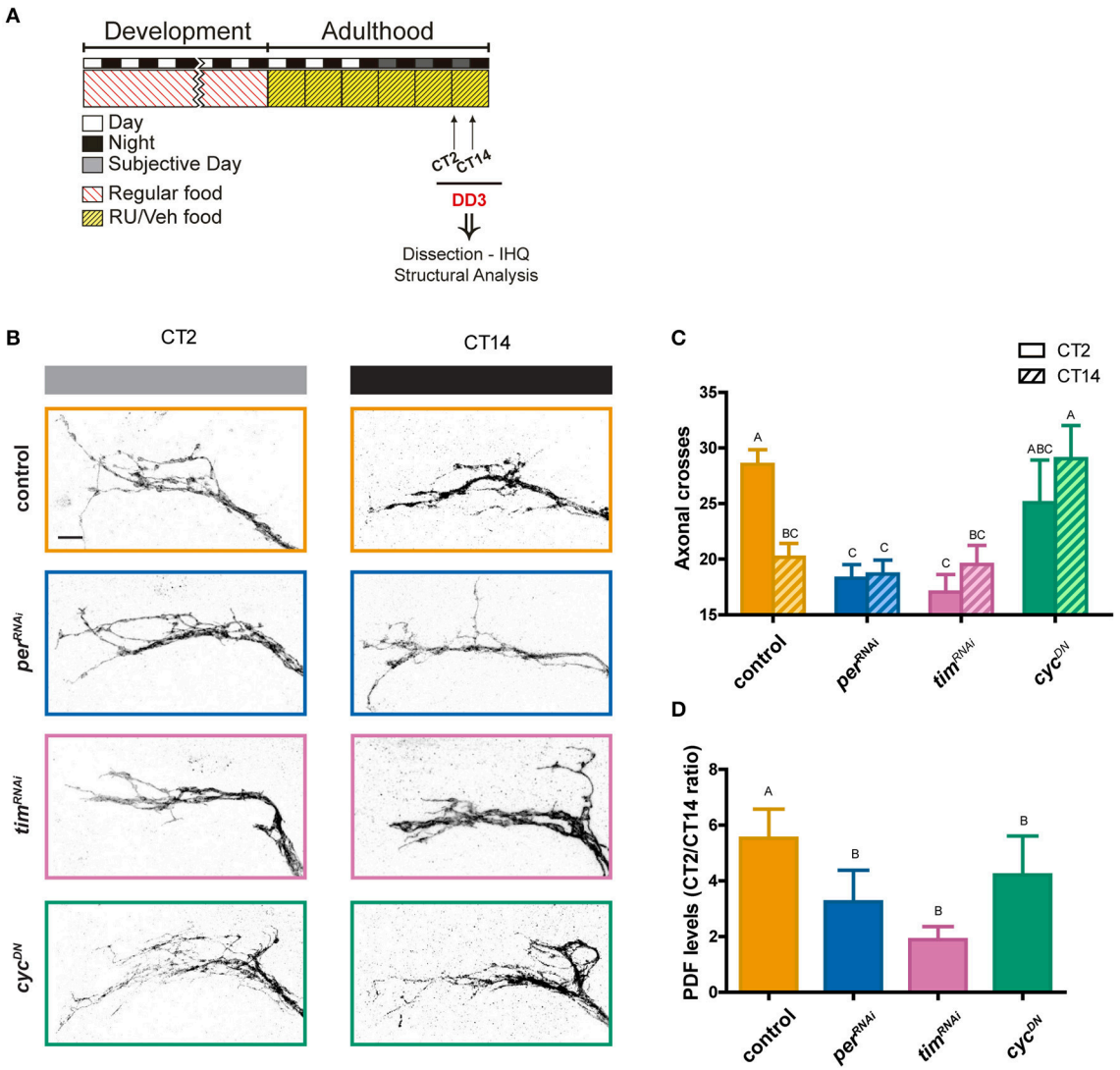

FIGURE 3 | Circadian structural plasticity is differentially altered by clock genes. **(A)** Schematic diagram illustrating the standard protocol. "Veh" and "RU" stand for "vehicle" and "RU486" containing fly food. **(B)** Representative confocal images of GFP immunoreactivity at the dorsal protocerebrum at the early subjective day (CT2, gray bar) and early subjective night (CT14, black bar) during the 3rd day of constant darkness (DD3). **(C)** Quantitation of total axonal crosses. Control flies display circadian structural remodeling of axonal terminals while animals with a deregulated clock show no differences across the day. Data represents the average of 3 experiments; a minimum of 27 brains were analyzed per CT/genotype. Different letters indicate statistically different treatments with a $p < 0.05$ (Two-way ANOVA with a Tukey *post-hoc* test, $n = 8–10$, $N = 3$). Controls in vehicle are not different from controls in RU containing food (Depetris-Chauvin et al., 2011). **(D)** Quantitation of PDF immunoreactivity at the dorsal protocerebrum at CT2 and CT14 on DD3. For a more direct comparison, PDF levels are shown as the ratio between CT2 and CT14. Control flies (orange), exhibit circadian oscillation of PDF levels, while different clock deregulation genotypes were significantly different from the control. Different letters indicate statistical differences with a $p < 0.05$ (Kruskal–Wallis One-way ANOVA, followed by Conover *Post-hoc* test, $n = 8–10$, $N = 3$).

we downregulated *per* or *tim* levels in the post-developmental brain, once the whole circadian network was established and well connected. Interestingly, adult-specific downregulation of either gene resulted in a similar structure, which at all times resembled the less complex arborization pattern exhibited by controls at night. These results reinforce the notion that the structural differences associated to loss of function mutations could uncover additional processes in which these proteins participate during early development or during establishment of the circadian circuitry.

The Contribution of Glial Clocks to LNv Structural Plasticity

Over the years it was established that glia, and particularly neuron-glia communications, plays an active role in the control of rhythmic outputs, affecting PDF immunoreactivity at the dorsal terminals and concomitantly, rhythmic behavior (Suh and Jackson, 2007; Ng et al., 2011; Ng and Jackson, 2015). Surprisingly, affecting glial clocks *per se* did not impact on rhythmic patterns of locomotor activity, at least upon chronic downregulation of PER levels (Ng et al., 2011).

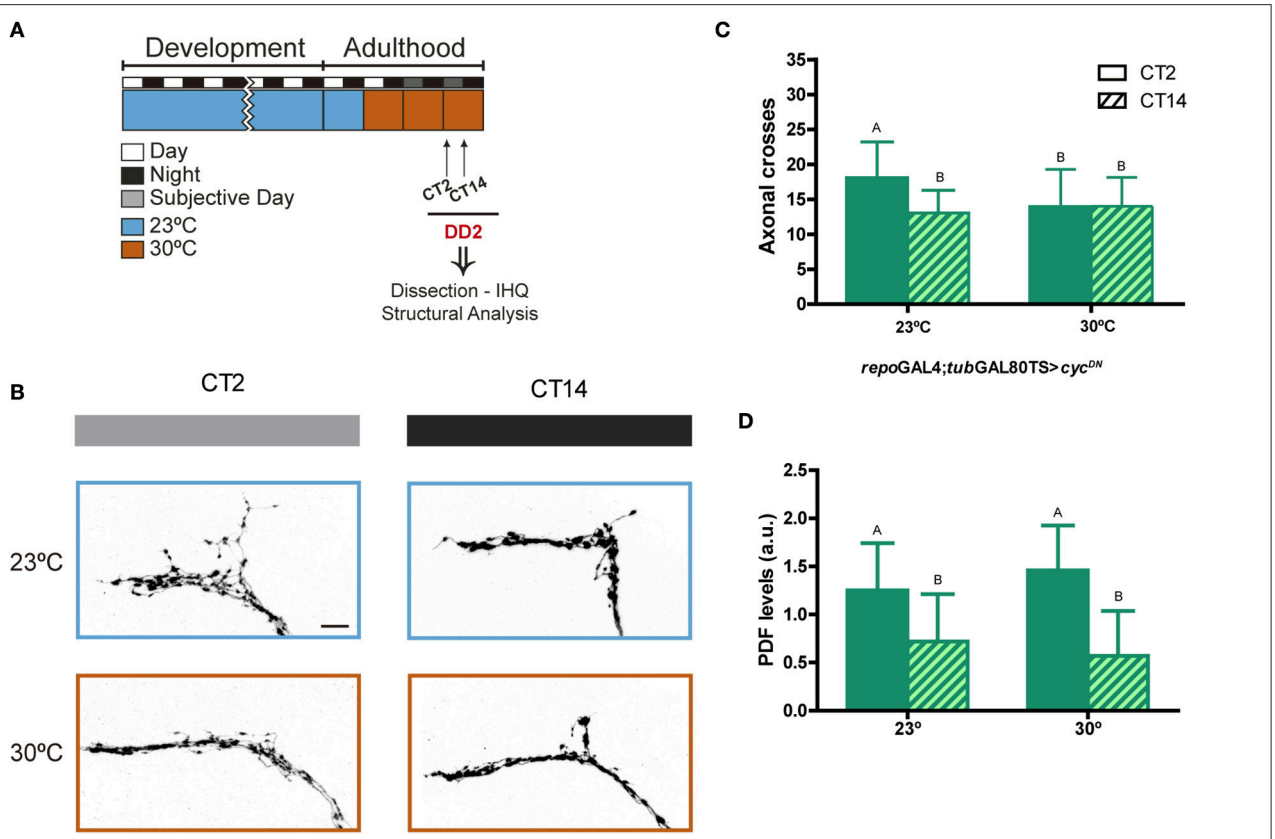

FIGURE 4 | Clocks in glia are required for circadian remodeling of neuronal terminals. **(A)** Schematic diagram illustrating the standard protocol; the restrictive condition is highlighted in light-blue (23°C), and the permissive condition is shown in orange (30°C). **(B)** Representative confocal images of dsRed immunoreactivity at the dorsal protocerebrum of flies containing UAS-cyc^{DN} under *repo*-Gal4;*tub*-$Gal80^{TS}$; *pdf*RED enables visualization of the axonal terminals. Brains were dissected at the early subjective day (CT2, gray bar) and early subjective night (CT14, black bar) during the 2nd day of constant darkness (DD2), which corresponds to the 3rd day of permissive condition (30°C). Control flies (always maintained at 23°C) are indicated in light-blue. **(C)** Quantitation of total axonal crosses of *repo*-Gal4;*tub*-$Gal80^{TS}$ > cyc^{DN}. Control flies (kept at 23°C) display circadian structural remodeling of axonal terminals while animals induced at 30°C show no differences along the day. Data represents the average of 3 experiments; a minimum of 27 brains were analyzed per CT/genotype. Different letters indicate statistical differences with a $p < 0.05$ (Two-way ANOVA with a Tukey *post-hoc* test, $n = 8$–10, $N = 3$). **(D)** Quantitation of PDF immunoreactivity at the dorsal protocerebrum from brains dissected at CT2 and CT14 on DD3. Control flies (23°C), exhibit circadian oscillation of PDF levels; those expressing cyc^{DN} at 30°C were not significantly different from controls. Same letters indicate no statistically different conditions ($p > 0.05$) (Kruskal–Wallis One-way ANOVA, followed by a Conover *post-hoc* test, $n = 8$–10, $N = 2$). Data represents the average of 2 experiments; a minimum of 16 brains were analyzed per CT/genotype.

In the visual system circadian remodeling of neuronal terminals is likely driven by different circadian oscillators, and takes place in photoreceptor cells, the most abundant peripheral oscillator in the fly head, but also in non- clock cells such as the L1 and L2 monopolar neurons in the lamina (Weber et al., 2009). In the latter, circadian input driving remodeling likely derives from the photoreceptors, the PDF+ central clock neurons, as well as the surrounding glia (reviewed in Gorska-Andrzejak, 2013). Structural changes also correlate with changes in the abundance of a marker of presynaptic active zones (Bruchpilot, BRP; Gorska-Andrzejak et al., 2013). Interestingly, it was reported that blocking clock function in glia alters, though it does not obliterate, the daily changes in BRP accumulation in the lamina cartridges (Gorska-Andrzejak et al., 2013). In the house fly, glial cells change in size in the opposite phase compared to neurons, and remodeling is affected either when perturbing glial metabolism or, more dramatically, glial communication (Pyza and Gorska-Andrzejak, 2004).

To begin to assess whether clocks in glia would contribute to the structural remodeling of the LNv projections, panglial CYC^{DN} expression was restricted to the adult. Interestingly, acute (for 2 days) disruption of glial clocks completely abolished circadian plasticity, underscoring their active contribution to the remodeling process. However, under those conditions, PDF immunoreactivity at the dorsal terminals exhibited no differences compared to controls, unexpectedly uncoupling both clock outputs. Despite a subtle effect derived from a short term blockage of CLK/CYC function cannot be ruled out, our results suggest that structural remodeling of the LNv terminals is even more sensitive to the alterations in glial physiology than PDF levels themselves.

Glia-to-neuron communication actively participates in the circadian regulation of terminal remodeling despite the

mechanisms remain to be uncovered. One possible scenario would depend on circadian release of gliotransmitters, as it has been shown to take place in mammalian astrocytes (i.e., ATP; Burkeen et al., 2011; Marpegan et al., 2011), or other ligands known to mediate neuro-glial communication [obvious candidates to test belong to the Fibroblast growth factor (FGF) and Bone morphogenetic protein BMP signaling pathways; Awasaki et al., 2011; Fuentes-Medel et al., 2012; Stork et al., 2014]. These molecules could alter excitability of the neuronal terminals, ultimately affecting activity-dependent mechanisms known to be required for structural plasticity (Sivachenko et al., 2013), or be more directly involved in the remodeling process.

Circadian structural remodeling has also been described in the mammalian suprachiasmatic nucleus (SCN). Interestingly, antiphasic cyclical changes in glial coverage of VIP and AVP neurons were reported in the rat SCN and were proposed to contribute to synchronization of the clock to the light-dark cycle (Becquet et al., 2008; Girardet et al., 2010). Although, little is known about circadian structural remodeling in the mammalian brain the pervasive conservation of the mechanisms underlying the molecular clock as well as those underlying synaptic plasticity would predict conservation on this phenomenon as well.

AUTHOR CONTRIBUTIONS

AH, JD, MFC designed experiments. AH, JD performed and analyzed experiments. AH, JD, MFC wrote the manuscript.

ACKNOWLEDGMENTS

We are indebted to members of the Ceriani lab for helpful discussion. We would like to specially thank Justin Blau (NYU. USA), the Bloomington (USA) Stock Center and the Vienna Drosophila Resource Center for fly stocks. MFC is a member of the Argentine Research Council (CONICET). AH and JD are supported by fellowships from CONICET. This work was supported by a grant from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Argentina, PICT2015-2041 (to MFC) and by a FIRCA-NIH grant (1R03TW008342) to MFC. The funders had no role in study design, 8 data collection and analysis, decision to publish, or preparation of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2017.00918/full#supplementary-material

REFERENCES

Appelbaum, L., Wang, G., Yokogawa, T., Skariah, G. M., Smith, S. J., Mourrain, P., et al. (2010). Circadian and homeostatic regulation of structural synaptic plasticity in hypocretin neurons. *Neuron* 68, 87–98. doi: 10.1016/j.neuron.2010.09.006

Awasaki, T., Huang, Y., O'connor, M. B., and Lee, T. (2011). Glia instruct developmental neuronal remodeling through TGF-beta signaling. *Nat. Neurosci.* 14, 821–823. doi: 10.1038/nn.2833

Bainton, R. J., Tsai, L. T., Schwabe, T., Desalvo, M., Gaul, U., and Heberlein, U. (2005). Moody encodes two GPCRs that regulate cocaine behaviors and blood-brain barrier permeability in Drosophila. *Cell* 123, 145–156. doi: 10.1016/j.cell.2005.07.029

Barnes, C. A. (2001). Plasticity in the aging central nervous system. *Int. Rev. Neurobiol.* 45, 339–354. doi: 10.1016/S0074-7742(01)45018-5

Becquet, D., Girardet, C., Guillaumond, F., Francois-Bellan, A. M., and Bosler, O. (2008). Ultrastructural plasticity in the rat suprachiasmatic nucleus. Possible involvement in clock entrainment. *Glia* 56, 294–305. doi: 10.1002/glia.20613

Bernardinelli, Y., Nikonenko, I., and Muller, D. (2014). Structural plasticity: mechanisms and contribution to developmental psychiatric disorders. *Front. Neuroanat.* 8:123. doi: 10.3389/fnana.2014.00123

Borycz, J., Borycz, J. A., Loubani, M., and Meinertzhagen, I. A. (2002). tan and ebony genes regulate a novel pathway for transmitter metabolism at fly photoreceptor terminals. *J. Neurosci.* 22, 10549–10557.

Bosler, O., Girardet, C., Franc, J. L., Becquet, D., and Francois-Bellan, A. M. (2015). Structural plasticity of the circadian timing system. An overview from flies to mammals. *Front. Neuroendocrinol.* 38, 50–64. doi: 10.1016/j.yfrne.2015.02.001

Bozorgmehr, T., Ardiel, E. L., Mcewan, A. H., and Rankin, C. H. (2013). Mechanisms of plasticity in a *Caenorhabditis elegans* mechanosensory circuit. *Front. Physiol.* 4:88. doi: 10.3389/fphys.2013.00088

Burkeen, J. F., Womac, A. D., Earnest, D. J., and Zoran, M. J. (2011). Mitochondrial calcium signaling mediates rhythmic extracellular ATP accumulation in suprachiasmatic nucleus astrocytes. *J. Neurosci.* 31, 8432–8440. doi: 10.1523/JNEUROSCI.6576-10.2011

Ceriani, M. F., Hogenesch, J. B., Yanovsky, M., Panda, S., Straume, M., and Kay, S. A. (2002). Genome-wide expression analysis in Drosophila reveals genes controlling circadian behavior. *J. Neurosci.* 22, 9305–9319.

