Glial Regulation of Circadian Behavior

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Abstract

Circadian rhythms are ubiquitous throughout nature. The molecular oscillators that generate these rhythms are components of nearly every part of biological life. While neuronal circadian circuitry is well studied, knowledge about the contribution of glial cells is currently limited. Recent studies in *Drosophila* and rodents have identified astrocytes as particularly important components of circadian rhythms. Manipulations of clock genes, calcium signaling, glutamate signaling, and vesicle secretion in astrocytes resulted in abnormal circadian behavior. While we now know that astrocytes are important for maintaining circadian rhythms, many questions remain about how these cells interact with circadian circuitry. Given that we know astrocytes communicate with neurons to regulate behavior, I focused my studies on identifying the signals used by these cells.

My thesis describes two approaches towards identifying glial components important for behavioral rhythmicity. First, I describe a genome-wide microRNA (miRNA)-based screen to identify brain glial cell processes required for circadian behavior. To identify glial miRNAs that regulate circadian rhythmicity, I employed a collection of "miR-sponges" to inhibit miRNA function in a glia-specific manner. My initial screen identified 20 glial miRNAs that regulate circadian behavior. I studied two miRNAs – miR-263b and miR-274 – in detail and found that both function in adult astrocytes to regulate behavior. Astrocyte-specific inhibition of miR-263b or miR-274 in adults acutely impairs circadian locomotor activity rhythms with no apparent effect on glial or clock cell viability. To identify potential RNA targets of miR-263b and miR-274, I screened 35 predicted miRNA targets, employing RNAi directed against each target. I

found that glial knockdown of two putative miR-274 targets – CG4328 and MESK2 – resulted in significantly decreased rhythmicity. Homology of the miR-274 targets to mammalian counterparts suggests mechanisms that might be relevant for the glial regulation of rhythmicity.

A second approach to understanding glial functions utilized cell type-specific profiling of fly astrocytes to identify RNAs showing circadian changes in abundance. I used a tagged ribosomal subunit to affinity purify RNA collected from astrocytes across two days for RNA-sequencing. Dr. Amy Yu performed the initial processing of the sequencing data. I then performed qualitative analysis of the results. 724 RNAs were found to exhibit circadian changes in abundance. Of those cycling genes, 576 were determined to be high-confidence astrocyte genes. I confirmed the list included cycling of core clock genes and known astrocyte genes. I performed gene ontology analysis to identify overrepresented categories of biological processes and cellular components. Among the overrepresented biological processes were multiple development related processes along with the category of circadian rhythm. Using the FlyBase annotations, I identified a number of genes with circadian or transmitter-related signaling functions. The largest categories of cellular components were ATP binding and structural components of ribosomes. In looking at the overall landscape of astrocyte protein synthesis, I observed two major phases of translation throughout the day, similar to a previous gene profiling study on clock cells.

Together, these studies add to our knowledge of glial functions in regulating circadian behavior. Insights into how glial cells interact with neurons to modulate behavior advance our understanding of pathologies that may result from glial dysfunction.

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List of Abbreviations

5-HT1B	5-hydroxytryptamine (serotonin) receptor 1B
3’ UTR	3’ Untranslated Region
ADCY6	Adenylyl cyclase VI
AGO	Argonaut
ASPD	Advanced Sleep Phase Disorder
ATP	Adenosine Triphosphate
BMAL1	Brain and Muscle ARNT-Like 1
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CK1ε	Casein Kinase 1 epsilon
CK2	Casein Kinase 2
CLOCK	Circadian Locomotor Output Cycles Kaput
CNS	Central nervous system
CREB	cAMP Response Element Binding Protein
CRY	CRYPTOCHROME
CT	Circadian Time
CWO	CLOCKWORKORANGE
CYC	CYCLE
DAM	*Drosophila* Activity Monitor
DBT	DOUBLETIME
DD	Constant Darkness
Dilp	*Drosophila* Insulin-like Peptides
DInR	*Drosophila* Insulin Receptor
DN	Dorsal Neuron
DNA	Deoxyribonucleic Acid
dn-SNARE	Dominant Negative Soluble N-ethylmaleimide-sensitive Factor Activating Protein Receptor
DSPD	Delayed Sleep Phase Syndrome
EAAT1	Excitatory Amino Acid Transporter 1
EBONY	N-b-alanyl-biogenic Amine Synthetase
EGFP	Enhanced Green Fluorescent Protein
ERK	Extracellular Signal-regulated Kinase
FACS	Fluorescent Activated Cell Sorting
FASPS	Familial Advanced Sleep Phase Syndrome
FMRP1	Fragile X protein
GABA	Gamma-Aminobutryric Acid
GAT	GABA Trransporter
GO	Gene Ontology
GS	Glutamine Synthetase
GTP	Guanosine triphosphate
IPCs	Insulin Producing Cells
ISL-2	Insulin Gene Enhancer Protein
JET	JETLAG