Chaturvedi, R., Reddig, K., and Li, H. S. (2014). Long-distance mechanism of neurotransmitter recycling mediated by glial network facilitates visual function in Drosophila. *Proc. Natl. Acad. Sci. U.S.A.* 111, 2812–2817. doi: 10.1073/pnas.1323714111

Chen, W. F., Maguire, S., Sowcik, M., Luo, W., Koh, K., and Sehgal, A. (2015). A neuron-glia interaction involving GABA transaminase contributes to sleep loss in sleepless mutants. *Mol. Psychiatry* 20, 240–251. doi: 10.1038/mp.2014.11

Damulewicz, M., Rosato, E., and Pyza, E. (2013). Circadian regulation of the Na+/K+-ATPase alpha subunit in the visual system is mediated by the pacemaker and by retina photoreceptors in *Drosophila melanogaster*. *PLoS ONE* 8:e73690. doi: 10.1371/journal.pone.0073690

Depetris-Chauvin, A., Berni, J., Aranovich, E. J., Muraro, N. I., Beckwith, E. J., and Ceriani, M. F. (2011). Adult-specific electrical silencing of pacemaker neurons uncouples molecular clock from circadian outputs. *Curr. Biol.* 21, 1783–1793. doi: 10.1016/j.cub.2011.09.027

Depetris-Chauvin, A., Fernandez-Gamba, A., Gorostiza, E. A., Herrero, A., Castano, E. M., and Ceriani, M. F. (2014). Mmp1 processing of the PDF neuropeptide regulates circadian structural plasticity of pacemaker neurons. *PLoS Genet.* 10:e1004700. doi: 10.1371/journal.pgen.1004700

Fernández, M. P., Berni, J., and Ceriani, M. F. (2008). Circadian remodeling of neuronal circuits involved in rhythmic behavior. *PLoS Biol.* 6:e69. doi: 10.1371/journal.pbio.0060069

Frank, M. G., and Cantera, R. (2014). Sleep, clocks, and synaptic plasticity. *Trends Neurosci.* 37, 491–501. doi: 10.1016/j.tins.2014.06.005

Fuentes-Medel, Y., Ashley, J., Barria, R., Maloney, R., Freeman, M., and Budnik, V. (2012). Integration of a retrograde signal during synapse formation by glia-secreted TGF-beta ligand. *Curr. Biol.* 22, 1831–1838. doi: 10.1016/j.cub.2012.07.063

Girardet, C., Becquet, D., Blanchard, M. P., Francois-Bellan, A. M., and Bosler, O. (2010). Neuroglial and synaptic rearrangements associated with photic entrainment of the circadian clock in the suprachiasmatic nucleus. *Eur. J. Neurosci.* 32, 2133–2142. doi: 10.1111/j.1460-9568.2010.07520.x

Gorostiza, E. A., Depetris-Chauvin, A., Frenkel, L., Pirez, N., and Ceriani, M. F. (2014). Circadian pacemaker neurons change synaptic contacts across the day. *Curr. Biol.* 24, 2161–2167. doi: 10.1016/j.cub.2014.07.063

Gorska-Andrzejak, J. (2013). Glia-related circadian plasticity in the visual system of Diptera. *Front. Physiol.* 4:36. doi: 10.3389/fphys.2013.00036

Gorska-Andrzejak, J., Makuch, R., Stefan, J., Gorlich, A., Semik, D., and Pyza, E. (2013). Circadian expression of the presynaptic active zone protein Bruchpilot in the lamina of *Drosophila melanogaster*. *Dev. Neurobiol.* 73, 14–26. doi: 10.1002/dneu.22032

Holtmaat, A., and Svoboda, K. (2009). Experience-dependent structural synaptic plasticity in the mammalian brain. *Nat. Rev. Neurosci.* 10, 647–658. doi: 10.1038/nrn2699

Im, S. H., Li, W., and Taghert, P. H. (2011). PDFR and CRY signaling converge in a subset of clock neurons to modulate the amplitude and phase of circadian behavior in Drosophila. *PLoS ONE* 6:e18974. doi: 10.1371/journal.pone.0018974

Lear, B. C., Zhang, L., and Allada, R. (2009). The neuropeptide PDF acts directly on evening pacemaker neurons to regulate multiple features of circadian behavior. *PLoS Biol.* 7:e1000154. doi: 10.1371/journal.pbio.1000154

Lerner, I., Bartok, O., Wolfson, V., Menet, J. S., Weissbein, U., Afik, S., et al. (2015). Clk post-transcriptional control denoises circadian transcription both temporally and spatially. *Nat. Commun.* 6:7056. doi: 10.1038/ncomms8056

Lin, Y., Stormo, G. D., and Taghert, P. H. (2004). The neuropeptide pigment-dispersing factor coordinates pacemaker interactions in the Drosophila circadian system. *J. Neurosci.* 24, 7951–7957. doi: 10.1523/JNEUROSCI.2370-04.2004

Liu, H., Zhou, B., Yan, W., Lei, Z., Zhao, X., Zhang, K., et al. (2014). Astrocyte-like glial cells physiologically regulate olfactory processing through the modification of ORN-PN synaptic strength in Drosophila. *Eur. J. Neurosci.* 40, 2744–2754. doi: 10.1111/ejn.12646

Marpegan, L., Swanstrom, A. E., Chung, K., Simon, T., Haydon, P. G., Khan, S. K., et al. (2011). Circadian regulation of ATP release in astrocytes. *J. Neurosci.* 31, 8342–8350. doi: 10.1523/JNEUROSCI.6537-10.2011

McGuire, S. E., Roman, G., and Davis, R. L. (2004). Gene expression systems in Drosophila: a synthesis of time and space. *Trends Genet.* 20, 384–391. doi: 10.1016/j.tig.2004.06.012

Mehnert, K. I., Beramendi, A., Elghazali, F., Negro, P., Kyriacou, C. P., and Cantera, R. (2007). Circadian changes in Drosophila motor terminals. *Dev. Neurobiol.* 67, 415–421. doi: 10.1002/dneu.20332

Mehnert, K. I., and Cantera, R. (2008). A peripheral pacemaker drives the circadian rhythm of synaptic boutons in Drosophila independently of synaptic activity. *Cell Tissue Res.* 334, 103–109. doi: 10.1007/s00441-008-0670-0

Mehnert, K. I., and Cantera, R. (2011). Circadian rhythms in the morphology of neurons in Drosophila. *Cell Tissue Res.* 344, 381–389. doi: 10.1007/s00441-011-1174-x

Melom, J. E., and Littleton, J. T. (2013). Mutation of a NCKX eliminates glial microdomain calcium oscillations and enhances seizure susceptibility. *J. Neurosci.* 33, 1169–1178. doi: 10.1523/JNEUROSCI.3920-12.2013

Ng, F. S., and Jackson, F. R. (2015). The ROP vesicle release factor is required in adult *Drosophila glia* for normal circadian behavior. *Front. Cell. Neurosci.* 9:256. doi: 10.3389/fncel.2015.00256

Ng, F. S., Tangredi, M. M., and Jackson, F. R. (2011). Glial cells physiologically modulate clock neurons and circadian behavior in a calcium-dependent manner. *Curr. Biol.* 21, 625–634. doi: 10.1016/j.cub.2011.03.027

Ozkaya, O., and Rosato, E. (2012). The circadian clock of the fly: a neurogenetics journey through time. *Adv. Genet.* 77, 79–123. doi: 10.1016/B978-0-12-387687-4.00004-0

Park, J. H., Helfrich-Forster, C., Lee, G., Liu, L., Rosbash, M., and Hall, J. C. (2000). Differential regulation of circadian pacemaker output by separate clock genes in Drosophila. *Proc. Natl. Acad. Sci. U.S.A.* 97, 3608–3613. doi: 10.1073/pnas.97.7.3608

Petsakou, A., Sapsis, T. P., and Blau, J. (2015). Circadian rhythms in rho1 activity regulate neuronal plasticity and network hierarchy. *Cell* 162, 823–835. doi: 10.1016/j.cell.2015.07.010

Pyza, E., and Gorska-Andrzejak, J. (2004). Involvement of glial cells in rhythmic size changes in neurons of the housefly's visual system. *J. Neurobiol.* 59, 205–215. doi: 10.1002/neu.10307

Pyza, E., and Meinertzhagen, I. A. (1999). Daily rhythmic changes of cell size and shape in the first optic neuropil in *Drosophila melanogaster*. *J. Neurobiol.* 40, 77–88. doi: 10.1002/(SICI)1097-4695(199907)40:1<77::AID-NEU7>3.0.CO;2-0

Ruiz, S., Ferreiro, M. J., Casanova, G., Olivera, A., and Cantera, R. (2010). Synaptic vesicles in motor synapses change size and distribution during the day. *Synapse* 64, 14–19. doi: 10.1002/syn.20699

Ruiz, S., Ferreiro, M. J., Menhert, K. I., Casanova, G., Olivera, A., and Cantera, R. (2013). Rhythmic changes in synapse numbers in *Drosophila melanogaster* motor terminals. *PLoS ONE* 8:e67161. doi: 10.1371/journal.pone.0067161

Rutila, J. E., Suri, V., Le, M., So, W. V., Rosbash, M., and Hall, J. C. (1998). CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of Drosophila period and timeless. *Cell* 93, 805–814. doi: 10.1016/S0092-8674(00)81441-5

Seugnet, L., Suzuki, Y., Donlea, J. M., Gottschalk, L., and Shaw, P. J. (2011). Sleep deprivation during early-adult development results in long-lasting learning deficits in adult Drosophila. *Sleep* 34, 137–146. doi: 10.1093/sleep/34.2.137

Sivachenko, A., Li, Y., Abruzzi, K. C., and Rosbash, M. (2013). The transcription factor mef2 links the Drosophila core clock to fas2, neuronal morphology, and circadian behavior. *Neuron* 79, 281–292. doi: 10.1016/j.neuron.2013.05.015

Stork, T., Sheehan, A., Tasdemir-Yilmaz, O. E., and Freeman, M. R. (2014). Neuron-glia interactions through the Heartless FGF receptor signaling pathway mediate morphogenesis of Drosophila astrocytes. *Neuron* 83, 388–403. doi: 10.1016/j.neuron.2014.06.026

Stuart, A. E., Borycz, J., and Meinertzhagen, I. A. (2007). The dynamics of signaling at the histaminergic photoreceptor synapse of arthropods. *Prog. Neurobiol.* 82, 202–227. doi: 10.1016/j.pneurobio.2007.03.006

Suh, J., and Jackson, F. R. (2007). Drosophila ebony activity is required in glia for the circadian regulation of locomotor activity. *Neuron* 55, 435–447. doi: 10.1016/j.neuron.2007.06.038

Tanoue, S., Krishnan, P., Krishnan, B., Dryer, S. E., and Hardin, P. E. (2004). Circadian clocks in antennal neurons are necessary and sufficient for olfaction rhythms in Drosophila. *Curr.Biol.* 14, 638–649. doi: 10.1016/j.cub.2004.04.009

Weber, P., Kula-Eversole, E., and Pyza, E. (2009). Circadian control of dendrite morphology in the visual system of Drosophila melanogaster. *PLoS ONE* 4:e4290. doi: 10.1371/journal.pone.0004290

Yoshii, T., Wulbeck, C., Sehadova, H., Veleri, S., Bichler, D., Stanewsky, R., et al. (2009). The neuropeptide pigment-dispersing factor adjusts period and phase of Drosophila's clock. *J. Neurosci.* 29, 2597–2610. doi: 10.1523/JNEUROSCI.5439-08.2009

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

MINI REVIEW
published: 22 December 2017
doi: 10.3389/fphys.2017.01087

Dynamic Neuron-Glia Interactions in an Oscillatory Network Controlling Behavioral Plasticity in the Weakly Electric Fish, *Apteronotus leptorhynchus*

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The involvement of glial cells in the regulation of physiological functions is being increasingly recognized, yet their role in plasticity of neural oscillators has remained largely elusive. An excellent model system to address the latter function is the pacemaker nucleus of the weakly electric fish, *Apteronotus leptorhynchus*. This brainstem oscillator drives the fish's electric organ discharge in a one-to-one fashion, with median frequencies of 880 Hz in males and 740 Hz in females. Morphometric analysis of the pacemaker nucleus has shown that astrocytes outnumber mature neurons seven-fold, and oscillator neurons even 200-fold. A similar dominance of astrocytes occurs among the adult-born cells that differentiate into glia and neurons. The astrocytes form a dense meshwork of cells interconnected by gap junctions. The degree of association of astrocytic fibers with the neural oscillator cells, and the gap-junction coupling between individual astrocytes, exhibit a sexual dimorphism, which parallels the sexual dimorphisms in the output frequency of the pacemaker nucleus, and ultimately in the electric organ discharge of the fish. It is hypothesized that the dynamics in astroglial structure mediate differences in the capacity to buffer potassium, which increases during the generation of action potentials. These differences, in turn, affect the excitability of the neural oscillator cells, and thus the output frequency of the pacemaker nucleus. Comparison of the pacemaker nucleus with other brain oscillators suggests that modulation of the output activity is one of the chief functions of the interaction of glia with the neural oscillator cells.

Keywords: neural oscillator, neuron-glia interaction, plasticity, astrocytes, gliogenesis, structural reorganization, pacemaker nucleus, *Apteronotus leptorhynchus*

OPEN ACCESS

Edited by:
Jolanta Górska-Andrzejak, Jagiellonian University, Poland

Reviewed by:
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Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: *30 October 2017*
Accepted: *11 December 2017*
Published: *22 December 2017*

Citation:
Zupanc GKH (2017) Dynamic Neuron-Glia Interactions in an Oscillatory Network Controlling Behavioral Plasticity in the Weakly Electric Fish, Apteronotus leptorhynchus. Front. Physiol. 8:1087. doi: 10.3389/fphys.2017.01087

INTRODUCTION

Since Maiken Nedergaard and Philip G. Haydon and associates reported in two independent papers in 1994 that stimulation of astrocytes results in elevated levels of Ca^{2+} in adjacent neurons (Nedergaard, 1994; Parpura et al., 1994), a plethora of studies have led to the notion that glial cells, particularly astrocytes, play an active role in the modulation of physiological functions of neural circuits. These studies have indicated that the modulatory actions of astrocytes can be grouped into two broad categories, according to the duration of their effects (Hatton, 1997; Theodosis et al., 2008; Haydon and Nedergaard, 2015). Transient changes, which operate on a second scale, are

based on molecular signals exchanged between astrocytes and neurons. Long-term changes, on the other hand, occur over minutes, hours, or days and involve restructuring of glial morphology, in addition to molecular-signaling.

Despite the recognition of astrocytes as key players in brain plasticity, their role in functional plasticity of neural oscillators has received surprisingly little attention. A neural oscillator well characterized in terms of its behavioral function and the plasticity associated with the sexually dimorphic output pattern is the pacemaker nucleus in the medulla oblongata of the weakly electric fish *Apteronotus leptorhynchus* (Dye and Meyer, 1986). Although the existence of glia in this nucleus has been known since its early morphological description (Elekes and Szabo, 1985), their potential role in the regulation of the oscillatory activity has been examined only in recent years (Sîrbulescu et al., 2014; Zupanc et al., 2014). In the following, I will summarize the key findings, discuss them in the broader context of glia-neuron interactions in oscillatory networks, and propose a model to explain how astrocytes might modulate the activity of neurons in the pacemaker nucleus to produce a sexually dimorphic behavior.

CELLULAR COMPOSITION OF THE PACEMAKER NUCLEUS

Like all apteronotids, *A. leptorhynchus* possesses an electric organ formed by modified axons of spinal motor neurons, which are commonly referred to as electromotor neurons. The synchronous depolarization of these electrocytes results in electric organ discharges (EODs) in a species-specific frequency range of 650–1,000 Hz (de Oliveira-Castro, 1955; Bennett, 1971; Waxman et al., 1972). Within this frequency band, males discharge at higher frequencies than females (**Figure 1A**; Meyer et al., 1987; Zupanc et al., 2014).

The EOD frequency is controlled, in a one-to-one fashion, by the frequency of the oscillatory network defined by two of the neuronal cell types of the pacemaker nucleus, pacemaker cells and relay cells (Elekes and Szabo, 1985; Dye and Heiligenberg, 1987). While the pacemaker cells are restricted to the pacemaker nucleus, the relay cells project down the spinal cord to form electrotonic junctions with the electromotor neurons.

Although pacemaker and relay cells are the only neuronal cells known to be involved in the generation of the oscillatory activity of the pacemaker nucleus, they are—with 87 and 20, respectively—a diminishing minority among neurons. The vast majority of neurons (all of which have been identified by their immunoreactivity against the neuron-specific marker protein Hu C/D) are small interneurons (**Figure 2A**; Elekes and Szabo, 1985; Turner and Moroz, 1995; Zupanc et al., 2014). They total ∼5,300 in mid-aged adult fish (Elekes and Szabo, 1985; Turner and Moroz, 1995; Sîrbulescu et al., 2014; Zupanc et al., 2014). Nevertheless, even when considering all three types of neurons combined, they comprise only a minute fraction of the roughly 80,000 cells whose cellular identity is known. (The pacemaker nucleus of a mid-aged fish is formed by an estimated 200,000 cells; the identity of ∼120,000 cells remains unknown). Besides neurons, stem/progenitor cells (∼35,000; see section "Development of Astrocytes and Neurons in the Adult Pacemaker Nucleus," below) and glia (∼40,000; Sîrbulescu et al., 2014) have been identified. The latter express a variety of glial marker proteins, including S100, glial fibrillary acidic protein (GFAP), glutamine synthetase, and vimentin, with a large degree of overlap among the individual expression patterns.

The above morphometric data indicate that, among identified cells, glia outnumber the three types of neurons more than 7-fold, and the pacemaker and relay cells even more than 200-fold. A glia-neuron ratio of 7:1, as found among identified cells in the pacemaker nucleus, is, by any means, extremely high. For comparison, in the spinal cord of *A. leptorhynchus* S100-expressing glia and Hu C/D-expressing neurons coexist in a 2:1 ratio (Sîrbulescu et al., 2017). Importantly, these two ratios are directly comparable, as they have been determined in the pacemaker nucleus and the spinal cord by employing identical immunostaining and image processing protocols. On the other hand, the 2:1 ratio in the spinal cord of *A. leptorhynchus* is in line with measures in various regions of rat and primate brains, which yield typical glia-to-neuron ratios between 0.5 and 2 (Hilgetag and Barbas, 2009; von Bartheld et al., 2016).

DEVELOPMENT OF ASTROCYTES AND NEURONS IN THE ADULT PACEMAKER NUCLEUS

Morphometric analysis of the pacemaker nucleus of fish of various ages has demonstrated an age-related increase in the total number of cells during adulthood, from ∼100,000 cells in young adults to 300,000 in adults toward the end of their lives (Sîrbulescu et al., 2014). However, whereas the small interneuron population grows at the same rate as the total number of pacemaker nucleus cells, no age-dependent increase in cell numbers has been found among pacemaker and relay cells—their numbers are remarkably constant over adult life.

The continuous growth of the pacemaker nucleus during adulthood is the result of continuous generation of new neurons and glial cells from adult stem/progenitor cells, most of which express the stem-cell markers Sox2 and Meis 1/2/3 (Sîrbulescu et al., 2014). However, only a minor fraction (2–3%) of them are mitotically active, whereas the rest are quiescent at any given time point. Further characterization of the stem/progenitor cells has shown that the vast majority of them express S100 and assume morphological properties of radial glia. Thus, these observations suggest that radial glia-like precursors that express Sox2 and Meis 1/2/3 serve as the adult stem/progenitor cells, which give rise to the new neurons and glia in the pacemaker nucleus. Radial glia have also been identified as endogenous multipotent stem cells in the adult mammalian brain (Doetsch et al., 1999; Laywell et al., 2000; Seri et al., 2001; Bonaguidi et al., 2011).

Combination of labeling of S-phase cells with a single pulse of the thymidine analog 5-bromo-2′-deoxyuridine, and immunostaining against the neuron-specific marker protein

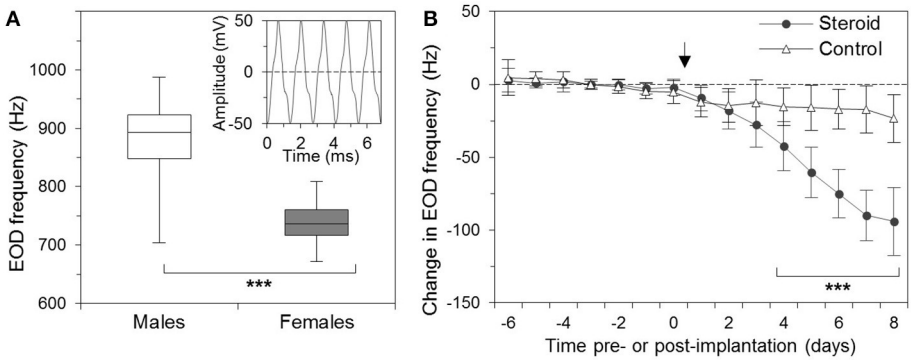

FIGURE 1 | Sexual dimorphism of the electric organ discharge (EOD) of the weakly electric fish, *Apteronotus leptorhynchus*. **(A)** Box-and-whisker plot of the EOD frequencies of males (35) and females (48), adjusted to an ambient temperature of 26°C. Whiskers mark the minimum and maximum values in each group, and boxes indicate the interquartile ranges. Middle lines denote medians. The difference in the EOD frequency between males and females is highly significant (***). The inset shows a trace of the EOD signal. **(B)** Effect of β-estradiol on the EOD frequency of *Apteronotus leptorhynchus*. The EOD frequency was determined on 7 consecutive days before the operation (days −6 through 0) and on 8 days following the surgery (days 1 through 8). Fish received implants with (circles; $n = 14$) or without (triangles; $n = 17$) β-estradiol immediately following the measurement of the EOD frequency on day 0 (indicated by arrow). For each fish, the changes in EOD frequency were quantified relative to its average frequency over the 7-day pre-implantation period. The dashed line indicates no change from this baseline. Starting with day 4 after the implantation, the EOD frequencies of the β-estradiol-treated fish were significantly different from the discharge frequencies of the controls as well as from the pre-implantation frequencies (***) (From: Zupanc et al., 2014. Copyright permission not required due to author's own work).

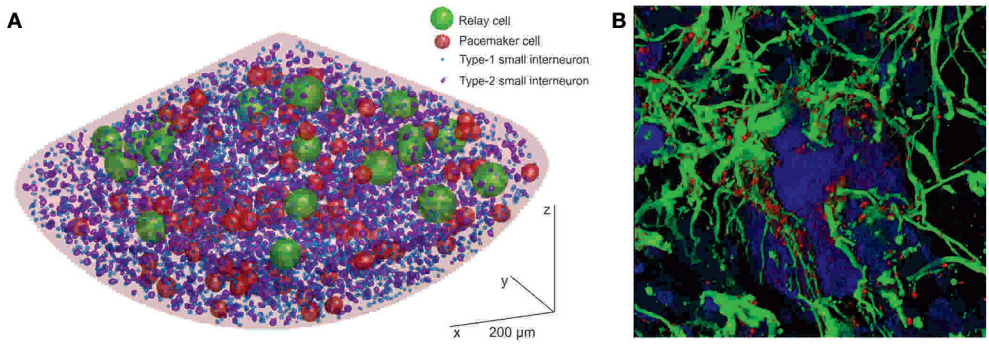

FIGURE 2 | Cellular structure of the pacemaker nucleus of *Apteronotus leptorhynchus*. **(A)** Three-dimensional model of the neural organization of the pacemaker nucleus, based on a statistical-mapping approach. Neurons form three classes—relay cells, pacemaker cells, and small interneurons—distinguished by the sizes of their cell bodies. The small interneurons can be further divided into subtypes 1 and 2. Glial cells are not shown because, due to their abundance, they would cover essentially the entire area of the nucleus, making it impossible to visualize the neuronal cells in this representation. **(B)** Association of glia with neurons in the pacemaker nucleus. The image is based on a three-dimensional shadow projection of a z-stack of confocal images. Neurons expressing Hu C/D (blue) are embedded in a dense meshwork of GFAP-expressing astrocytes (green). The glia express the gap-junction protein connexin-43 in a punctate pattern (red). In the center of the image, a pacemaker cell (P), surrounded by a dense astrocytic syncytium, is shown. Scale bar = 5 µm (After: Sîrbulescu et al., 2014. Copyright permission not required due to author's own work).

Hu C/D and the glia-specific marker protein S100 after various chase times, has revealed some notable details of the developmental pattern of the adult-born cells in the pacemaker nucleus (Sîrbulescu et al., 2014). At early stages of the cellular development, about equal portions of the progeny acquire neuronal and astrocytic identities. However, starting at about 10 days, the number of young neurons decreases, while the number of cells that differentiate into astrocytes continuously increases, resulting, at 100 days of cellular age, in a glia-to-neuron ratio of 4:1. Such a ratio is in line with the dominance of astrocytes observed among the entire population of mature cells in the pacemaker nucleus.

GLIAL DYNAMICS IN THE PACEMAKER NUCLEUS ASSOCIATED WITH BEHAVIORAL PLASTICITY

The abundance of astrocytes, relative to the number of neurons, and in particular to the number of pacemaker and relay cells, prompts the question of what role glia play in the pacemaker nucleus. Some important insights toward finding an answer to this question have been provided by analysis of the structural interconnection of pacemaker and relay cells with astrocytes, and the dynamics of this system. Three-dimensional reconstruction of pacemaker nucleus tissue based on confocal

laser scanning micrographs has revealed a dense meshwork of astrocytic fibers closely associated with the pacemaker and relay cells (**Figure 2B**; Zupanc et al., 2014), reminiscent of the close apposition of glial processes to pacemaker and relay cells revealed by electron microscopy (Elekes and Szabo, 1985). These S100-immunopositive astrocytes express high levels of connexin-43, a member of the connexin family of transmembrane gap junction proteins that plays a critical role in the formation of the astrocytic syncytium (Giaume and Liu, 2012). Thus, the morphological data suggest that the pacemaker and relay cells are embedded in a dense meshwork of astrocytes interconnected by gap junctions to form an astrocytic syncytium.

The astrocytic syncytium undergoes dynamic changes in concert with alterations in the EOD frequency. As mentioned above, males and females occupy different frequency domains within the species-specific EOD range. At a water temperature of 26°C, the mean EOD frequency of males is ∼880 Hz, whereas the mean frequency of females is ∼740 Hz (**Figure 1A**). This sexual dimorphism is controlled by steroid hormones. Chronic administration of β-estradiol results in both males and females in a gradual decrease of the EOD frequency (**Figure 1B**; Meyer et al., 1987; Schaefer and Zakon, 1996; Zupanc et al., 2014).

The sexual dimorphism in the fish's EOD frequency is paralleled by changes in the morphology and expression pattern of the astrocytes in the pacemaker nucleus (Zupanc et al., 2014). In females, the pacemaker nucleus is occupied by numerous, intensely labeled GFAP-immunoreactive fibers. In males, the number and intensity of labeling of such fibers is significantly lower. This overall difference extends to individual pacemaker and relay cells. Quantitative analysis has shown that the total GFAP labeling associated with the area covered by pacemaker and relay cells is significantly higher in females than in males. Similarly, the relative area covered by connexin-43 immunoreactivity in the region around pacemaker cells is almost 3-times larger in females than in males.

The changes in astrocytic architecture that occur as part of the development of the sexual dimorphism in the structure and function of the pacemaker nucleus could be due to the generation of new astrocytes, or the outgrowth of existing astrocytes, or both, and are probably controlled by estradiol. Evidence in support of both estradiol-induced gliogenesis and astrocytic remodeling has been obtained in mammals. In mice, sex differences have been demonstrated in cell-cycle regulation of hematopoietic stem cells. Such cells divide significantly more frequently in females than in males—an effect that can be mimicked by administration of estradiol in both males and females (Nakada et al., 2014). In the basal hypothalamus of mammals, the portion of neuronal somata covered by astrocytic processes fluctuates over the estrous cycle, being high when estrogen levels are elevated, and low when estrogen levels have decreased (García-Segura et al., 1994). The retraction and elongation of astrocytic processes over the surfaces of neurons can be mimicked by administration of estradiol to ovariectomized animals, an effect that occurs as rapidly as within 2 h after the steroid hormone application (Langle et al., 2003). It is likely that this modulatory effect of estradiol on glial structure is direct, as estrogen receptors have been found in astrocytic cells of hypothalamic areas (Langub and Watson, 1992).

WHAT IS THE FUNCTION OF ASTROCYTES IN THE PACEMAKER NUCLEUS?

The relative abundance of astrocytes, the dominance of gliogenesis over neurogenesis, the close association of astrocytic processes with pacemaker and relay cells, the structural reorganization of the astrocytic syncytium occurring in concert with sexual dimorphism-related changes in the oscillatory output frequency of the pacemaker nucleus—taken together, these observations point to a role of astrocytes in mediating the function of the pacemaker nucleus as the master oscillator in the neural network that controls the EOD frequency. However, despite this compelling correlative evidence, the role of the astrocytic meshwork in the regulation of the pacemaker frequency, and thus the EOD frequency, has remained elusive.

Given the high-frequency firing of the pacemaker and relay cells and the resulting continuous release of potassium from these cells into the extracellular space, it is plausible to hypothesize that one of the chief functions of the astrocytic meshwork in the pacemaker nucleus is to buffer the increases in K^+ concentrations. Potassium buffering by glia was first suggested over half a century ago (Hertz, 1965). Since then, a large body of experimental evidence has established astrocytic buffering of excess K^+ as a key concept of glial function in the nervous system (Kofuji and Newman, 2004). It is thought that the uptake of extracellular K^+ is primarily achieved via Na^+/K^+-ATPase, but K^+ channels have also been implicated in this function. It is, therefore, worth noting that Kv1.2 potassium channels have been identified through immunohistochemical staining in thin fibers resembling the astrocytic processes that wrap around pacemaker and relay cells, but are absent from these neurons (Smith and Zakon, 2000). Following uptake of extracellular K^+, the abundant coupling of astrocytes via gap junctions, as indicated by the intense connexin-43 immunoreactivity associated with astrocytic cells (Zupanc et al., 2014), might serve as a mechanism to aid redistribution of K^+ from sites of excessive levels to sites of lower K^+ concentrations within the pacemaker nucleus.

The astrocytic syncytium surrounding the pacemaker and relay cells may be involved not only in elimination of excess K^+ ions from the extracellular space, but also in regulating the output frequency of the pacemaker because a major determinant of the membrane potential is the extracellular K^+ concentration. An increase in the extracellular K^+ concentration leads to a reduction in the K^+ equilibrium potential, whereas a decrease in the extracellular K^+ concentration results in an increase in the K^+ equilibrium potential. In either case, these changes affect the excitability of neurons by altering the difference between the resting potential and the threshold potential at which voltage-gated sodium channels are activated (Wang et al., 2012).

Some important insights into the role of astrocytic buffering of K^+ in the generation of high-frequency oscillations has been gained by studying epilepsy and experimental models of this condition. Epileptic seizures are characterized by high-frequency oscillations (Bragin et al., 1999; Jirsch et al., 2006; Pearce et al., 2013). Impairment of K^+ buffering induced in astrocytes by altering the expression of inwardly rectifying K^+ channels

enhances the susceptibility for epileptic seizures (Steinhäuser et al., 2012). Similarly, seizures can also be triggered by increases in the extracellular K^+ concentration (Traynelis and Dingledine, 1988; Rangroo Thrane et al., 2013).

Conversely, enhancement of the efficiency in K^+ uptake and inter-astrocytic redistribution will reduce the excitability of neural networks by widening the gap between the resting membrane potential and the threshold potential at which action potentials are triggered. Such increase in astrocytic buffering capacity is predicted to result in a reduction in output frequency of neural oscillators. In the pacemaker nucleus, enhanced buffering capacity might be achieved by increasing the surface area of pacemaker and relay cells covered by astrocytic processes, and by strengthening gap junction coupling between individual astrocytes.

MODEL OF THE DEVELOPMENT OF THE SEXUALLY DIMORPHIC EOD FREQUENCY

Based on the above empirical evidence and theoretical considerations, the following working hypothesis emerges to mechanistically explain the development of the sexual dimorphism in EOD frequency of *A. leptorhynchus*: Differences in the association of the astrocytic syncytium with pacemaker and relay cells in the pacemaker nucleus develop as part of sexual differentiation under the direct control of steroid hormones, foremost estradiol. In females, the larger surface area of pacemaker and relay cells covered by astrocytic processes, accompanied by increases in specific types of K^+ channel and/or in Na^+/K^+-ATPase expression, and the stronger gap-junction coupling of the individual astrocytes within the astrocytic syncytium, serve as mechanisms to increase astrocytic K^+ buffering. As a result, the levels of extracellular K^+ are reduced. This reduction leads to a decrease in the excitability of the pacemaker and relay cells, thereby lowering the oscillator frequency of the pacemaker nucleus, and ultimately the EOD frequency of the fish.

CONCLUSIONS

Involvement of glial cells in neural oscillations has been suspected since circadian fluctuations in GFAP labeling in the suprachiasmatic nucleus of hamsters (Lavialle and Servière, 1993; Lavialle et al., 2001) and in the abundance of clock gene products in glial cells of *Drosophila* (Siwicki et al., 1988; Zerr et al., 1990; Ewer et al., 1992) were first reported. Despite accumulating (mostly circumstantial) evidence, the precise role of glia in the regulation of oscillatory activity has remained largely a matter of speculation. A key question is whether glia in different oscillatory networks share similar functions and mechanisms that mediate these functions, despite major differences in cellular organization and output patterns. Besides the pacemaker nucleus of *A. leptorhynchus*, two other oscillatory systems in which the role of glia has been studied are circadian clocks and olfactory glomeruli. While the period of the pacemaker nucleus oscillations is on the order of 1 ms, corresponding values of circadian rhythms approximate 1 day. Mitral cells in individual olfactory glomeruli of mice exhibit oscillations in membrane potential with period lengths somewhat in between these two extremes, namely ∼5 s (Roux et al., 2015).

Notwithstanding the diversity of these systems, functional studies support the notion that the role of glia in these oscillatory networks is similar. Manipulation of clock genes specifically in astrocytes within the suprachiasmatic nucleus of mice has indicated that the astrocytic circadian clock, although neither necessary nor sufficient for sustaining circadian rhythms, interacts with the neuronal clock system to modulate the period length, and thus the frequency, of the circadian rhythm (Tso et al., 2017). Similarly, disruption by toxins of the metabolism of glial cells in the visual clock system of dipterans does not abolish circadian rhythms but affects the amplitude of circadian fluctuations in the size of neurons within this system (Pyza and Górska-Andrzejak, 2004). Lastly, combination of genetic and pharmacological approaches in the olfactory bulb system has shown that the activity of gap-junction channels interconnecting astrocytes modulates the amplitude and the firing rate of mitral cells (Roux et al., 2015).

Taken together, structural and functional studies suggest that astrocytes, although not essential for the generation of neural activity in oscillatory networks, play a critical role in the modulation of the output pattern of such networks. They appear to exert this function by undergoing both short-term and long-term plastic changes in morphological structure, ion channel expression, and/or gap-junction coupling between individual astrocytic cells. Although many details of the modulatory action of glia remain to be elucidated, it becomes increasingly clear that biologically realistic models of plasticity of oscillatory neural networks will have to take the role of glia-neuron interactions in this phenomenon into account.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

REFERENCES

Bennett, M. V. L. (1971). "Electric organs," in *Fish Physiology*, *Vol. 5, Sensory Systems and Electric Organs*, eds W. S. Hoar and D. J. Randall (New York, NY: Academic Press), 347–491. doi: 10.1016/S1546-5098(08)60051-5

Bonaguidi, M. A., Wheeler, M. A., Shapiro, J. S., Stadel, R. P., Sun, G. J., Ming, G. L., et al. (2011). *In vivo* clonal analysis reveals self-renewing and multipotent adult neural stem cell characteristics. *Cell* 145, 1142–1155. doi: 10.1016/j.cell.2011.05.024

Bragin, A., Engel, J. Jr., Wilson, C. L., Fried, I., and Mathern, G. W. (1999). Hippocampal and entorhinal cortex high-frequency oscillations (100–500 Hz) in human epileptic brain and in kainic acid-treated rats with chronic seizures. *Epilepsia* 40, 127–137. doi: 10.1111/j.1528-1157.1999.tb02065.x

de Oliveira-Castro, G. (1955). Differentiated nervous fibers that constitute the electric organ of *Sternarchus albifrons*. *Linn. An. Acad. Bras. Cienc.* 27, 557–564.

Doetsch, F., Caillé, I., Lim, D. A., García-Verdugo, J. M., and Alvarez-Buylla, A. (1999). Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97, 703–716. doi: 10.1016/S0092-8674(00)80783-7

Dye, J., and Heiligenberg, W. (1987). Intracellular recording in the medullary pacemaker nucleus of the weakly electric fish, *Apteronotus*, during modulatory behaviors. *J. Comp. Physiol. A* 161, 187–200. doi: 10.1007/BF00615240

Dye, J. C., and Meyer, J. H. (1986). "Central control of the electric organ discharge in weakly electric fish," in *Electroreception,* eds T. H. Bullock and W. Heiligenberg (New York, NY: John Wiley), 71–102.

Elekes, K., and Szabo, T. (1985). Synaptology of the medullary command (pacemaker) nucleus of the weakly electric fish (*Apteronotus leptorhynchus*) with particular reference to comparative aspects. *Exp. Brain Res.* 60, 509–520. doi: 10.1007/BF00236936

Ewer, J., Frisch, B., Hamblen-Coyle, M. J., Rosbash, M., and Hall, J. C. (1992). Expression of the period clock gene within different cell types in the brain of *Drosophila* adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. *J. Neurosci.* 12, 3321–3349.

García-Segura, L. M., Chowen, J. A., Párducz, A., and Naftolin, F. (1994). Gonadal hormones as promoters of structural synaptic plasticity: cellular mechanisms. *Prog. Neurobiol.* 44, 279–307. doi: 10.1016/0301-0082(94)90042-6

Giaume, C., and Liu, X. (2012). From a glial syncytium to a more restricted and specific glial networking. *J. Physiol.* 106, 34–39. doi: 10.1016/j.jphysparis.2011.09.001

Hatton, G. I. (1997). Function-related plasticity in hypothalamus. *Annu. Rev. Neurosci.* 20, 375–397. doi: 10.1146/annurev.neuro.20.1.375

Haydon, P. G., and Nedergaard, M. (2015). How do astrocytes participate in neural plasticity? *Cold Spring Harb. Perspect. Biol.* 7:a020438. doi: 10.1101/cshperspect.a020438

Hertz, L. (1965). Possible role of neuroglia: a potassium-mediated neuronal-neuroglial-neuronal impulse transmission system. *Nature* 206, 1091–1094. doi: 10.1038/2061091a0

Hilgetag, C. C., and Barbas, H. (2009). Are there ten times more glia than neurons in the brain? *Brain Struct. Funct.* 213, 365–366. doi: 10.1007/s00429-009-0202-z

Jirsch, J. D., Urrestarazu, E., LeVan, P., Olivier, A., Dubeau, F., and Gotman, J. (2006). High-frequency oscillations during human focal seizures. *Brain* 129, 1593–1608. doi: 10.1093/brain/awl085

Kofuji, P., and Newman, E. A. (2004). Potassium buffering in the central nervous system. *Neuroscience* 129, 1045–1056. doi: 10.1016/j.neuroscience.2004.06.008

Langle, S. L., Poulain, D. A., and Theodosis, D. T. (2003). Induction of rapid, activity-dependent neuronal-glial remodelling in the adult rat hypothalamus *in vitro*. *Eur. J. Neurosci.* 18, 206–214. doi: 10.1046/j.1460-9568.2003.02741.x

Langub, M. C. Jr., and Watson, R. E. Jr. (1992). Estrogen receptor-immunoreactive glia, endothelia, and ependyma in guinea pig preoptic area and median eminence: electron microscopy. *Endocrinology* 130, 364–372. doi: 10.1210/endo.130.1.1727710

Lavialle, M., Begue, A., Papillon, C., and Vilaplana, J. (2001). Modifications of retinal afferent activity induce changes in astroglial plasticity in the hamster circadian clock. *Glia* 34, 88–100. doi: 10.1002/glia.1044

Lavialle, M., and Servière, J. (1993). Circadian fluctuations in GFAP distribution in the Syrian hamster suprachiasmatic nucleus. *Neuroreport* 4, 1243–1246. doi: 10.1097/00001756-199309000-00008

Laywell, E. D., Rakic, P., Kukekov, V. G., Holland, E. C., and Steindler, D. A. (2000). Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. *Proc. Natl. Acad. Sci. U.S.A.* 97, 13883–13888. doi: 10.1073/pnas.250471697

Meyer, J. H., Leong, M., and Keller, C. H. (1987). Hormone-induced and maturational changes in electric organ discharges and electroreceptor tuning in the weakly electric fish *Apteronotus*. *J. Comp. Physiol. A* 160, 385–394. doi: 10.1007/BF00613028

Nakada, D., Oguro, H., Levi, B. P., Ryan, N., Kitano, A., Saitoh, Y., et al. (2014). Oestrogen increases haematopoietic stem-cell self-renewal in females and during pregnancy. *Nature* 505, 555–558. doi: 10.1038/nature12932

Nedergaard, M. (1994). Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science* 263, 1768–1771. doi: 10.1126/science.8134839

Parpura, V., Basarsky, T. A., Liu, F., Jeftinija, K., Jeftinija, S., and Haydon, P. G. (1994). Glutamate-mediated astrocyte-neuron signalling. *Nature* 369, 744–747. doi: 10.1038/369744a0

Pearce, A., Wulsin, D., Blanco, J. A., Krieger, A., Litt, B., and Stacey, W. C. (2013). Temporal changes of neocortical high-frequency oscillations in epilepsy. *J. Neurophysiol.* 110, 1167–1179. doi: 10.1152/jn.01009.2012

Pyza, E., and Górska-Andrzejak, J. (2004). Involvement of glial cells in rhythmic size changes in neurons of the housefly's visual system. *J. Neurobiol.* 59, 205–215. doi: 10.1002/neu.10307

Rangroo Thrane, V., Thrane, A. S., Wang, F., Cotrina, M. L., Smith, N. A., Chen, M., et al. (2013). Ammonia triggers neuronal disinhibition and seizures by impairing astrocyte potassium buffering. *Nat. Med.* 19, 1643–1648. doi: 10.1038/nm.3400

Roux, L., Madar, A., Lacroix, M. M., Yi, C., Benchenane, K., and Giaume, C. (2015). Astroglial connexin 43 hemichannels modulate olfactory bulb slow oscillations. *J. Neurosci.* 35, 15339–15352. doi: 10.1523/JNEUROSCI.0861-15.2015

Schaefer, J. E., and Zakon, H. H. (1996). Opposing actions of androgen and estrogen on *in vitro* firing frequency of neuronal oscillators in the electromotor system. *J. Neurosci.* 16, 2860–2868.

Seri, B., García-Verdugo, J. M., McEwen, B. S., and Alvarez-Buylla, A. (2001). Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J. Neurosci.* 21, 7153–7160.