L-VGCC	L-type Voltage Gated Calcium Channel
LD	12hr:12hr Light Dark
lLN_v	Large Ventral Lateral Neuron
LN_d	Dorsolateral Neuron
Loq	Loquacious
LPN	Lateral Posterior Neuron
LTP	Long-term Potentiation
MAPK	Mitogen Activated Protein Kinase
MESK2	Misexpression Suppressor of KSR 2
miRNA	MicroRNA
mIPSC	Miniature Inhibitory Postsynaptic Current
miR-SP	miRNA Sponge
mGluR	Metabotropic Glutamate Receptor
mRNA	Messenger RNA
NDRG	N-myc Downstream Regulated Gene
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
PACT	Protein activator of protein kinase RNA
PBST	Phosphate Buffered Saline with Triton
PDF	Pigment-dispersing Factor
PDFR	Pigment-dispersing Factor Receptor
PDP1ε	Pyruvate Dehydrogenase Phosphatase Catalytic Subunit 1 Epsilon
PFA	Paraformaldehyde
PER	PERIOD
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
qPCR	Quantitative Polymerase-Chain Reaction
RAN-GTP	Ras-related nuclear protein-GTP
RI	Rhythmicity Index
RISC	RNA-induced Silencing Complex
RNA	Ribonucleic Acid
RNAi	RNA inhibitor
RNA Pol II	RNA Polymerase II
RNA-Seq	RNA Sequencing
sLN_v	Small Ventral Lateral Neuron
SCN	Suprachiasmatic Nucleus
SERCA	Sarco-endoplasmic Reticulum Calcium ATPase
SGG	SHAGGY
SNP	Single Nucleotide Polymorphism
SNAP23	Synaptosomal-associated protein 23
SNARE	Soluble N-ethylmaleimide-sensitive Factor Activating Protein Receptor
SSRI	Selective Serotonin Reuptake Inhibitor
TARGET	Temporal and Regional Gene Expression Targeting
TIM	TIMELESS
TPR	Temperature Preference Rhythm
TRAP	Translating Ribosome Affinity Purification

TRBP	TAR RNA-binding protein
TTFL	Transcriptional/Translational Feedback Loop
UAS	Upstream Activating Sequence
VGLUT	Vesicular Glutamate Transporter
VIP	Vasoactive Intestinal Polypeptide
VRI	VRILLE
ZT	Zeitgeber Time

Chapter 1: Introduction

1.1 Circadian rhythms

Humans have devised time keeping tools for many millennia, from obelisks and sundials to stop watches and atomic clocks. We are fascinated with the idea of controlling time as demonstrated with creative works such as “Back to the Future” or “The Time Machine.” As we can all attest to, the perception of time fluctuates depending on influences such as experience or state of mind. Deadlines appear to make time speed up while the treadmill may make time slow down. Despite the fluidity in how we perceive time, our bodies contain precise clocks evolved from billions of years of time keeping. Circadian clocks are ubiquitous and have been found in everything from bread mold to humans. Circadian rhythms help us survive by allowing us to anticipate and to adapt to day and night. For example, some animals will awaken before dawn, so they are ready to hunt prey. This phenomenon, termed anticipation, does not require these animals to first experience light. Similarly (and for better or worse), modern humans can adjust to awakening without their alarm clocks.