Sîrbulescu, R. F., Ilieş, I., Meyer, A., and Zupanc, G. K. H. (2017). Additive neurogenesis supported by multiple stem cell populations mediates adult spinal cord development: a spatiotemporal statistical mapping analysis in a teleost model of indeterminate growth. *Dev. Neurobiol.* 77, 1269–1307. doi: 10.1002/dneu.22511

Sîrbulescu, R. F., Ilieş, I., and Zupanc, G. K. (2014). Quantitative analysis reveals dominance of gliogenesis over neurogenesis in an adult brainstem oscillator. *Dev. Neurobiol.* 74, 934–952. doi: 10.1002/dneu.22176

Siwicki, K. K., Eastman, C., Petersen, G., Rosbash, M., and Hall, J. C. (1988). Antibodies to the period gene product of *Drosophila* reveal diverse tissue distribution and rhythmic changes in the visual system. *Neuron* 1, 141–150. doi: 10.1016/0896-6273(88)90198-5

Smith, G. T., and Zakon, H. H. (2000). Pharmacological characterization of ionic currents that regulate the pacemaker rhythm in a weakly electric fish. *J. Neurobiol.* 42, 270–286. doi: 10.1002/(SICI)1097-4695(20000205)42:2<270::AID-NEU10>3.0.CO;2-V

Steinhäuser, C., Seifert, G., and Bedner, P. (2012). Astrocyte dysfunction in temporal lobe epilepsy: K^+ channels and gap junction coupling. *Glia* 60, 1192–1202. doi: 10.1002/glia.22313

Theodosis, D. T., Poulain, D. A., and Oliet, S. H. (2008). Activity-dependent structural and functional plasticity of astrocyte-neuron interactions. *Physiol. Rev.* 88, 983–1008. doi: 10.1152/physrev.00036.2007

Traynelis, S. F., and Dingledine, R. (1988). Potassium-induced spontaneous electrographic seizures in the rat hippocampal slice. *J. Neurophysiol.* 59, 259–276.

Tso, C. F., Simon, T., Greenlaw, A. C., Puri, T., Mieda, M., and Herzog, E. D. (2017). Astrocytes regulate daily rhythms in the suprachiasmatic nucleus and behavior. *Curr. Biol.* 27, 1055–1061. doi: 10.1016/j.cub.2017.02.037

Turner, R. W., and Moroz, L. L. (1995). Localization of nicotinamide adenine dinucleotide phosphate-diaphorase activity in electrosensory and electromotor systems of a gymnotiform teleost, *Apteronotus leptorhynchus*. *J. Comp. Neurol.* 356, 261–274. doi: 10.1002/cne.903560209

von Bartheld, C. S., Bahney, J., and Herculano-Houzel, S. (2016). The search for true numbers of neurons and glial cells in the human brain: a review of 150 years of cell counting. *J. Comp. Neurol.* 524, 3865–3895. doi: 10.1002/cne.24040

Wang, F., Smith, N. A., Xu, Q., Fujita, T., Baba, A., Matsuda, T., et al. (2012). Astrocytes modulate neural network activity by Ca^{2+}-dependent uptake of extracellular K^+. *Sci. Signal.* 5:ra26. doi: 10.1126/scisignal.2002334

Waxman, S. G., Pappas, G. D., and Bennett, M. V. L. (1972). Morphological correlates of functional differentiation of nodes of Ranvier along single fibers in the neurogenic electric organ of the knife fish Sternarchus. *J. Cell Biol.* 53, 210–224. doi: 10.1083/jcb.53.1.210

Zerr, D. M., Hall, J. C., Rosbash, M., and Siwicki, K. K. (1990). Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of *Drosophila*. *J. Neurosci.* 10, 2749–2762.

Zupanc, G. K. H., Ilieș, I., Sîrbulescu, R. F., and Zupanc, M. M. (2014). Large-scale identification of proteins involved in the development of a sexually dimorphic behavior. *J. Neurophysiol.* 111, 1646–1654. doi: 10.1152/jn.00750.2013

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 20 February 2019
doi: 10.3389/fphys.2019.00133

The RNA Helicase BELLE Is Involved in Circadian Rhythmicity and in Transposons Regulation in *Drosophila melanogaster*

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OPEN ACCESS

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Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: 31 October 2017
Accepted: 04 February 2019
Published: 20 February 2019

Citation:
Cusumano P, Damulewicz M, Carbognin E, Caccin L, Puricella A, Specchia V, Bozzetti MP, Costa R and Mazzotta GM (2019) The RNA Helicase BELLE Is Involved in Circadian Rhythmicity and in Transposons Regulation in Drosophila melanogaster. Front. Physiol. 10:133. doi: 10.3389/fphys.2019.00133

Circadian clocks control and synchronize biological rhythms of several behavioral and physiological phenomena in most, if not all, organisms. Rhythm generation relies on molecular auto-regulatory oscillations of interlocked transcriptional-translational feedback loops. Rhythmic clock-gene expression is at the base of rhythmic protein accumulation, though post-transcriptional and post-translational mechanisms have evolved to adjust and consolidate the proper pace of the clock. In *Drosophila*, BELLE, a conserved DEAD-box RNA helicase playing important roles in reproductive capacity, is involved in the small RNA-mediated regulation associated to the piRNA pathway. Here, we report that BELLE is implicated in the circadian rhythmicity and in the regulation of endogenous transposable elements (TEs) in both nervous system and gonads. We suggest that BELLE acts as important element in the piRNA-mediated regulation of the TEs and raise the hypothesis that this specific regulation could represent another level of post-transcriptional control adopted by the clock to ensure the proper rhythmicity.

Keywords: RNA helicase BELLE, *Drosophila*, post-transcriptional control, circadian clock, piRNA-mediated transposons regulation

INTRODUCTION

Circadian clocks have evolved in most, if not all, organisms to time their physiology and behavior to the most appropriate phases of the day–night cycles (Young and Kay, 2001; Dubruille and Emery, 2008; Peschel and Helfrich-Forster, 2011). They control and synchronize the biological rhythms of a vast array of behavioral and physiological phenomena, including visual sensitivity, hormone secretion, locomotion, hatching, eclosion, ecdysteroid synthesis, sperm release, gene expression, and sleep wake cycle (Sandrelli et al., 2008; Allada and Chung, 2010; Schiesari et al., 2011). Biological clocks are self-sustained in the absence of environmental cues. Rhythm generation occurs in single cells and is based on molecular auto-regulatory oscillations of interlocked transcriptional-translational feedback loops. In *Drosophila*, the transcription factors CLOCK (CLK) and CYCLE (CYC) activate, as heterodimer, the expression of the clock genes *period* (*per*) and *timeless* (*tim*). The correspondent proteins, PER and TIM, are post-translational regulated and eventually accumulate in the cytoplasm and enter the nucleus, where they ultimately inhibit their own transcription by

repressing CLK-CYC activity. Two other interlocked feedback loops regulating the expression of the *Clock* gene and CLK/CYC mediated transcription, respectively, contribute to the robustness of the circadian oscillations (reviewed in Ozkaya and Rosato, 2012).

Rhythmic clock-gene expression is important to generate rhythmic protein accumulation; however, cycling protein levels do not depend only on cycling mRNAs but post-transcriptional and post-translational mechanisms have also evolved to adjust and consolidate the proper pace of the clock (Kojima et al., 2011). Among these, alternative splicing, mRNA nuclear export, polyadenilation, regulation of translation and degradation, play fundamental roles in the post-transcriptional regulation of circadian gene expression (Majercak et al., 1999, 2004; Collins et al., 2004; Boothroyd et al., 2007; Kojima et al., 2012; Huang et al., 2013; MacGregor et al., 2013; Robles et al., 2014; Montelli et al., 2015).

In recent years, a role for small, non-coding RNAs (miRNA) as post-transcriptional regulators of circadian rhythmicity in *Drosophila* has been also established. They regulate the proper development and maintenance of circadian rhythms acting either directly on the expression of clock genes or indirectly on signaling outputs (reviewed in Xue and Zhang, 2018).

Another class of small RNA, the piRNAs, has recently been associated to the age-dependent rhythmicity (Kuintzle et al., 2017). In particular, putative primary piRNA transcripts corresponding to transposons exhibit a *de novo* oscillation in old flies and circadian clock components appear to regulate these late rhythmicity (Kuintzle et al., 2017). piRNAs are small molecules protecting animal cells from the de-regulation of transposons and other repetitive genetic elements, preserving genome stability (Li et al., 2009; Thomson and Lin, 2009; Le Thomas et al., 2014). The piRNA biogenesis starts with the transcription of genomic clusters dispersed in the genome, producing the primary piRNA precursor transcripts (Brennecke et al., 2007). The piRNA pathway was first discovered in the gonads (Vagin et al., 2006; Aravin et al., 2007; Brennecke et al., 2007; Specchia et al., 2008, 2010, 2017; Specchia and Bozzetti, 2009; Bozzetti et al., 2015; Iwasaki et al., 2015; Sahin et al., 2016), but later it was observed in the nervous system of both *Drosophila* and mice (Lee et al., 2011; Perrat et al., 2013).

belle emerged in a screen for genes involved in the RNAi pathway: it binds to Ago1 and Ago2, involved in the small RNA-mediated regulation (Kim et al., 2005; Ulvila et al., 2006; Zhou et al., 2008). Very recently, a role of *belle* in the regulation on specific P-derived constructs in the ovary has been described (Lo et al., 2016), and therefore it is now considered a "bona fide" piRNA gene. *belle* encodes a DEAD-box RNA helicase: it is the closest paralog of *vasa* and plays roles in the reproductive system allowing fertility in *Drosophila melanogaster*. Like other members of the DEAD box family, Vasa and BELLE are supposed to have ATPase, RNA binding, and RNA unwinding activities (Liang et al., 1994; Sengoku et al., 2006). BELLE is essential for both male and female reproductive capacity and its function in gonads is conserved during evolution. It localizes at the perinuclear region, called "nuage," of the germ cells in male and female gonads (Johnstone et al., 2005; Kibanov et al., 2011), where the majority of key components regulating transposable elements (TEs) are located (Lim and Kai, 2007; Klattenhoff and Theurkauf, 2008; Kibanov et al., 2011; Nagao et al., 2011).

Here, we have identified for BELLE important roles as putative circadian clock component and regulator of TEs in brain and in gonads. These apparently unrelated functions suggest the hypothesis that BELLE might be a key element in small RNA (piRNA)-mediated regulation of the TEs and that this regulatory pathway is involved in circadian rhythmicity.

MATERIALS AND METHODS

Fly Strains

The following strains of *Drosophila melanogaster* were used: w^{1118}, *CantonS*, *yw;tim*-Gal4 (Emery et al., 1998), *UAS-belle*RNAi (VDRC #6299), *GMR*-Gal4 (BDSC #8440), *ninaE*-Gal4 (BDSC #1104), *repo*-Gal4 (BDSC #7415), *pdf*-Gal4 (BDSC #6900), *Eaat1*-Gal4 (BDSC #8849), *Lama*-Gal4 (BDSC #35543), per^0 (Smith and Konopka, 1981), *yw*;P{EPgy2}$bel^{EY08943}$ (BDSC #19945); P{PZ}bel^{cap-1} ry^{506}/TM3, ry^{RK} Sb^1 Ser^1 (BDSC #11778), *bel::GFP* (Morin et al., 2001) (Kyoto Stock Centre #*ZCL2696*), *UAS-CD8-GFP* (BDSC #5137). Co-IP was performed with *yw; tim-GAL4/+; UAS-HAcry/+* (Dissel et al., 2004). Flies were maintained on a standard cornmeal medium under LD 12:12 regime and at constant 23°C.

Co-immunoprecipitation, Mass Spectrometry Analysis, and Western Blot

Head extracts from overexpressing HACRY flies raised in 12:12 light:dark cycles and collected at Zeitgeber Time 24 (ZT24), before lights on, and after a 15-min light pulse were subjected to coimmunoprecipitation and Mass Spectrometry analysis as previously described (Mazzotta et al., 2013). Immunocomplexes were analyzed by Western blot using the following antibodies: rabbit polyclonal anti-BELLE (1:2000) (Johnstone et al., 2005) and mouse anti-HA (1:5000; Sigma Aldrich, St. Louis, MO, United States). Anti-rabbit IgG HRP (1:3000; BioRad Laboratories, Hercules, CA, United States) and anti-mouse IgG HRP (1:5000; Sigma Aldrich, St. Louis, MO, United States) were used as secondary antibodies.

RNA Extraction and qRT-PCR

RNA was extracted using the reagent Trizol (Invitrogen) as indicated in manufacturer's instructions. For the analysis of *belle* expression, around 50 heads from flies reared in LD cycles for at least 3 days and collected at the indicated time points were used. For the analysis of transposons 30 mg of male or female gonadal tissues or heads were used. 1 μg of total RNA was used as a template for oligonucleotide dT-primed reverse transcription using SuperScriptIII RNaseH-reverse transcriptase (Invitrogen), according to manufacturer's instructions. qRT-PCR was performed in the 7500 Real-TimePCR System (Applied Biosystem) using SYBR green (Celbio) according to the manufacturer's instruction. For quantification of the transcripts, we used the $2\Delta\Delta Ct$ method as previously reported

(Specchia et al., 2010). The primers used are listed in **Supplementary Table S1**.

Immunocytochemistry

For the analysis of BELLE localization, heads from 7 days old males were fixed in 4% paraformaldehyde in phosphate buffer saline (PBS; pH 7.4) for 4 h, then they were washed in PBS twice and cryoprotected by incubation in 12.5% sucrose for 10 min and in 25% sucrose at 4°C overnight. Material was embedded in Tissue-Tek, frozen in liquid nitrogen, and cryostat 20 μm sections were cut. The sections were washed in PBS for 30 min and five times in phosphate buffer (PB) with an addition of 0.2% Triton X-100 (PBT). After that, sections were incubated in 5% normal goat serum (NGS) with an addition of 0.5% bovine serum albumin (BSA) for 30 min first at room temperature, and then incubated with primary antibodies anti-REPO (mouse, 1:500, Hybridoma Bank), anti-Ebony (rabbit, 1:750, a gift from Dr. B. T. Hovemann, Ruhr Universität Bochum) and anti-GFP (rabbit, 1:1000, Novus Biologicals) for 24 h. Afterward, sections were washed six times in PBT/BSA, and blocked in 5% NGS for 45 min. After that, goat anti-mouse (conjugated with Cy3) and goat anti-rabbit (conjugated with Alexa 488) secondary antibodies (both 1:500, Jackson Immuno Research) were applied overnight in 4°C. Finally, sections were washed twice in BSA, six times in PBT, and twice in PBS. Then, cryosections were mounted in Vectashield medium (Vector) and examined with a Zeiss Meta 510 Laser Scanning Microscope. About 30 brains or cryosections were checked.

For the analysis of PER in clock-neurons, flies were synchronized by LD cycles at least for 3 days and then collected under LD or DD conditions at the indicated time points. Whole flies were quickly fixed for 2 h in PFA 4%. About 10–12 brains were dissected in PBS and then treated as previously described (Vanin et al., 2012). Average fluorescence values are reported.

Male and Female Fertility Analysis

One young male was mated to three control virgin females, and three virgin females were mated with three control males. Ten individual males and 30 females were tested for each genotype. After 4 days, the crosses were transferred to a fresh vial. The parental flies were removed from the last vial after an additional 4 days. The number of the adult progeny from each vial was counted.

Behavioral Analysis

Locomotor activity of individual flies was recorded for 3 days in LD and 7 days in DD using the Drosophila Activity Monitoring System (TriKinetics, Inc., Waltham, MA, United States). Data were collected every 5 min and then analyzed in 30 min bins using spectral analysis and autocorrelation (Zordan et al., 2007). Morning anticipations was detected fly-by-fly examining the activity mean of 3 days under LD conditions (Vanin et al., 2012) and the Morning Index was calculated as in Seluzicki et al. (2014).

Statistical Analyses

For comparisons between two measurements a two-tailed Student's *t*-test was used to show the significance level of the replicated experiments. Expression profile's significance was evaluated by non-parametric ANOVA, Kruskal–Wallis test (p-value < 0.05) whereas putative period and phase of oscillation were identified by RAIN non-parametric test for independent samples (adjusted p-value < 0.05) (Thaben and Westermark, 2014).

RESULTS

dCRY Interacts With the RNA Helicase BELLE

In an experiment aimed at the identification of new genes involved in the circadian machinery in *Drosophila*, a coimmunoprecipitation assay followed by mass spectrometry analysis was performed on transgenic flies overexpressing a hemagglutinin (HA)-tagged form of dCRY (HACRY) raised in 12:12 light:dark cycles and collected at Zeitgeber Time 24 (ZT24), before lights on, and after a 15-min light pulse (Mazzotta et al., 2013).

A ∼85-kDa species was observed in the sample in the dark that was not present in the negative control (**Figure 1A**). This protein band was digested in-gel, and the peptide mixtures analyzed by liquid chromatography–mass spectrometry (LC-MS)/MS using an ESI-QTOF mass spectrometer (Wilm et al., 1996). Analysis of the MS/MS data using the MASCOT software yielded the identification of BELLE (**Supplementary Table S2**), a DEAD box RNA helicase involved in RNA metabolism at several levels (Linder and Jankowsky, 2011).

The presence of BELLE in the complex with HACRY was also confirmed by Western blot with an anti-BELLE antibody (Johnstone et al., 2005). By this procedure, BELLE was also detected after a 15 min of light pulse (**Figure 1B**). The same blot was hybridized also with an anti-HA antibody, in order to assess the specificity of the interaction (**Figure 1B**). The signal corresponding to BELLE was more intense in head collected in the dark; in fact after 15 min of light pulse the amount of BELLE was lower, in line with that of HACRY that decreased due to the well-known light-dependent degradation of CRY (Peschel et al., 2009).

The RNA Helicase BELLE Is Expressed in the Fly Brain

It is known that BELLE is expressed in germ cells throughout all developmental stages and in larval neurons (Johnstone et al., 2005; Rolls et al., 2007), but little information was available regarding its expression in the adult fly head.

In order to characterize BELLE expression in the fly brain, we performed an immunocytochemistry experiment on flies carrying a GFP exon trap in *belle* (*bel::GFP*) (Morin et al., 2001). Flies were maintained in standard light–dark conditions, brains were dissected and an anti-GFP antibody was used to detect BEL::GFP expression. We observed BELLE expression in clock neurons and in glial cells in the optic lobe, with a predominantly cytoplasmic localization (**Figures 2**, **3**). In clock neurons, BELLE was expressed in either the small or the

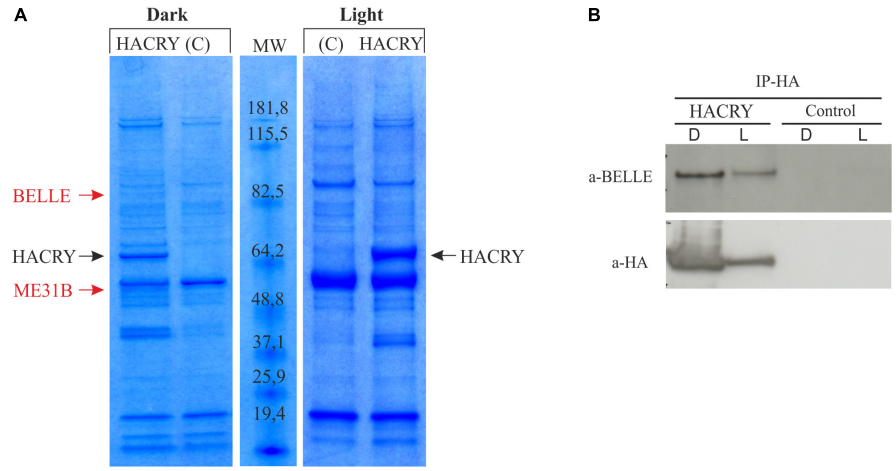

FIGURE 1 | dCRY interacts with BELLE. **(A)** Coomassie blue-stained gel of heads protein extracts coimmunoprecipitated with an anti-HA antibody. HACRY-overexpressing flies (HACRY, *yw;tim-GAL4/+; UASHAcry/+*) and relative controls (C, *yw;tim-GAL4*) were reared in 12:12 light: dark and collected in the dark (ZT24) and in the light (ZT24 + 15-min light pulse). Molecular masses of markers are indicated (BenchMark Pre-Stained Protein Ladder; Invitrogen). MW, molecular weight. Bands corresponding to HACRY are indicated in black, while stained proteins excised and characterized by mass spectrometry are indicated in red. **(B)** Co-immunoprecipitation and Western blot confirming the interaction between HACRY and BELLE in HACRY-overexpressing flies (HACRY, *yw;tim-GAL4/+; UASHAcry/+*). *tim-GAL4* flies were used as control. Heads were collected as in **(A)**. Membranes were probed with anti-BELLE and anti-HA antibodies.

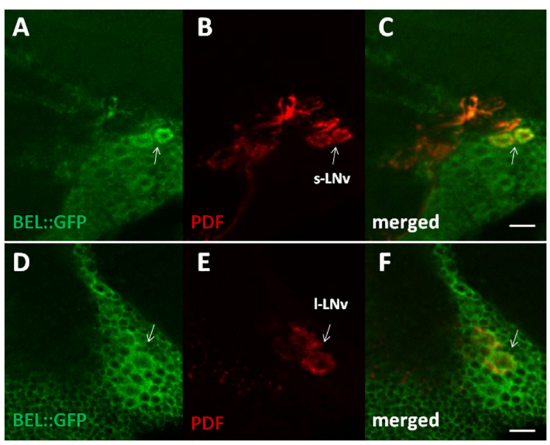

FIGURE 2 | BELLE localizes in the main pacemaker cells in the brain of *Drosophila melanogaster*. Confocal images of whole mount of adult brain expressing BELLE fused with green fluorescent protein (*bel::GFP*). Scale bar: 10 μm. **(A,D)** Anti-GFP immunostaining (BEL::GFP). **(B,E)** Anti-PDF was used to label PDF-expressing ventral lateral neurons. Depends on optical section small LNv (s-LNv) or large LNv (l-LNv) are visualized. **(C,F)** BEL::GFP and PDF immune staining co-localize in the area of LNv cell bodies.

large ventral lateral neurons (s-LNvs and l-LNvs respectively) (**Figure 2**), PDF-expressing cells that act in promoting and governing the circadian locomotor behavior. In glia we observed BELLE expression in the epithelial glia and a weak labeling also in the lamina (**Figures 3D–F**): position and shape of these GFP- labeled cells suggest they may be R1-R6 photoreceptor terminals (**Figures 3D–F**); however, anti-REPO staining showed glia cells nuclei at the top of each structure (**Figure 3G**). Observed columnar shape is also similar to anti-EBONY staining (**Figure 3H**), which is a marker for epithelial glia (Richard et al., 2002), suggesting that BELLE may be located in epithelial glia in the lamina. Moreover, we observed immunostaining for BEL::GFP also in giant inner chiasm glia, which connects the medulla with the lobula complex in the optic lobe (Kremer et al., 2017). In these cells, both BELLE and PER are expressed (**Supplementary Figure S1**).

Belle Expression Shows Circadian Features

In order to characterize the temporal expression of *belle*, mRNA and protein levels were analyzed during the 24 h. Wild-type flies *white*^{1118} were synchronized by 12:12 LD cycles and collected every 3 h, either in LD regime or at the third day after being transferred to constant darkness (DD).

belle expression appeared to oscillate both in LD and in DD conditions. In particular, in LD cycles the level of differential expression during the 24 h was just below the limit of significance (Kruskal–Wallis *p*-value 0.06); nevertheless, the algorithm RAIN suggested the presence of a rhythmic expression pattern (adjusted *p*-value 2,61E-02) with a peak at ZT12 (**Figure 4A**). In constant darkness *belle* mRNA showed differential expression around the 24 h (Kruskal–Wallis *p*-value 0.03), and a peak at CT63 was identified (RAIN *p*-value 1,92E-05) (**Figure 4B**). These results are in line with a previous microarray study showing a peak of expression for *belle* at ZT17 (Claridge-Chang et al., 2001). In *per*^{0} mutant, *belle* oscillation is maintained in LD cycles, although with a peak at ZT3 (Kruskal–Wallis *p*-value 0.02, RAIN *p*-value 4,91E-07) (**Figure 4A**), while it was lost in constant conditions (Kruskal–Wallis *p*-value 0.609) (**Figure 4B**).

The analysis of BELLE in fly heads by western blot showed no significant protein oscillation, neither in LD nor in DD (Kruskal–Wallis *p*-values 0.9 and 0.8, respectively) (**Supplementary**

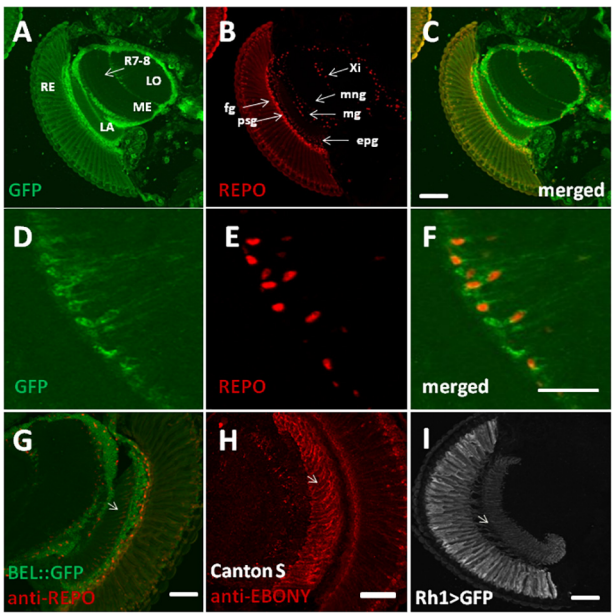

FIGURE 3 | BELLE localizes in the visual system of *Drosophila melanogaster*. Confocal images of horizontal cryosection of adult brain expressing BELLE fused with green fluorescent protein (*bel::GFP*). Anti-REPO was used to label all glia nuclei. **(A)** BEL::GFP signal is localized in the optic neuropils: lamina (LA), medulla (ME), and lobula (LO). Weak signal is visible in R7 and R8 photoreceptor terminals (R7-8) in the medulla. Signal in the retina (RE) is probably due to autofluorescence. **(B)** Anti-REPO staining allowed recognized different glia types: fenestrated glia (fg), pseudocartridge glia (psg), epithelial glia (epg), medulla glia (mg), marginal glia (mng), and inner chiasm glia (Xi). **(C)** Merged signal showed localization of BELLE in glial cells. Bar 50 μm. **(D–F)** Higher magnification of inner chiasm glia showed that BELLE is localized mostly in the cytoplasm. Bar 20 μm. **(G–I)** Localization of BELLE in the first optic neuropile, called lamina, analyzed on confocal images of horizontal cryosection of adult brain. Bar: 50 μm. **(G)** Anti-GFP and Anti-REPO immunostaining showing that BEL::GFP signal is higher in the distal lamina (close to retina), where interneurons cell bodies are located. Weak labeling is also visible across the lamina, forming regular columns. Glia nuclei staining overlaps with observed BELLE location, suggesting that this signal originates from epithelial glia. **(H)** Section of wild-type *CantonS* brain stained with anti-EBONY to label epithelial glial cells in the lamina and their specific columnar shape (see Richard et al., 2002). **(I)** Anti-GFP staining on section of Rh1 > GFP brains showing R1–R6 photoreceptors terminals in the lamina.

Information and Supplementary Figure S2). This result is in line with that obtained by co-immunoprecipitation (**Figure 1B**): the lower amount of BELLE pulled down in light compared to dark uniquely depends on the light-mediated degradation of CRY.

BELLE Affects Circadian Locomotor Activity

In order to unravel a possible involvement of *belle* in the generation of circadian rhythmicity in *Drosophila*, we depleted the expression of the gene by RNAi in both glia and clock neurons.

When we knocked down *belle* with the pan-glial driver *repo*-Gal4 (Awasaki and Ito, 2004) and *lama*-Gal4, a driver for glial precursor cells, lamina precursor cells and lamina neurons (Apitz and Salecker, 2016), we observed developmental lethality. The same result was obtained by KD in all canonical clock neurons and clock relevant glial cells expressing *timeless* (with a *tim*-Gal4) (Emery et al., 1998). This result is in agreement to what reported for the null allele of *belle* (Johnstone et al., 2005). When we depleted *belle* in PDF expressing neurons and a restricted subset of glial cells (with *pdf*-Gal4 and *Eaat1*-Gal4, respectively) (Renn et al., 1999; Rival et al., 2004), flies were viable and endogenous rhythmicity was not altered (**Table 1**); only a slight impairment in the morning anticipation of locomotor activity in LD was observed (**Table 2**). In contrast, RNAi against *belle* in the photoreceptor cells (*GMR*-Gal4 and *ninaE*-Gal4) did not affect either vitality or behavior (**Supplementary Table S3**).

We then decided to investigate a possible role for *belle* in the circadian machinery using two viable mutant lines generated by p-element insertion in the regulatory region ($belle^{cap-1}$ and $belle^{EY08943}$) (Bellen et al., 2004). Flies were entrained for 3 days in light dark cycles at constant temperature (23°) followed by 7 days of constant darkness. Either mutant exhibited an impairment of the locomotor behavior (**Figure 5**): in LD conditions, both lines presented the canonical bimodal profile, but a severe loss of the morning anticipation of the locomotor activity was displayed (**Figure 5A** and **Table 3**). A high percentage of flies showed also an arrhythmic behavior in DD, although no defects were monitored concerning the period, which was comparable to wild-type (**Figure 5B** and **Table 4**).

Altered PER Cycling in Circadian Pacemaker Neurons in Belle Mutants

We performed PER staining on brains dissected every 4 h from flies collected under LD cycles and during the 5th day of DD conditions, in order to assess whether the impairment of the circadian locomotor activity of the *belle* mutants could be due to an altered PER cycling in the pacemaker neurons. PDF expressing cells were identified by co-staining for this peptide.

Mutant lines showed defects in some clusters of neurons. Under LD conditions, $belle^{cap-1}$ flies exhibited a significant reduction of oscillation amplitude in both s-LNvs and l-LNvs, particularly marked at ZT0 (**Figure 6**). Similarly, in constant darkness, a general decrease of PER staining was observed compared to wild-type, which was particularly enhanced during the subjective night/beginning of the subjective day (**Figure 7**). Quantification of PER levels indicated that the greatest effect occurs in the small PDF cells (s-LNvs) (**Figure 7**), those cells that normally show high PER expression and also govern the behavioral phenotype in constant conditions (Grima et al., 2004). Besides a decrease of its levels, in these cells the accumulation of PER is slow down, with a peak delayed of about 4 h with respect to control (**Figure 7**). In large PDF neurons (l-LNvs) PER cycling is dampened, similarly to control and in accordance to previous observations (Grima et al., 2004).

The $bel^{EY08943}$ mutant exhibited a less striking phenotype: a significant difference was observed in the PDF positive s-LNvs only in constant darkness, where the oscillation of PER was

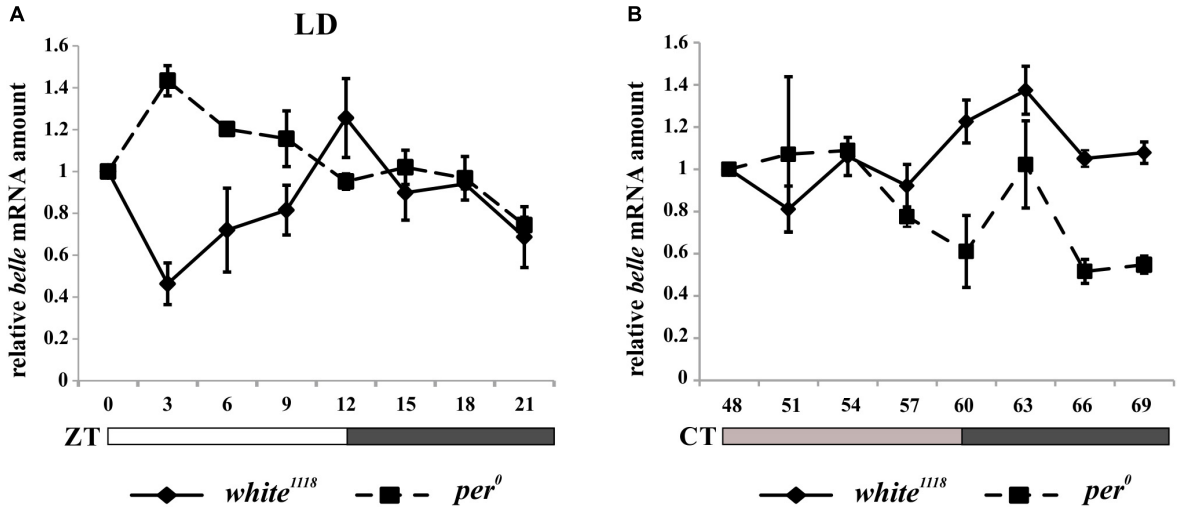

FIGURE 4 | *belle* mRNA expression shows circadian features. qRT-PCR on heads from *white*^{1118} and *per*^{0} flies reared in 12.12 LD **(A)** or over the third day in DD **(B)**. Relative abundance of *belle* mRNA was defined as a ratio with *rp49*. Values represent mean levels ± SEM of three biological replicates. The value corresponding to CT 54 in *per*^{0} flies represents mean level of two biological replicates.

TABLE 1 | Locomotor activity of *belle* Knock-Down flies under constant darkness (DD).

Genotype	N Tot	N Alive	N R	% R	τ		*SEM*
w;pdf-Gal4/+;UAS-RNAi-belle/+	63	55	48	87.27	24.95	±	0.12
w;Eaat-Gal4/+;UAS-RNAi-belle/+	32	31	27	87.10	24.01	±	0.07
w;pdf-Gal4/+	30	27	26	96.30	24.49	±	0.09
w;Eaat-Gal4/+	30	29	26	89.66	24.08	±	0.06
w;;UAS-RNAi-belle/+	47	39	33	84.62	23.96	±	0.07

R: rhythmic flies. The experiments were performed at 23°C.

TABLE 2 | Locomotor activity of *belle* Knock-Down flies in entrainment conditions (LD).

Genotype	MA (%)	EA (%)	MI		*SEM*	*N*
w;pdf-Gal4/+;UAS-RNAi-belle/+	54.55	92.73	0.069[a]	±	0.025	55
w;Eaat-Gal4/+;UAS-RNAi-belle/+	67.74	90.32	0.274	±	0.034	31
w;pdf-Gal4/+	74.05	100	0.141	±	0.038	27
w;Eaat-Gal4/+	86.21	100	0.254	±	0.038	29
w;;UAS-RNAi-belle/+	82.05	100	0.109	±	0.022	39

t-Test was performed. [a]: ns vs. w;pdf-Gal4/+and w;;UAS-RNAi-belle/+. MA: morning anticipation; EA: evening anticipation. MA and EA were detected, fly-by-fly, examining the bout of activity prior to light transitions. MI: Morning Index. The experiments were performed at 23°C.

4 h delayed compared to control (**Supplementary Figure S3**), similarly to what described in *bel*$^{cap-1}$ flies (see above).

Interestingly, both *belle* mutants were characterized by a reduction in the number of l-LNvs: a high percentage of brains, in fact, presented three neurons instead of the canonical four (83.93, 76.28, and 6.78% in *bel* $^{cap-1}$, *bel* ^{EY08943}, and *white* ^{1118}, respectively).

Belle Influences the Expression of Specific TEs in the Fly Heads and Gonads

As BELLE co-localizes with its paralog Vasa, a key component of the piRNA pathway-mediated regulation of the TEs, in ovaries and testes (Johnstone et al., 2005), we decided to further investigate about a possible involvement of *belle* in the piRNA-mediated regulation of TEs elements.

We analyzed the RNA expression of specific transposons in heads of adult *belle* ^{EY08943} and *belle* ^{cap1} flies. In *belle* ^{EY08943} two of them, *roo* and *blood*, showed a significant reduction in their expression compared to control (**Figure 8**, left panel). In addition, *R1* also exhibited a slight reduction in the RNA amount, though not significant. In *belle* $^{cap-1}$ the TE transcripts were greatly reduced, also compared to *belle* ^{EY08943}, and even *R1* exhibited a significant reduction compared to control (**Figure 8**, right panel). These results are in disagreement with other piRNA mutants, that normally show an increased amount of the TE transcripts (Vagin et al., 2006; Brennecke et al., 2007; Ghildiyal and Zamore, 2009;

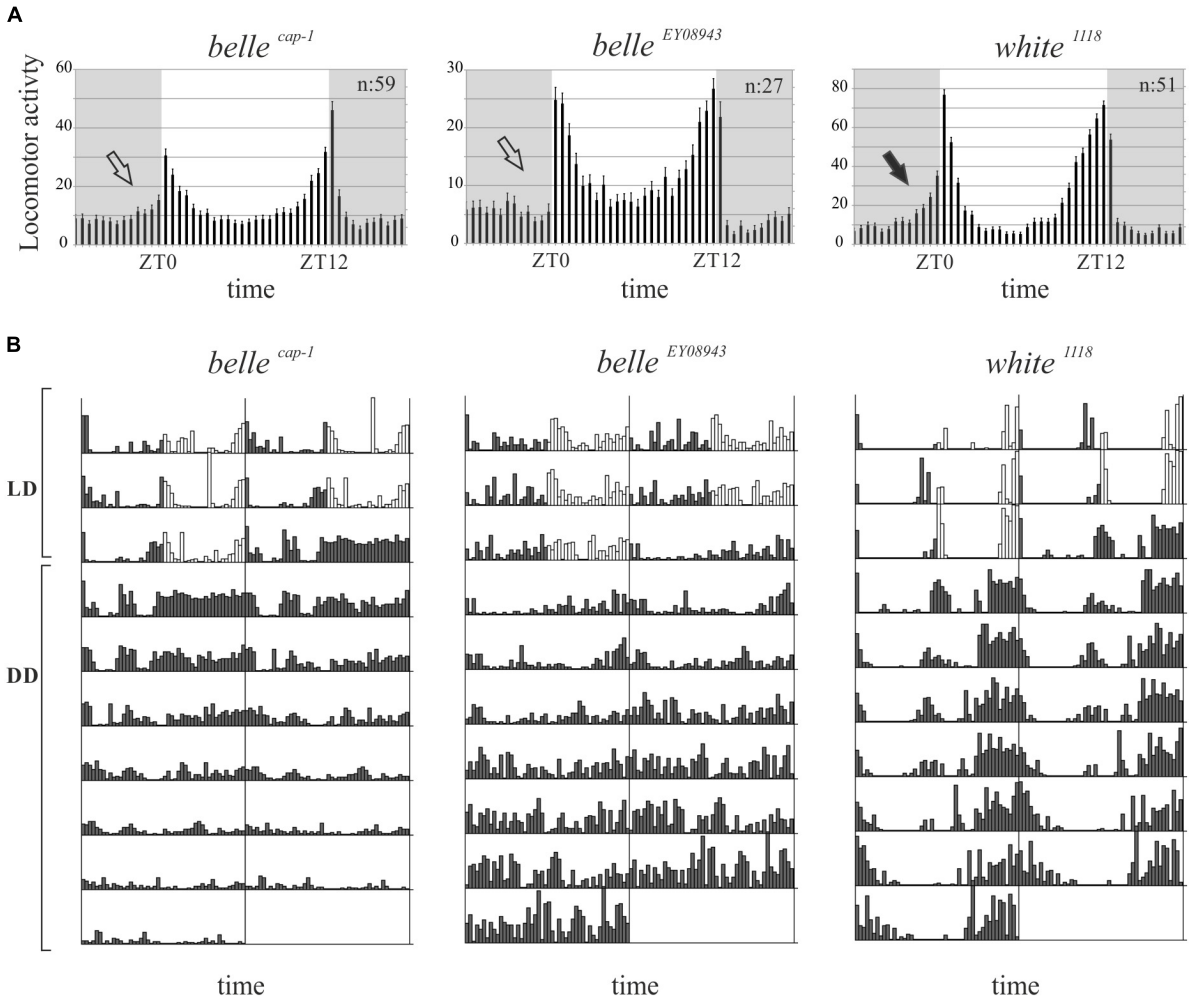

FIGURE 5 | BELLE affects circadian locomotor activity. **(A)** Activity means of flies over 3 days of LD cycles ± SEM. N: number of flies; gray boxes: dark phase. *Belle* mutants lost the morning anticipation (empty arrows) compared to control (black arrow). ZT0: lights-on, ZT12: lights-off. **(B)** Representative double-plotted locomotor activity of a single fly recorded for 3 days under LD and 7 days under DD conditions (gray bars: dark phase; white bars: light phase). LD: light–dark cycles; DD: constant darkness.

TABLE 3 | Locomotor activity of *belle* mutants in entrainment conditions (LD).

Genotype	MA (%)	EA (%)	MI		*SEM*	*N*
belle $^{cap-1}$ [a]	42.37	81.36	0.022[a]	±	0.033	59
belle ^{EY08943} [b]	14.81	70.37	0.026[b]	±	0.041	27
white ^{1118}	94.12	98.04	0.179	±	0.024	51

t-Test was performed. [a,b]*: $p < 0.005$ compared to white ^{1118} control. MA: morning anticipation; EA: evening anticipation. MA and EA were detected, fly-by-fly, examining the bout of activity prior to light transitions. MI: Morning Index. The experiments were performed at 23°C.*

TABLE 4 | Locomotor activity of *belle* mutants in constant darkness (DD).

Genotype	N Tot	N Alive	N R	% R	τ		*SEM*
belle $^{cap-1}$ [a]	85	59	40	67.80	24.24	±	0.10
belle ^{EY08943} [b]	46	27	8	29.63	24.36	±	0.25
white ^{1118}	54	51	45	88.24	24.44	±	0.12

R: rhythmic flies. The experiments were performed at 23°C.