In 1729, Jean-Jacque d’Ortous deMairan observed the rhythmic opening and closing of the heliotrope plant kept in constant darkness, giving the first clue to the existence of an endogenous timekeeper. Modern chronobiology began in 1971, when Ron Konopka and Seymour Benzer isolated *Drosophila* mutants with long (28 hours), short (19 hours) or no circadian periods (Konopka and Benzer, 1971). The mutations causing these altered circadian periods were revealed to be in the clock gene *period* (*per*). Underlying the importance of chronobiology, the 2017 Nobel Prize in physiology or medicine was awarded to Jeffrey Hall, Michael Rosbash and Michael Young for their work deciphering

the molecular mechanisms that control circadian rhythm using *Drosophila*. Jeffrey Hall, in collaboration with Michael Rosbash at Brandeis University, and separately Michael Young at Rockefeller University, isolated the *per* gene and rescued the arrhythmicity of the per^0 mutant by adding back wild-type *per* (Bargiello et al., 1984; Zehring et al., 1984). Hall and Rosbash discovered that both the protein PER and the gene *per* are rhythmically expressed (Hardin et al., 1990; Siwicki et al., 1988). Localization studies found PER in the nucleus, providing evidence that PER may inhibit its own transcription (Liu et al., 1992). To get into the nucleus, Young discovered that TIMELESS (TIM), the product of *tim*, binds to PER, translocates into the nucleus and inhibits the translation of *per* (Vosshall et al., 1994). DOUBLETIME (DBT), a kinase, was found to regulate the accumulation of PER (Price et al., 1998). All together, these bodies of work represent the beginning of how the molecular mechanisms of circadian rhythms were unraveled.

Drosophila melanogaster, otherwise known as the fruit fly, is an archetypal model system for the study of circadian rhythms. The condensed life span allows for quick and expansive generation of flies, a situation ideal for genetic screens. As there are only four pairs of chromosomes, genetic manipulation is simplified compared to other experimental models. The concise, but heavily conserved genome allows for detailed study of molecular pathways with less concern about compensation due to genetic redundancy. For example, there are three *per* genes in mammals but only one in *Drosophila*. *Drosophila* has two predominant rhythmic behaviors: locomotor behavior and eclosion. In 12-hour light: 12-hour dark conditions (LD 12:12), *Drosophila* exhibits a bimodal activity pattern peaking at dawn and dusk. In between the activity during the day is their "siesta" and during the night is night sleep. Sleep in *Drosophila* has characteristics

resembling human sleep, for example, increased arousal thresholds and sleep rebound after sleep deprivation (Hendricks et al., 2000; Shaw et al., 2000).

The experimental toolkit for *Drosophila* research facilitates simple and specific in *vivo* manipulations that are relevant for the study of behavior. There exist multiple binary expression systems, the most common being the GAL4/Upstream Activating Sequence (UAS) system (Brand and Perrimon, 1993). The yeast transcription factor GAL4, under the control of a promoter, binds to the UAS to promote transcription of the downstream gene or sequence. This allows for spatial control of genes or RNAi. Additionally, the repressor GAL80 can inhibit Gal4 function (Lee and Luo, 1999). This inhibition can be made spatially specific to restrict UAS expression in a cell-type specific manner. We can achieve temporal control of UAS expression by utilizing a temperature sensitive GAL80, $GAL80^{ts}$ (McGuire et al., 2004). At low temperatures, $Gal80^{ts}$ inhibits Gal4, but at high temperatures, $Gal80^{ts}$ is inhibited and Gal4 can drive expression of the UAS-transgene. Simultaneous manipulations in different cell populations can also be performed by utilizing multiple binary expression systems in concert, for example, the LexA/LexAop or the Q-system (Lai and Lee, 2006; Potter et al., 2010).

To be considered a circadian rhythm, three criteria must be satisfied: 1) the rhythm must persist in free-running conditions (where there are no external time cues) and have a period of about approximated one day (~21-29 hours), 2) the rhythms can be entrained by external cues such as light and temperature and 3) the rhythms are temperature compensated, meaning they proceed at the same rate no matter the temperature. The single day circadian period evolved in response to the rotation of the earth; thus, it is not a coincidence it takes about 24 hours for one full rotation and our circadian rhythms also

typically run at around 24 hours. The requirement for a rhythm that continues without zeitgebers, or time-givers, is necessary for biological phenomena such as anticipation and feeding. For example, bats awaken before dusk so that they are ready to hunt for food when it is dark. It is important that the rhythms are entrainable, meaning we can adjust our biological rhythms to new circumstances such as a different time zone. Temperature compensation is important to maintain rhythms even as temperature fluctuates. Biochemical reactions occur faster at higher temperatures, but clocks have mechanisms to compensate so that they do not run faster or slower depending on internal body temperature. Together, these three requirements describe a system that is precise enough to keep time without cues, yet flexible enough to adjust to exogenous influences. Practically speaking, this means clocks will run the same in every season no matter the longitude.