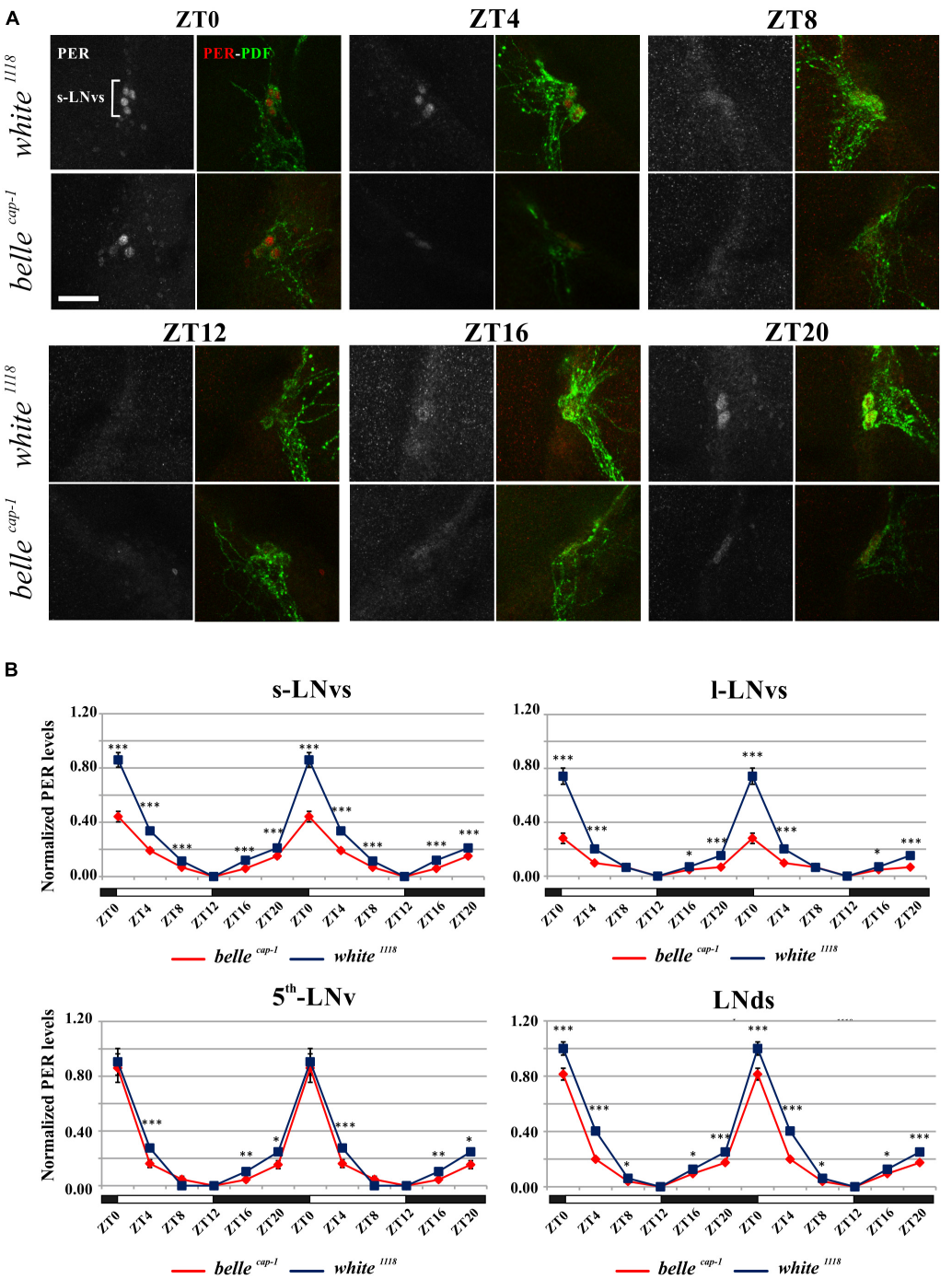

FIGURE 6 | Altered PER expression in *belle* $^{cap-1}$ mutant in entrainment conditions. PER oscillations in the clock Lateral Neurons of *belle* $^{cap-1}$ mutant flies in LD cycles. Flies were collected at the indicated time points after 3 days in LD and stained for PER and PDF. **(A)** Representative confocal stack images of PER staining in s-LNvs. *belle* $^{cap-1}$ mutants displayed lower PER expression in circadian pacemaker neurons, compared to control (*white* ^{1118}). Scale bar: 25 μm. **(B)** PER quantification in clock Lateral Neurons. Peak value was set to 1 and the rest of the values were normalized accordingly. Data are shown as double-plotted. *t*-Test: $^{***}p < 0.005$, $^{**}p < 0.01$, $^{*}p < 0.05$.

Khurana and Theurkauf, 2010; Specchia et al., 2010; Piacentini et al., 2014; Bozzetti et al., 2015). Nevertheless, based on our observations, a role for *belle* in the regulation of the TEs expression can still be hypothesized.

In order to better elucidate this involvement, we decided to investigate *belle* function in the gonads, where the role of the different components of the piRNA pathway is almost entirely known (Vagin et al., 2006; Brennecke et al., 2007;

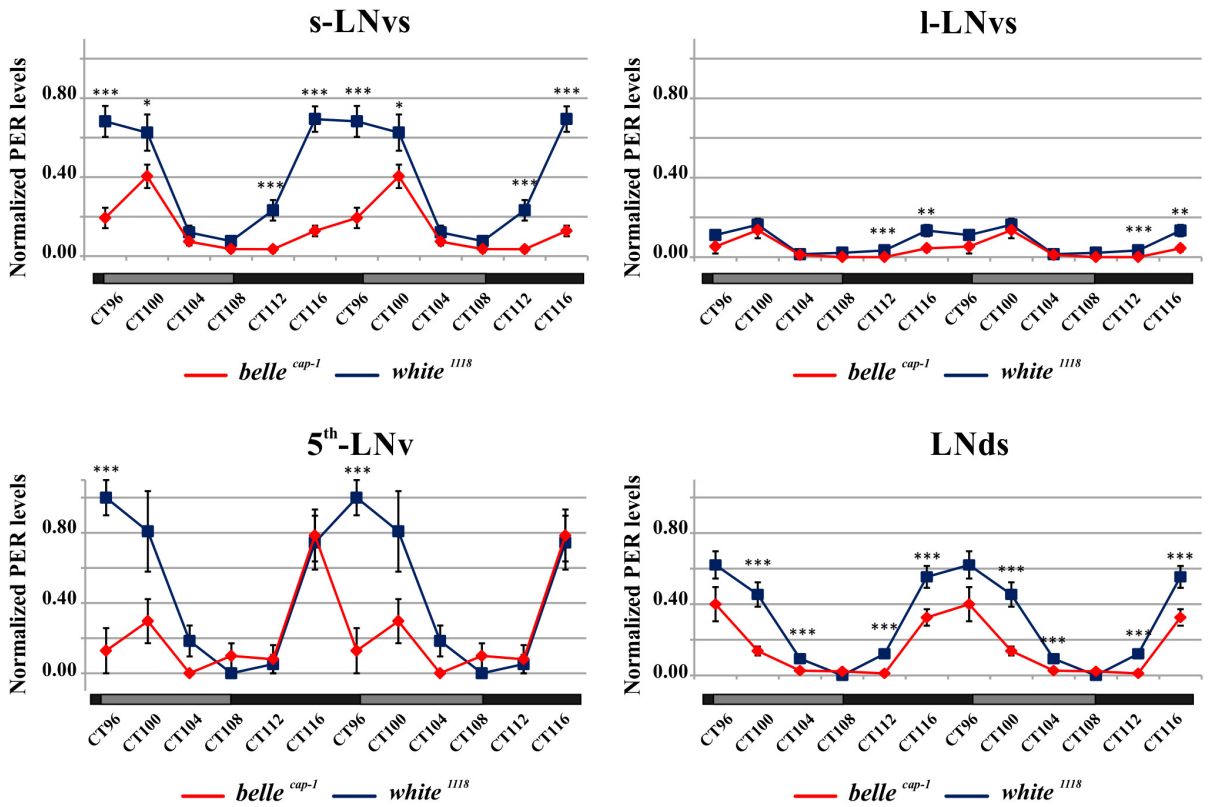

FIGURE 7 | Altered PER expression in *belle* $^{cap-1}$ mutant in constant conditions. PER quantifications in the clock Lateral Neurons of *belle* $^{cap-1}$ mutant flies in constant darkness. Flies were first entrained for 2 days in LD and then analyzed after 4 days of DD. Only arrhythmic flies were collected every 4 h and stained for PER expression during the 5th day of DD. Peak value was set to 1 and the rest of the values were normalized accordingly. Data are shown as double-plotted. *t*-Test: $^{***}p < 0.005$, $^{**}p < 0.01$, $^{*}p < 0.05$.

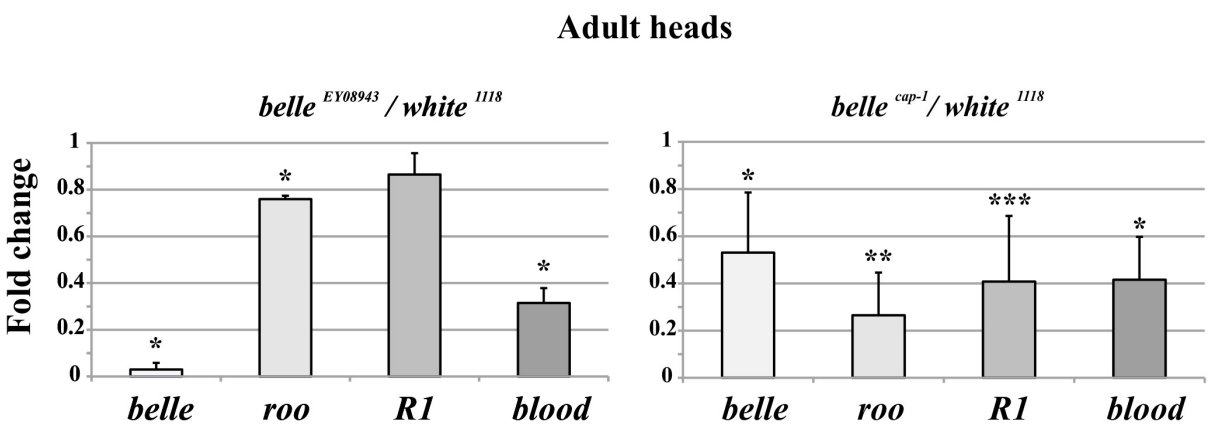

FIGURE 8 | Altered TEs expression in *belle* mutant heads. qRT-PCR on heads from *belle* ^{EY08943}, *belle* $^{cap-1}$, and *white* ^{1118} flies reared in 12:12 LD cycles and collected at ZT3. Represented are relative amount of *belle* and three transposable elements (TEs) (*roo, R1,* and *blood*) in *belle* ^{EY08943} **(A)** and in *belle* $^{cap-1}$ **(B)** compared to *white* ^{1118} control, to which an arbitrary value of 1 was assigned. Represented are mean ± SD of three biological replicates. $^{***}p < 0.005$, $^{**}p < 0.01$, $^{*}p < 0.05$.

Klattenhoff and Theurkauf, 2008; Li et al., 2009; Malone et al., 2009; Khurana and Theurkauf, 2010; Senti and Brennecke, 2010; Czech et al., 2013; Handler et al., 2013; Muerdter et al., 2013; Iwasaki et al., 2015). Our group has identified some key components of the piRNA pathway, like *aubergine*, *hsp83*, *dFmr1* (Specchia et al., 2008, 2010, 2017; Specchia and Bozzetti, 2009; Bozzetti et al., 2015) and demonstrated that TEs and repetitive sequences like *Stellate* are

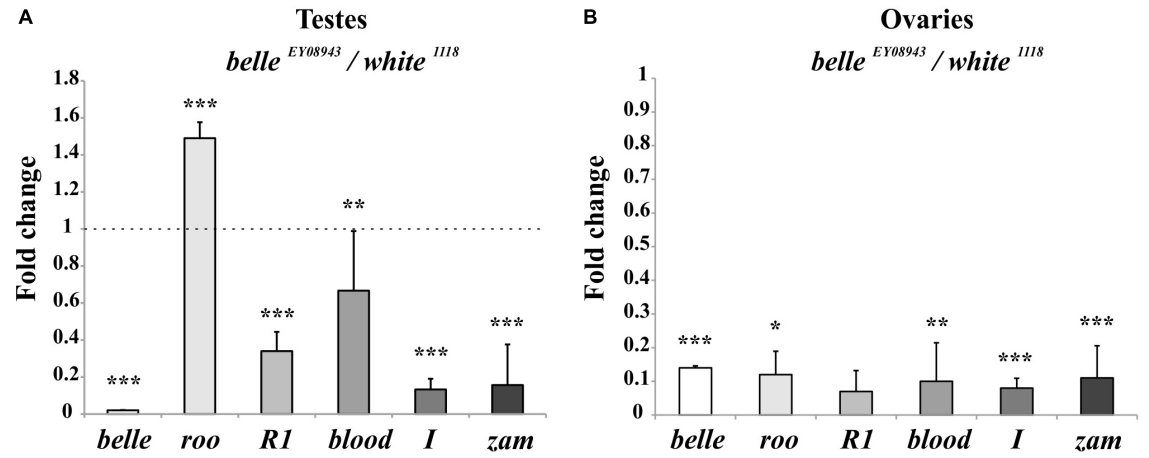

FIGURE 9 | Altered TEs expression in *belle* mutant gonads. qRT-PCR on ovaries **(A)** and testes **(B)** from *belle* ^{EY08943} and *white* ^{1118} flies reared in 12:12 LD cycles and collected at ZT3. Represented relative mRNA levels of *belle* and five TEs (*roo, R1*, *blood*, *I-element,* and *ZAM*) in *belle* ^{EY08943} compared to *white* ^{1118}, control, to which an arbitrary value of 1 was assigned. Represented are mean ± SD of three biological replicates. ***$p < 0.005$, **$p < 0.01$, *$p < 0.05$.

transcriptionally activated as a consequence of mutations in these piRNA genes (Aravin et al., 2001, 2003; Nishida et al., 2007; Specchia and Bozzetti, 2009; Bozzetti et al., 2012, 2015; Malone et al., 2015; Sahin et al., 2016).

We first tested the expression of some transposons in gonads: a dramatic reduction of transposons transcript was observed in ovaries from *belle* mutants in comparison to wild-type (**Figure 9B** and **Supplementary Figure S4**), similarly to what observed in heads. A similar reduction was also observed in *belle* ^{EY08943} testes, with the exception of *roo*, whose transcript increases in *belle* ^{EY08943} compared to control (**Figure 9A**).

We have also performed a fertility test, showing that mutant males are completely sterile with respect to controls (average progeny of 128), while females exhibit a severe reduction of fertility (average progeny of 6,6 in mutants with respect to 43,4 in control) (**Supplementary Figure S5**). This observation is in agreement to what reported for other alleles of *belle* (Johnstone et al., 2005).

DISCUSSION

Belle Is a Putative Circadian Clock Component

Aiming at finding new molecular component of the clock machinery in *Drosophila*, we have identified the DEAD-box RNA helicase BELLE as interactor of CRY. DEAD (Asp-Glu-Ala-Asp)-box RNA helicases are highly conserved proteins known to play central roles in essentially every stages of RNA metabolism, in both nucleus and cytoplasm (Linder and Fuller-Pace, 2013). BELLE has been implicated in splicing and translational regulation (Worringer et al., 2009; Ihry et al., 2012) and, more recently, in miRNA and siRNA pathways (Pek and Kai, 2011). It is essential for viability, organismal growth, and fertility (Johnstone et al., 2005).

The involvement of a RNA helicase in the circadian clock is not uncommon. In *Neurospora crassa*, in fact, two members of DEAD-box RNA helicase family play important roles in the circadian machinery: FRH (Frequency-interacting RNA Helicase), that regulates the stability of FREQUENCY (FRQ), thus acting on both negative and positive circadian feedback (Cheng et al., 2005; Guo et al., 2010; Shi et al., 2010; Lauinger et al., 2014), and PRD-1 (PERIOD-1), ortholog of human DDX5 and DDX17, specifically regulating circadian rhythmicity also in FRQ-less oscillators (Emerson et al., 2015; Adhvaryu et al., 2016).

BELLE has been extensively studied for its crucial role in the *Drosophila* gonads, where it is essential for viability and fertility (Johnstone et al., 2005; Kibanov et al., 2011). However the presence of BELLE has been reported also in *Drosophila* neurons, specifically in the neuronal cell body (Beckham et al., 2008), and proteomic data describe its expression in adult fly heads (Aradska et al., 2015).

We have depicted BELLE expression in both PDF expressing clock neurons and in the glial cells in the optic lobe. It is reported that adult glial cells contain a molecular clock and evidences about their role in the modulation of circadian rhythmicity are continuously accumulating (Siwicki et al., 1988; Zerr et al., 1990; Ewer et al., 1992; Pyza and Gorska-Andrzejak, 2004; Suh and Jackson, 2007; Damulewicz et al., 2013; Ng and Jackson, 2015; Ng et al., 2016). Nevertheless, the signaling between glia and the clock neurons that control the locomotor behavior is not yet fully understood. Many PER-expressing glial cells, such as epithelial glial, are located in proximity of clock neurons or their processes (Suh and Jackson, 2007), suggesting a direct communication. Epithelial glia affects rhythmic behavior also by regulating neurotransmitter metabolism through Ebony (Suh and Jackson, 2007). BELLE is mostly localized in the cytoplasm: this observation is in accordance with previous reports of a predominantly cytoplasmic distribution for BELLE in fly gonads (Johnstone et al., 2005) and its localization in cytoplasmic processing bodies (P-bodies), where it is supposed

to act as translational repressor (Beckham et al., 2008). At the transcriptional level, we observed a clear daily rhythm for *belle* mRNA either in LD and DD; the phase of the oscillation is altered in per^0 flies in LD, while it is abolished in constant conditions, suggesting that the circadian clock controls the expression of this helicase.

These results are perfectly in line with previously reported observations: in fact, a microarray study showed a peak of expression for *belle* at ZT17 (Claridge-Chang et al., 2001) and, in a chromatin immunoprecipitation (ChIP) analysis, *belle* was found to be a direct target of CLK in fly heads (Abruzzi et al., 2011). The peak-time of *belle* mRNA in the late day/early night is in agreement with a direct control of CLOCK in the expression of this gene, since it is reported that CLK-CYC bind to E-box of their target genes in the late day (reviewed in Peschel and Helfrich-Forster, 2011).

The effect of down-regulation of *belle* in glia and in TIM-expressing cells, lethality and arrested growth at larval stage, respectively, is in agreement with previously reported observation (Johnstone et al., 2005). In fact, strong alleles of *belle* are zygotic lethal and larvae arrest their development at stage L1, which is prolonged up to 5 days (Johnstone et al., 2005). This larval growth arrest phenotype is common to other genes encoding for translation factors, such as elongation factors *elF4A* and *elF4E* (Galloni and Edgar, 1999; Lachance et al., 2002), for which a lethality was observed also when knocked down in TIM cells (Bradley et al., 2012). The down-regulation of *belle* in a restricted subset of clock neurons or glial cells resulted in the loss of the morning anticipation in about 60% of flies reared in entrainment conditions, suggesting a possible role for BELLE in the regulation of morning activity. This phenotype was much stronger in two viable allele of *belle*: in LD cycles, in fact, a high proportion of flies were devoid of morning anticipatory, further indicating that the small ventral lateral neurons, that constitute the morning oscillator (Grima et al., 2004), are affected in these flies. In constant conditions the percentage of rhythmic flies in the two *belle* mutants was significantly lower compared to wild-type, reinforcing the fact that the activity of lateral ventral neurons, that drive also the endogenous behavioral rhythmicity (Helfrich-Forster, 1998; Renn et al., 1999; Lin et al., 2004), are impaired by *belle* mutation.

We sought to analyze PER cycling in clock neurons of *belle* mutants, in order to determine whether an altered PER expression could account for the behavioral defects of these flies. In fact PER oscillation in circadian pacemaker neurons is a hallmark of a functioning molecular oscillator, and an impairment of LNvs activity results in a de-synchronization of PER cycling within different groups of circadian neurons (Renn et al., 1999; Peng et al., 2003; Lin et al., 2004).

Although with a different magnitude between the two mutants, a general decrease of PER staining was observed compared to control. In entrainment conditions, in *belle* $^{cap-1}$ flies the amplitude of PER oscillation was significantly reduced in both s-LNvs and l-LNvs, and the difference was particularly marked at ZT0. In constant conditions, s-LNvs displayed the stronger effect, with a PER reduction more pronounced at the end of subjected night/beginning of subjective day, when the protein is expressed at maximum levels. Moreover, in these cells the accumulation of PER was also slow down, reaching maximum levels about 4 h later than the control. The *belle* ^{EY08943} mutant exhibited a less striking phenotype: only in constant darkness PDF positive s-LNvs displayed a 4 h delay compared to control. Altered PER oscillation in PDF-positive LNvs may account for the defects in the morning anticipation of the locomotor activity of *belle* mutants. These flies did not display particular defects in PDF projections/arborizations, but a reduction in the number of large ventral lateral neurons (l-LNvs) was observed. The altered PER cycling in clock neurons is reminiscent of the phenotype observed in mutants for RNA binding proteins involved at different levels in the post-transcriptional regulation of circadian clock, such as the translational factors NAT1 (Bradley et al., 2012) and ATAXIN2 (ATX2) (Lim and Allada, 2013; Zhang et al., 2013). ATX-2 is a RNA-binding protein that very recently has been shown to sustain circadian behavior in *Drosophila* functioning as both activator and repressor of translation, and this switch is mediated by complex formation with two specific factors, LSM12 and ME31B/DDX6, respectively (Lim and Allada, 2013). *Lsm12* mutant flies exhibited a dampened PER cycling in circadian pacemaker neurons, that resulted in a lengthening of the locomotor activity period (Lee et al., 2017). ME31B is the homolog of human DDX6, a RNA helicase belonging to the same family of DEAD-box ATP-dependent helicase of BELLE, and flies in which the expression of this gene was depleted exhibit very poor rhythmicity (Lee et al., 2017). All these similarities led us to hypothesize that also BELLE could play a role in the post-transcriptional control of the circadian clock in *Drosophila*, probably in mechanisms in which the aforementioned factors are also involved. This idea is supported by the fact that we have found ME31B in our Co-IP and MS analysis in the same complex as BELLE in fly heads (**Figure 1** and **Supplementary Table S2**), and that the two proteins belongs to the same translational repression complex that in *Drosophila* embryo is responsible for both repression and degradation of non-localized *nanos* mRNA, in a mechanism that involves also the Piwi-interacting RNA (piRNA) machinery (Gotze et al., 2017).

BELLE Has a Role in the Regulation of the TEs in the Nervous System and in Gonads

BELLE has a well-studied role in the *Drosophila* gonads (Johnstone et al., 2005; Kibanov et al., 2011; Kotov et al., 2016). In this study we have observed that loss of *belle* function impinges on the expression of TEs either in the brain or in the gonads, with an impact on the fertility of both males and females. We have observed a reduction of the transposons' transcription in *belle* mutant, in comparison to wild-type; notably, other piRNA mutants normally exhibit an increased TEs' expression (Aravin et al., 2007; Specchia et al., 2008, 2017; Specchia and Bozzetti, 2009; Senti and Brennecke, 2010). This observation well-agrees with the recently reported data on the effect of *belle* mutants in the silencing of P-transposon-derived constructs in ovaries,

depending on *de novo* piRNAs generated from the transgenes in the region of their insertion (Lo et al., 2016). Our data led us to infer that *belle* regulates the transposons probably *via* the piRNA-mediated pathway. This hypothesis is somehow supported by the fact that Vasa and Spindle-E, two members of the same DEAD box protein family, have a well-recognized role in the piRNA pathway in ovaries and testes, though their loss of function results in an up-regulation of TEs (Lasko, 2013; Iwasaki et al., 2015; Specchia et al., 2017), as opposite to that of *belle*. They also co-localize at the "nuage" of the germline where, Argonaute, Tudor and many other factors are also located for an efficient TE silencing (Lasko and Ashburner, 1990; Lim and Kai, 2007; Lasko, 2013; Bozzetti et al., 2015). However, in contrast to the majority of piRNA-related genes (Nishida et al., 2007; Specchia et al., 2008, 2010, 2017; Specchia and Bozzetti, 2009; Bozzetti et al., 2015; Sahin et al., 2016), *belle* seems not to be involved in the *crystal-Stellate* regulatory pathway, since mutant flies did not exhibited Stellate-made crystalline aggregates in the spermatocytes (**Supplementary Information and Supplementary Figure S6**). This observation well-agrees with the demonstration that *belle* ^{EY08943} mutant does not show an increase of RNA for TEs and repetitive sequences like *Stellate*.

A possible role of BELLE in the piRNA pathway is only starting to be elucidated: our experiments suggest that it might act in maintaining precise levels of TE RNAs, probably regulating the activity of other piRNA components.

The involvement of *belle* in both circadian rhythmicity and piRNA mediated transposon regulation suggests association between these two biological processes. This hypothesis is supported by indirect, though reasonable, observations.

First of all, circadian components have been linked to piRNA factors in the gonads. In fact, the mammalian CLOCK and BMAL1 transcription factors have been associated to the chromatoid body (CB) (Peruquetti et al., 2012), a cytoplasmic electron dense structure in the male germline with similarities to *Drosophila* polar granules and "nuage" of the germ cells (Grivna et al., 2006), where many components of different RNA metabolism pathways, like piRNAs, are located (Tanaka et al., 2000; Kotaja et al., 2006; Nagamori et al., 2011).

Secondly, a piRNA gene plays essential roles in neuronal development and circadian regulation. Our group has recently identified *dFmr1*, a gene with well-characterized functions in development of nervous system (Schenck et al., 2002; Napoli et al., 2008; Aitken and Lorsch, 2012; Doll and Broadie, 2016) and in regulation of circadian rhythmicity (Dockendorff et al., 2002; McBride et al., 2005; Santos et al., 2014), as a piRNA gene playing a crucial role in the gonads, where it ensures fertility and piRNA-regulated genome stability (Bozzetti et al., 2015; Specchia et al., 2017). Nevertheless, a role of this gene in the piRNA-mediated regulation in the nervous system has not been reported yet. *dFmr1* mutants display an altered rhythmicity in both eclosion and locomotor activity, due to an effect on the output factor CREB (cAMP response element binding protein) rather than the molecular oscillator (Dockendorff et al., 2002).

Third, circadian transposon regulation ensures genome integrity during aging. It is well-known that TEs activation induces an age-dependent loss of neuronal functions in *Drosophila* brain (Li et al., 2013). Very recently, a set of putative primary piRNA transcripts fully overlapping transposons in antisense were shown to display a clock-controlled *de novo* oscillation in old flies, raising the hypothesis that circadian piRNA expression could represent a new strategy adopted by the clock to preserve genome integrity during aging (Kuintzle et al., 2017).

A clear involvement of the piRNA pathway in the circadian rhythmicity has not been demonstrated so far. However, we have described an emerging role of *belle* in both circadian rhythmicity and transposon regulation, that leads to the attractive hypothesis that piRNA-mediated regulation could be another level of post-transcriptional control adopted by the clock to ensure the proper rhythmicity.

AUTHOR CONTRIBUTIONS

RC, GMM, and MPB conceived and supervised the study. PC, MD, EC, LC, VS, and AP performed experiments and analyzed the data. PC, MD, GMM, RC, VS, and MPB discussed results and wrote the manuscript.

FUNDING

This work was funded by grants from: University of Padova to GMM (Grant No. CPDA099390/09) and PC ("Progetto Giovani 2010"_Grant GRIC101061), European Community (6th Framework Project EUCLOCK No. 018741), National Research Council of Italy and MIUR (EPIGEN Flagship Project – Subproject 4), INsecTIME Marie Curie Initial Training Network (Grant No. PITN-GA-2012-316790) to RC, TELETHON (Grant No. GG14181) to MPB, Polish National Science Centre (Narodowe Centrum Nauki, NCN _ Grant No. UMO-2014/15/D/NZ3/05207) to MD.

Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537), Vienna Drosophila Resource Center (VDRC) and Kyoto Stock Centre were used in this study.

ACKNOWLEDGMENTS

We thank Moyra Mason (University of Padova) for her work on CoIP and MS; Federica Sandrelli (University of Padova) for her initial help with locomotor activity analysis; Alberto Biscontin (University of Padova) for help with statistical analyses; Prof. Paul Lasko (Department of Biology, McGill University, Montreal, QC, Canada) for anti-BELLE antibody.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00133/full#supplementary-material

REFERENCES

Abruzzi, K. C., Rodriguez, J., Menet, J. S., Desrochers, J., Zadina, A., Luo, W., et al. (2011). *Drosophila* CLOCK target gene characterization: implications for circadian tissue-specific gene expression. *Genes Dev.* 25, 2374–2386. doi: 10.1101/gad.174110.111

Adhvaryu, K., Firoozi, G., Motavaze, K., and Lakin-Thomas, P. (2016). PRD-1, a component of the circadian system of *Neurospora crassa*, is a member of the DEAD-box RNA Helicase family. *J. Biol. Rhythms* 31, 258–271. doi: 10.1177/0748730416639717

Aitken, C. E., and Lorsch, J. R. (2012). A mechanistic overview of translation initiation in eukaryotes. *Nat. Struct. Mol. Biol.* 19, 568–576. doi: 10.1038/nsmb.2303

Allada, R., and Chung, B. Y. (2010). Circadian organization of behavior and physiology in *Drosophila*. *Annu. Rev. Physiol.* 72, 605–624. doi: 10.1146/annurev-physiol-021909-135815

Apitz, H., and Salecker, I. (2016). Retinal determination genes coordinate neuroepithelial specification and neurogenesis modes in the *Drosophila* optic lobe. *Development* 143, 2431–2442. doi: 10.1242/dev.135004

Aradska, J., Bulat, T., Sialana, F. J., Birner-Gruenberger, R., Erich, B., and Lubec, G. (2015). Gel-free mass spectrometry analysis of *Drosophila melanogaster* heads. *Proteomics* 15, 3356–3360. doi: 10.1002/pmic.201500092

Aravin, A. A., Hannon, G. J., and Brennecke, J. (2007). The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. *Science* 318, 761–764. doi: 10.1126/science/.1146484

Aravin, A. A., Lagos-Quintana, M., Yalcin, A., Zavolan, M., Marks, D., Snyder, B., et al. (2003). The small RNA profile during *Drosophila melanogaster* development. *Dev. Cell* 5, 337Ú350. doi: 10.1016/S1534-5807(03)00228-4

Aravin, A. A., Naumova, N. M., Tulin, A. V., Vagin, V. V., Rozovsky, Y. M., and Gvozdev, V. A. (2001). Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the D. melanogaster germline. *Curr. Biol.* 11, 1017–1027. doi: 10.1016/S0960-9822(01)00299-8

Awasaki, T., and Ito, K. (2004). Engulfing action of glial cells is required for programmed axon pruning during *Drosophila metamorphosis*. *Curr. Biol.* 14, 668–677. doi: 10.1016/j.cub.2004.04.001

Beckham, C., Hilliker, A., Cziko, A. M., Noueiry, A., Ramaswami, M., and Parker, R. (2008). The DEAD-box RNA helicase Ded1p affects and accumulates in *Saccharomyces cerevisiae* P-bodies. *Mol. Biol. Cell* 19, 984–993. doi: 10.1091/mbc.E07-09-0954

Bellen, H. J., Levis, R. W., Liao, G., He, Y., Carlson, J. W., Tsang, G., et al. (2004). The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila* genes. *Genetics* 167, 761–781. doi: 10.1534/genetics.104.026427

Boothroyd, C. E., Wijnen, H., Naef, F., Saez, L., and Young, M. W. (2007). Integration of light and temperature in the regulation of circadian gene expression in *Drosophila*. *PLoS Genet.* 3:e54. doi: 10.1371/journal.pgen.0030054

Bozzetti, M. P., Fanti, L., Di Tommaso, S., Piacentini, L., Berloco, M., Tritto, P., et al. (2012). The "special" crystal-stellate system in *Drosophila melanogaster* reveals mechanisms underlying piRNA pathway-mediated canalization. *Gen. Res. Int.* 2012:324293. doi: 10.1155/2012/324293

Bozzetti, M. P., Specchia, V., Cattenoz, P. B., Laneve, P., Geusa, A., Sahin, H. B., et al. (2015). The *Drosophila* fragile X mental retardation protein participates in the piRNA pathway. *J. Cell Sci.* 128, 2070–2084. doi: 10.1242/jcs.161810

Bradley, S., Narayanan, S., and Rosbash, M. (2012). NAT1/DAP5/p97 and atypical translational control in the *Drosophila* circadian oscillator. *Genetics* 192, 943–957. doi: 10.1534/genetics.112.143248

Brennecke, J., Aravin, A. A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R., et al. (2007). Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. *Cell* 128, 1089–1103. doi: 10.1016/j.cell.2007.01.043

Cheng, P., He, Q., Wang, L., and Liu, Y. (2005). Regulation of the Neurospora circadian clock by an RNA helicase. *Genes Dev.* 19, 234–241. doi: 10.1101/jad.1266805

Claridge-Chang, A., Wijnen, H., Naef, F., Boothroyd, C., Rajewsky, N., and Young, M. W. (2001). Circadian regulation of gene expression systems in the *Drosophila* head. *Neuron* 32, 657Ű671. doi: 10.1016/S0896-6273(01)00515-3

Collins, B. H., Rosato, E., and Kyriacou, C. P. (2004). Seasonal behavior in *Drosophila melanogaster* requires the photoreceptors, the circadian clock, and phospholipase C. *Proc. Natl. Acad. Sci. U.S.A.* 101, 1945–1950. doi: 10.1073/pnas.0308240100

Czech, B., Preall, J. B., McGinn, J., and Hannon, G. J. (2013). A transcriptome-wide RNAi screen in the *Drosophila* ovary reveals factors of the germline piRNA pathway. *Mol. Cell.* 50, 749–761. doi: 10.1016/j.molcel.2013.04.007

Damulewicz, M., Rosato, E., and Pyza, E. (2013). Circadian regulation of the Na+/K+-ATPase alpha subunit in the visual system is mediated by the pacemaker and by retina photoreceptors in *Drosophila melanogaster*. *PLoS One* 8:e73690. doi: 10.1371/journal.pone.0073690

Dissel, S., Codd, V., Fedic, R., Garner, K. J., Costa, R., Kyriacou, C. P., et al. (2004). A constitutively active cryptochrome in *Drosophila melanogaster*. *Nat. Neurosci.* 7, 834–840. doi: 10.1038/nn1285

Dockendorff, T. C., Su, H. S., McBride, S. M., Yang, Z., Choi, C. H., Siwicki, K. K., et al. (2002). *Drosophila* lacking dfmr1 activity show defects in circadian output and fail to maintain courtship interest. *Neuron* 34, 973–984. doi: 10.1016/S0896-6273(02)00724-9

Doll, C. A., and Broadie, K. (2016). Neuron class-specific requirements for fragile X mental retardation protein in critical period development of calcium signaling in learning and memory circuitry. *Neurobiol. Dis.* 89, 76–87. doi: 10.1016/j.nbd.2016.02.006

Dubruille, R., and Emery, P. (2008). A plastic clock: how circadian rhythms respond to environmental cues in *Drosophila*. *Mol. Neurobiol.* 38, 129–145. doi: 10.1007/s12035-008-8035-y

Emerson, J. M., Bartholomai, B. M., Ringelberg, C. S., Baker, S. E., Loros, J. J., and Dunlap, J. C. (2015). period-1 encodes an ATP-dependent RNA helicase that influences nutritional compensation of the Neurospora circadian clock. *Proc. Natl. Acad. Sci. U.S.A.* 112, 15707–15712. doi: 10.1073/pnas.1521918112

Emery, P., So, W. V., Kaneko, M., Hall, J. C., and Rosbash, M. (1998). CRY, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell* 95, 669–679. doi: 10.1016/S0092-8674(00)81637-2

Ewer, J., Frisch, B., Hamblen-Coyle, M. J., Rosbash, M., and Hall, J. C. (1992). Expression of the period clock gene within different cell types in the brain of *Drosophila* adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. *J. Neurosci.* 12, 3321–3349. doi: 10.1523/JNEUROSCI.12-09-03321.1992

Galloni, M., and Edgar, B. A. (1999). Cell-autonomous and non-autonomous growth-defective mutants of *Drosophila* melanogaster. *Development* 126, 2365–2375.

Ghildiyal, M., and Zamore, P. D. (2009). Small silencing RNAs: an expanding universe. *Nat. Rev. Gen.* 10, 94–108. doi: 10.1038/nrg2504

Gotze, M., Dufourt, J., Ihling, C., Rammelt, C., Pierson, S., Sambrani, N., et al. (2017). Translational repression of the *Drosophila* nanos mRNA involves the RNA helicase Belle and RNA coating by Me31B and Trailer hitch. *RNA* 23, 1552–1568. doi: 10.1261/rna.062208.117

Grima, B., Chelot, E., Xia, R., and Rouyer, F. (2004). Morning and evening peaks of activity rely on different clock neurons of the *Drosophila* brain. *Nature* 431, 869–873. doi: 10.1038/nature02935

Grivna, S. T., Beyret, E., Wang, Z., and Lin, H. (2006). A novel class of small RNAs in mouse spermatogenic cells. *Genes Dev.* 20, 1709–1714. doi: 10.1101/gad.1434406

Guo, J., Cheng, P., and Liu, Y. (2010). Functional significance of FRH in regulating the phosphorylation and stability of Neurospora circadian clock protein FRQ. *J. Biol. Chem.* 285, 11508–11515. doi: 10.1074/jbc.M109.071688

Handler, D., Meixner, K., Pizka, M., Lauss, K., Schmied, C., Gruber, F. S., et al. (2013). The genetic makeup of the *Drosophila* piRNA pathway. *Mol. Cell* 50, 762–777. doi: 10.1016/j.molcel.2013.04.031

Helfrich-Forster, C. (1998). Robust circadian rhythmicity of Drosophila melanogaster requires the presence of lateral neurons: a brain-behavioral study of disconnected mutants. *J. Comp. Physiol. A* 182, 435–453. doi: 10.1007/s003590050192

Huang, Y., Ainsley, J. A., Reijmers, L. G., and Jackson, F. R. (2013). Translational profiling of clock cells reveals circadianly synchronized protein synthesis. *PLoS Biol.* 11:e1001703. doi: 10.1371/journal.pbio.1001703

Ihry, R. J., Sapiro, A. L., and Bashirullah, A. (2012). Translational control by the DEAD Box RNA helicase belle regulates ecdysone-triggered transcriptional cascades. *PLoS Genet.* 8:e1003085. doi: 10.1371/journal.pgen.1003085

Iwasaki, Y. W., Siomi, M. C., and Siomi, H. (2015). PIWI-interacting RNA: its biogenesis and functions. *Annu. Rev. Biochem.* 84, 405–433. doi: 10.1146/annurev-biochem-060614-034258

Johnstone, O., Deuring, R., Bock, R., Linder, P., Fuller, M. T., and Lasko, P. (2005). Belle is a *Drosophila* DEAD-box protein required for viability and in the germ line. *Dev. Biol.* 277, 92–101. doi: 10.1016/j.ydbio.2004.09.009

Khurana, J. S., and Theurkauf, W. (2010). piRNAs, transposon silencing, and *Drosophila* germline development. *J. Cell Biol.* 191, 905–913. doi: 10.1083/jcb.201006034

Kibanov, M. V., Egorova, K. S., Ryazansky, S. S., Sokolova, O. A., Kotov, A. A., Olenkina, O. M., et al. (2011). A novel organelle, the piNG-body, in the nuage of *Drosophila* male germ cells is associated with piRNA-mediated gene silencing. *Mol. Biol. Cell* 22, 3410–3419. doi: 10.1091/mbc.E11-02-0168

Kim, D. S., Gusti, V., Pillai, S. G., and Gaur, R. K. (2005). An artificial riboswitch for controlling pre-mRNA splicing. *RNA* 11, 1667–1677. doi: 10.1261/rna.2162205

Klattenhoff, C., and Theurkauf, W. (2008). Biogenesis and germline functions of piRNAs. *Development* 135, 3–9. doi: 10.1242/dev.006486

Kojima, S., Sher-Chen, E. L., and Green, C. B. (2012). Circadian control of mRNA polyadenylation dynamics regulates rhythmic protein expression. *Genes Dev.* 26, 2724–2736. doi: 10.1101/gad.208306.112

Kojima, S., Shingle, D. L., and Green, C. B. (2011). Post-transcriptional control of circadian rhythms. *J. Cell Sci.* 124, 311–320. doi: 10.1242/jcs.065771

Kotaja, N., Bhattacharyya, S. N., Jaskiewicz, L., Kimmins, S., Parvinen, M., Filipowicz, W., et al. (2006). The chromatoid body of male germ cells: similarity with processing bodies and presence of Dicer and microRNA pathway components. *Proc. Natl. Acad. Sci. U.S.A.* 103, 2647–2652. doi: 10.1073/pnas.0509333103