1.1.1 Molecular clocks

The biological clocks within the central nervous system (central clocks) are regulated by transcriptional/translational feedback loops (TTFLs) that drive rhythmic changes in gene transcription and subsequent protein abundance (Fig. 1.1). By utilizing both transcription and translation to drive the clock, this system provides many avenues - transcriptional, post-transcriptional, translational and post-translational - for regulation and control of timing. At every step, there is structure to ensure that the clock runs properly.

In *Drosophila*, the central oscillators consist of the transcription factors CLOCK (CLK) and CYCLE (CYC) which form a heterodimer to positively regulate the transcription of *per* and *tim* by binding to E-box sequences (CACGTG) in their promoters

(Allada et al., 1998; Darlington et al., 1998; Glossop et al., 1999; Hao et al., 1997; Lee et al., 1998; Rutila et al., 1998). PER and TIM also form a heterodimer, accumulate in the cytoplasm, and then enter the nucleus where they negatively regulate their own transcription by inhibiting CLK and CYC (Darlington et al., 1998; Lee et al., 1998; Saez and Young, 1996). *per* and *tim* mRNA abundance peaks in the early evening and protein abundance peaks at the end of the night (Hardin et al., 1990; Hunter-Ensor et al., 1996; Sehgal et al., 1995; Zerr et al., 1990). In the cytoplasm, the kinase DBT binds to PER and phosphorylates it which results in PER degradation (Kloss et al., 1998, 2001; Price et al., 1998). As TIM accumulates in the cytoplasm, PER and TIM bind to inhibit the degradation of PER by DBT and together translocate to the nucleus to suppress transcription of *per* and *tim*. The kinase SHAGGY (SGG) promotes TIM phosphorylation and regulates the timing of PER/TIM nuclear translocation (Martinek et al., 2001). Another kinase, Casein Kinase 2 (CK2) is important in regulating the abundance and cellular distribution of both PER and TIM and consequently, regulates circadian period (Akten et al., 2003; J.-M. Lin et al., 2002; Meissner et al., 2008). CK2 can also directly target CLK to inhibit its degradation (Szabó et al., 2013). A second loop of control comes from CLK/CYC, which can also promote the transcription of *vrille* (*vri*) and *PAR domain protein 1 epsiolon* (*Pdp1ε*) (Cyran et al., 2003). VRI inhibits transcription of *Clk* while PDP1ε promotes transcription of *Clk* with the repression by VRI occurring first, as VRI abundance peaks 3-6 hours before PDP1ε (Cyran et al., 2003; Glossop et al., 2003). CLOCKWORKORANGE (CWO) represents a third loop as it inhibits transcription of CLK/CYC targets (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007; Richier et al., 2008).

Resetting the clock relies on light and the subsequent degradation of TIM (Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). This degradation is caused by interaction with the blue light photoreceptor CRYTOCHROME (CRY). After exposure to light, CRY undergoes a conformational change, allowing it to bind to TIM (Busza et al., 2004; Ceriani et al., 1999, 2002; Egan et al., 1999; Emery et al., 1998). TIM and CRY are then ubquitinated by the E3 ligase JETLAG (JET) and processed through the proteasomal pathway (Koh et al., 2006; Peschel et al., 2006).

In mammals, the central clock consists of a complex between Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle ARNT-Like 1 (BMAL1) which positively regulates the expression of multiple PERs and CRYs, which in turn functions as its own transcriptional repressor complex. Similar to *Drosophila*, there are many additional loops of regulation (Mohawk et al., 2012). Non-transcriptional oscillators have also been described in organisms from cyanobacteria to mammals and even in human blood cells (van Ooijen and Millar, 2012).