Kotov, A. A., Olenkina, O. M., Kibanov, M. V., and Olenina, L. V. (2016). RNA helicase Belle (DDX3) is essential for male germline stem cell maintenance and division in *Drosophila*. *Biochim. Biophys. Acta* 1863, 1093–1105. doi: 10.1016/j.bbamcr.2016.02.006

Kremer, M. C., Jung, C., Batelli, S., Rubin, G. M., and Gaul, U. (2017). The glia of the adult *Drosophila* nervous system. *Glia* 65, 606–638. doi: 10.1002/glia.23115

Kuintzle, R. C., Chow, E. S., Westby, T. N., Gvakharia, B. O., Giebultowicz, J. M., and Hendrix, D. A. (2017). Circadian deep sequencing reveals stress-response genes that adopt robust rhythmic expression during aging. *Nat. Commun.* 8:14529. doi: 10.1038/ncomms14529

Lachance, P. E., Miron, M., Raught, B., Sonenberg, N., and Lasko, P. (2002). Phosphorylation of eukaryotic translation initiation factor 4E is critical for growth. *Mol. Cell Biol.* 22, 1656–1663. doi: 10.1128/MCB.22.6.1656-1663.2002

Lasko, P. (2013). The DEAD-box helicase Vasa: evidence for a multiplicity of functions in RNA processes and developmental biology. *Biochim. Biophys. Acta* 1829, 810–816. doi: 10.1016/j.bbagrm.2013.04.005

Lasko, P. F., and Ashburner, M. (1990). Posterior localization of vasa protein correlates with, but is not sufficient for, pole cell development. *Genes Dev.* 4, 905–921. doi: 10.1101/gad.4.6.905

Lauinger, L., Diernfellner, A., Falk, S., and Brunner, M. (2014). The RNA helicase FRH is an ATP-dependent regulator of CK1a in the circadian clock of *Neurospora crassa*. *Nat. Commun.* 5:3598. doi: 10.1038/ncomms4598

Le Thomas, A., Toth, K. F., and Aravin, A. A. (2014). To be or not to be a piRNA: genomic origin and processing of piRNAs. *Genome Biol.* 15:204. doi: 10.1186/gb4154

Lee, E. J., Banerjee, S., Zhou, H., Jammalamadaka, A., Arcila, M., Manjunath, B. S., et al. (2011). Identification of piRNAs in the central nervous system. *RNA* 17, 1090–1099. doi: 10.1261/rna.2565011

Lee, J., Yoo, E., Lee, H., Park, K., Hur, J. H., and Lim, C. (2017). LSM12 and ME31B/DDX6 define distinct modes of posttranscriptional regulation by ATAXIN-2 protein complex in *Drosophila* circadian pacemaker neurons. *Mol. Cell* 66, 129–140.e7. doi: 10.1016/j.molcel.2017.03.004

Li, C., Vagin, V. V., Lee, S., Xu, J., Ma, S., Xi, H., et al. (2009). Collapse of germline piRNAs in the absence of Argonaute3 reveals somatic piRNAs in flies. *Cell* 137, 509–521. doi: 10.1016/j.cell.2009.04.027

Li, W., Cressy, M., Qin, H., Fulga, T., Van Vactor, D., and Dubnau, J. (2013). MicroRNA-276a functions in ellipsoid body and mushroom body neurons for naive and conditioned olfactory avoidance in *Drosophila*. *J. Neurosci.* 33, 5821–5833. doi: 10.1523/JNEUROSCI.4004-12.2013

Liang, L., Diehl-Jones, W., and Lasko, P. (1994). Localization of vasa protein to the Drosophila pole plasm is independent of its RNA-binding and helicase activities. *Development* 120, 1201–1211.

Lim, A. K., and Kai, T. (2007). Unique germ-line organelle, nuage, functions to repress selfish genetic elements in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 6714–6719. doi: 10.1073/pnas.0701920104

Lim, C., and Allada, R. (2013). ATAXIN-2 activates PERIOD translation to sustain circadian rhythms in *Drosophila*. *Science* 340, 875–879. doi: 10.1126/science.1234785

Lin, Y., Stormo, G. D., and Taghert, P. H. (2004). The neuropeptide pigment-dispersing factor coordinates pacemaker interactions in the *Drosophila* circadian system. *J. Neurosci.* 24, 7951–7957. doi: 10.1523/JNEUROSCI.2370-04.2004

Linder, P., and Fuller-Pace, F. V. (2013). Looking back on the birth of DEAD-box RNA helicases. *Biochim. Biophys. Acta* 1829, 750–755. doi: 10.1016/j.bbagrm.2013.03.007

Linder, P., and Jankowsky, E. (2011). From unwinding to clamping - the DEAD box RNA helicase family. *Nat. Rev. Mol. Cell Biol.* 12, 505–516. doi: 10.1038/nrm3154

Lo, P. K., Huang, Y. C., Poulton, J. S., Leake, N., Palmer, W. H., Vera, D., et al. (2016). RNA helicase Belle/DDX3 regulates transgene expression in *Drosophila*. *Dev. Biol.* 412, 57–70. doi: 10.1016/j.ydbio.2016.02.014

MacGregor, D. R., Gould, P., Foreman, J., Griffiths, J., Bird, S., Page, R., et al. (2013). HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 is required for circadian periodicity through the promotion of nucleo-cytoplasmic mRNA export in Arabidopsis. *Plant Cell* 25, 4391–4404. doi: 10.1105/tpc.113.114959

Majercak, J., Chen, W. F., and Edery, I. (2004). Splicing of the period gene 3'-terminal intron is regulated by light, circadian clock factors, and phospholipase C. *Mol. Cell. Biol.* 24, 3359–3372. doi: 10.1128/MCB.24.8.3359-3372.2004

Majercak, J., Sidote, D., Hardin, P. E., and Edery, I. (1999). How a circadian clock adapts to seasonal decreases in temperature and day length. *Neuron* 24, 219–230. doi: 10.1016/S0896-6273(00)80834-X

Malone, C. D., Brennecke, J., Dus, M., Stark, A., McCombie, W. R., Sachidanandam, R., et al. (2009). Specialized piRNA pathways act in germline and somatic tissues of the *Drosophila* ovary. *Cell* 137, 522–535. doi: 10.1016/j.cell.2009.03.040

Malone, C. D., Lehmann, R., and Teixeira, F. K. (2015). The cellular basis of hybrid dysgenesis and stellate regulation in *Drosophila*. *Curr. Opin. Genet. Dev.* 34, 88–94. doi: 10.1016/j.gde.2015.09.003

Mazzotta, G., Rossi, A., Leonardi, E., Mason, M., Bertolucci, C., Caccin, L., et al. (2013). Fly cryptochrome and the visual system. *Proc. Natl. Acad. Sci. U.S.A.* 110, 6163–6168. doi: 10.1073/pnas.1212317110

McBride, S. M., Choi, C. H., Wang, Y., Liebelt, D., Braunstein, E., Ferreiro, D., et al. (2005). Pharmacological rescue of synaptic plasticity, courtship behavior, and mushroom body defects in a Drosophila model of fragile X syndrome. *Neuron* 45, 753–764. doi: 10.1016/j.neuron.2005.01.038

Montelli, S., Mazzotta, G., Vanin, S., Caccin, L., Corra, S., De Pitta, C., et al. (2015). period and timeless mRNA Splicing Profiles under Natural Conditions in Drosophila melanogaster. *J. Biol. Rhythms* 30, 217–227. doi: 10.1177/0748730415583575

Morin, X., Daneman, R., Zavortink, M., and Chia, W. (2001). A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 98, 15050–15055. doi: 10.1073/pnas.261408198

Muerdter, F., Guzzardo, P. M., Gillis, J., Luo, Y., Yu, Y., Chen, C., et al. (2013). A genome-wide RNAi screen draws a genetic framework for transposon control and primary piRNA biogenesis in *Drosophila*. *Mol. Cell* 50, 736–748. doi: 10.1016/j.molcel.2013.04.006

Nagamori, I., Cruickshank, V. A., and Sassone-Corsi, P. (2011). Regulation of an RNA granule during spermatogenesis: acetylation of MVH in the chromatoid body of germ cells. *J. Cell Sci.* 124, 4346–4355. doi: 10.1242/jcs.096461

Nagao, A., Sato, K., Nishida, K. M., Siomi, H., and Siomi, M. C. (2011). Gender-specific hierarchy in nuage localization of PIWI-interacting RNA factors in *Drosophila*. *Front. Genet.* 2:55. doi: 10.3389/fgene.2011.00055

Napoli, I., Mercaldo, V., Boyl, P. P., Eleuteri, B., Zalfa, F., De Rubeis, S., et al. (2008). The fragile X syndrome protein represses activity-dependent translation through CYFIP1, a new 4E-BP. *Cell* 134, 1042–1054. doi: 10.1016/j.cell.2008.07.031

Ng, F. S., and Jackson, F. R. (2015). The ROP vesicle release factor is required in adult Drosophila glia for normal circadian behavior. *Front Cell Neurosci.* 9:256. doi: 10.3389/fncel.2015.00256

Ng, F. S., Sengupta, S., Huang, Y., Yu, A. M., You, S., Roberts, M. A., et al. (2016). TRAP-seq Profiling and RNAi-based genetic screens identify conserved glial genes required for adult *Drosophila* behavior. *Front. Mol. Neurosci.* 9:146. doi: 10.3389/fnmol.2016.00146

Nishida, K. M., Saito, K., Mori, T., Kawamura, Y., Nagami-Okada, T., Inagaki, S., et al. (2007). Gene silencing mechanisms mediated by aubergine piRNA complexes in *Drosophila* male gonad. *RNA* 13, 1911–1922. doi: 10.1261/rna.744307

Ozkaya, O., and Rosato, E. (2012). The circadian clock of the fly: a neurogenetics journey through time. *Adv. Genet.* 77, 79–123. doi: 10.1016/B978-0-12-387687-4.00004-0

Pek, J. W., and Kai, T. (2011). DEAD-box RNA helicase Belle/DDX3 and the RNA interference pathway promote mitotic chromosome segregation. *Proc. Natl. Acad. Sci. U.S.A.* 108, 12007–12012. doi: 10.1073/pnas.1106245108

Peng, Y., Stoleru, D., Levine, J. D., Hall, J. C., and Rosbash, M. (2003). *Drosophila* free-running rhythms require intercellular communication. *PLoS Biol.* 1:E13. doi: 10.1371/journal.pbio.0000013

Perrat, P. N., DasGupta, S., Wang, J., Theurkauf, W., Weng, Z., Rosbash, M., et al. (2013). Transposition-driven genomic heterogeneity in the *Drosophila* brain. *Science* 340, 91–95. doi: 10.1126/science.1231965

Peruquetti, R. L., de Mateo, S., and Sassone-Corsi, P. (2012). Circadian proteins CLOCK and BMAL1 in the chromatoid body, a RNA processing granule of male germ cells. *PLoS One* 7:e42695. doi: 10.1371/journal.pone.0042695

Peschel, N., Chen, K. F., Szabo, G., and Stanewsky, R. (2009). Light-dependent interactions between the *Drosophila* circadian clock factors cryptochrome, jetlag, and timeless. *Curr. Biol.* 19, 241–247. doi: 10.1016/j.cub.2008.12.042

Peschel, N., and Helfrich-Forster, C. (2011). Setting the clock–by nature: circadian rhythm in the fruitfly *Drosophila melanogaster*. *FEBS Lett.* 585, 1435–1442. doi: 10.1016/j.febslet.2011.02.028

Piacentini, L., Fanti, L., Specchia, V., Bozzetti, M. P., Berloco, M., Palumbo, G., et al. (2014). Transposons, environmental changes, and heritable induced phenotypic variability. *Chromosoma* 123, 345–354. doi: 10.1007/s00412-014-0464-y

Pyza, E., and Gorska-Andrzejak, J. (2004). Involvement of glial cells in rhythmic size changes in neurons of the housefly's visual system. *J. Neurobiol.* 59, 205–215. doi: 10.1002/neu.10307

Renn, S. C., Park, J. H., Rosbash, M., Hall, J. C., and Taghert, P. H. (1999). A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. *Cell* 99, 791–802. doi: 10.1016/S0092-8674(00)81676-1

Richard, A., Rybak, J., Stortkuhl, K., Meinertzhagen, I., and Hovemann, B. (2002). Ebony protein in the *Drosophila* nervous system: optic neuropile expression in glial cells. *J. Comp. Neurol.* 452, 93–102. doi: 10.1002/cne.10360

Rival, T., Soustelle, L., Strambi, C., Besson, M. T., Iché, M., and Birman, S. (2004). Decreasing glutamate buffering capacity triggers oxidative stress and neuropil degeneration in the *Drosophila* brain. *Curr. Biol.* 14, 599–605. doi: 10.1016/j.cub.2004.03.039

Robles, M. S., Cox, J., and Mann, M. (2014). In-vivo quantitative proteomics reveals a key contribution of post-transcriptional mechanisms to the circadian regulation of liver metabolism. *PLoS Genet.* 10:e1004047. doi: 10.1371/journal.pgen.1004047

Rolls, M. M., Satoh, D., Clyne, P. J., Henner, A. L., Uemura, T., and Doe, C. Q. (2007). Polarity and intracellular compartmentalization of Drosophila neurons. *Neural Dev.* 2:7. doi: 10.1186/1749-8104-2-7

Sahin, H. B., Karatas, O. F., Specchia, V., Tommaso, S. D., Diebold, C., Bozzetti, M. P., et al. (2016). Novel mutants of the aubergine gene. *Fly* 10, 81–90. doi: 10.1080/19336934.2016.1174355

Sandrelli, F., Costa, R., Kyriacou, C. P., and Rosato, E. (2008). Comparative analysis of circadian clock genes in insects. *Insect Mol. Biol.* 17, 447–463. doi: 10.1111/j.1365-2583.2008.00832.x

Santos, A. R., Kanellopoulos, A. K., and Bagni, C. (2014). Learning and behavioral deficits associated with the absence of the fragile X mental retardation protein: what a fly and mouse model can teach us. *Learn. Mem.* 21, 543–555. doi: 10.1101/lm.035956.114

Schenck, A., Van de Bor, V., Bardoni, B., and Giangrande, A. (2002). Novel features of dFMR1, the *Drosophila* orthologue of the fragile X mental retardation protein. *Neurobiol. Dis.* 11, 53–63. doi: 10.1006/nbdi.2002.0510

Schiesari, L., Kyriacou, C. P., and Costa, R. (2011). The hormonal and circadian basis for insect photoperiodic timing. *FEBS Lett.* 585, 1450–1460. doi: 10.1016/j.febslet.2011.02.026

Seluzicki, A., Flourakis, M., Kula-Eversole, E., Zhang, L., Kilman, V., and Allada, R. (2014). Dual PDF signaling pathways reset clocks via TIMELESS and acutely excite target neurons to control circadian behavior. *PLoS Biol.* 12:e1001810. doi: 10.1371/journal.pbio.1001810

Sengoku, T., Nureki, O., Nakamura, A., Kobayashi, S., and Yokoyama, S. (2006). Structural basis for RNA unwinding by the DEAD-box protein *Drosophila* Vasa. *Cell* 125, 287–300. doi: 10.1016/j.cell.2006.01.054

Senti, K. A., and Brennecke, J. (2010). The piRNA pathway: a fly's perspective on the guardian of the genome. *Trends Genet.* 26, 499–509. doi: 10.1016/j.tig.2010.08.007

Shi, M., Collett, M., Loros, J. J., and Dunlap, J. C. (2010). FRQ-interacting RNA helicase mediates negative and positive feedback in the Neurospora circadian clock. *Genetics* 184, 351–361. doi: 10.1534/genetics.109.111393

Siwicki, K. K., Eastman, C., Petersen, G., Rosbash, M., and Hall, J. C. (1988). Antibodies to the period gene product of *Drosophila* reveal diverse tissue distribution and rhythmic changes in the visual system. *Neuron* 1, 141–150. doi: 10.1016/0896-6273(88)90198-5

Smith, R. F., and Konopka, R. J. (1981). Circadian clock phenotypes of chromosome aberrations with a breakpoint at the per locus. *Mol. Gen. Genet.* 183, 243–251. doi: 10.1007/BF00270625

Specchia, V., Benna, C., Mazzotta, G. M., Piccin, A., Zordan, M. A., Costa, R., et al. (2008). aubergine gene overexpression in somatic tissues of aubergine(sting) mutants interferes with the RNAi pathway of a yellow hairpin dsRNA in *Drosophila melanogaster*. *Genetics* 178, 1271–1282. doi: 10.1534/genetics.107.078626

Specchia, V., and Bozzetti, M. P. (2009). Different aubergine alleles confirm the specificity of different RNAi pathways in *Drosophila melanogaster*. *Fly* 3, 170–172. doi: 10.4161/fly.8054

Specchia, V., D'Attis, S., Puricella, A., and Bozzetti, M. P. (2017). dFmr1 plays roles in small RNA pathways of *Drosophila melanogaster*. *Int. J. Mol. Sci.* 18:E1066. doi: 10.3390/ijms18051066

Specchia, V., Piacentini, L., Tritto, P., Fanti, L., D'Alessandro, R., Palumbo, G., et al. (2010). Hsp90 prevents phenotypic variation by suppressing the mutagenic activity of transposons. *Nature* 463, 662–665. doi: 10.1038/nature08739

Suh, J., and Jackson, F. R. (2007). *Drosophila* ebony activity is required in glia for the circadian regulation of locomotor activity. *Neuron* 55, 435–447. doi: 10.1016/j.neuron.2007.06.038

Tanaka, S. S., Toyooka, Y., Akasu, R., Katoh-Fukui, Y., Nakahara, Y., Suzuki, R., et al. (2000). The mouse homolog of *Drosophila* Vasa is required for the development of male germ cells. *Genes Dev.* 14, 841–853. doi: 10.1101/gad.14.7.841

Thaben, P. F., and Westermark, P. O. (2014). Detecting rhythms in time series with RAIN. *J. Biol. Rhythms* 29, 391–400. doi: 10.1177/07487304145530

Thomson, T., and Lin, H. (2009). The biogenesis and function of PIWI proteins and piRNAs: progress and prospect. *Annu. Rev. Cell Dev. Biol.* 25, 355–376. doi: 10.1146/annurev.cellbio.24.110707.175327

Ulvila, J., Parikka, M., Kleino, A., Sormunen, R., Ezekowitz, R. A., Kocks, C., et al. (2006). Double-stranded RNA is internalized by scavenger receptor-mediated endocytosis in Drosophila S2 cells. *J. Biol. Chem.* 281, 14370–14375. doi: 10.1074/jbc.M513868200

Vagin, V. V., Sigova, A., Li, C., Seitz, H., Gvozdev, V., and Zamore, P. D. (2006). A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* 313, 320–324. doi: 10.1126/science.1129333

Vanin, S., Bhutani, S., Montelli, S., Menegazzi, P., Green, E. W., Pegoraro, M., et al. (2012). Unexpected features of *Drosophila* circadian behavioural rhythms under natural conditions. *Nature* 484, 371–375. doi: 10.1038/nature10991

Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T., et al. (1996). Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* 379, 466–469. doi: 10.1038/379466a0

Worringer, K. A., Chu, F., and Panning, B. (2009). The zinc finger protein Zn72D and DEAD box helicase Belle interact and control maleless mRNA and protein levels. *BMC Mol. Biol.* 10:33. doi: 10.1186/1471-2199-10-33

Xue, Y., and Zhang, Y. (2018). Emerging roles for microRNA in the regulation of *Drosophila* circadian clock. *BMC Neurosci.* 19:1. doi: 10.1186/s12868-018-0401-8

Young, M. W., and Kay, S. A. (2001). Time zones: a comparative genetics of circadian clocks. *Nat. Rev. Genet.* 2, 702–715. doi: 10.1038/35088576

Zerr, D. M., Hall, J. C., Rosbash, M., and Siwicki, K. K. (1990). Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of *Drosophila*. *J. Neurosci.* 10, 2749–2762. doi: 10.1523/JNEUROSCI.10-08-02749.1990

Zhang, Y., Ling, J., Yuan, C., Dubruille, R., and Emery, P. (2013). A role for *Drosophila* ATX2 in activation of PER translation and circadian behavior. *Science* 340, 879–882. doi: 10.1126/science.1234746

Zhou, R., Hotta, I., Denli, A. M., Hong, P., Perrimon, N., and Hannon, G. J. (2008). Comparative analysis of argonaute-dependent small RNA pathways in *Drosophila*. *Mol. Cell.* 32, 592–599. doi: 10.1016/j.molcel.2008.10.018

Zordan, M. A., Benna, C., and Mazzotta, G. (2007). Monitoring and analyzing *Drosophila* circadian locomotor activity. *Methods Mol. Biol.* 362, 67–81. doi: 10.1007/978-1-59745-257-1_4

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