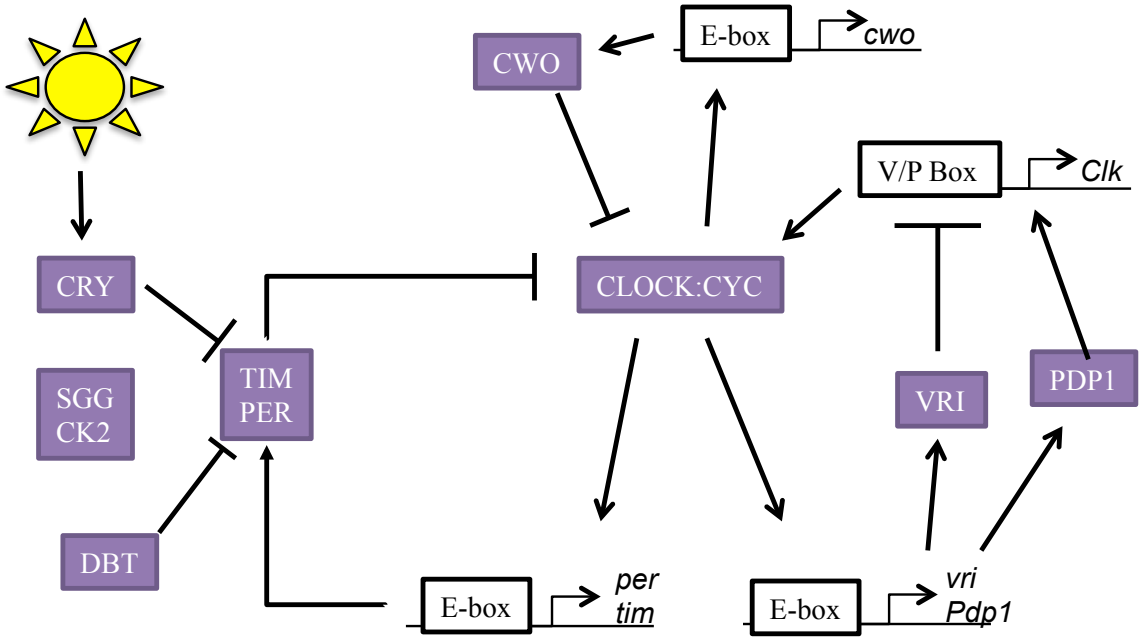


Figure 1.1: Simplified Schematic of *Drosophila* Core Oscillators.
As discussed in Chapter 1.1.1 Molecular clocks.

1.1.2 Neuronal pacemakers

In mammals, the central clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus where there are about 20,000 neurons. The SCN receives input from photoreceptors in the retina. Ablation of the SCN results in loss of behavioral rhythms (Stephan and Zucker, 1972). Vasoactive intestinal polypeptide (VIP), expressed in a small subset of SCN neurons, is responsible for mediating rhythmicity and synchrony between the mammalian clock neurons (Aton et al., 2005). Application of VIP to the SCN can interfere with the electrical firing rhythm of the SCN (Reed et al., 2001) and with locomotor behavioral rhythm (Piggins et al., 1995). There are also peripheral clocks that reside in various tissues throughout the body (Mohawk et al., 2012). They are dependent on the master clock in the SCN to keep synchrony. For example, rhythmic gene expression in the liver is dependent on the SCN, as destruction of the SCN eliminates these rhythms (Terazono et al., 2003).

In *Drosophila*, there are about 150 clock neurons, each with a molecular oscillator (composed of clock proteins such as PER and TIM)(Kaneko et al., 1997; Kaneko and Hall, 2000; Shafer et al., 2006). The clock neurons are divided into subgroups: the dorsal neurons (DNs) DN_1, DN_2 and DN_3, the dorsolateral neurons (LN_ds), the lateral posterior neurons (LPNs), the large ventrolateral neurons (lLN_vs), the small ventrolateral neurons (sLN_vs) and the PDF-negative sLN_vs. There are eight lLN_vs and eight sLN_vs that express pigment-dispersing factor (PDF), a neuropeptide essential for behavioral rhythmicity (Renn et al., 1999). The lLN_vs project to the optic lobes and accessory medulla and the sLN_vs project to the dorsalmedial brain region (Helfrich-Förster et al., 2007). PDF is the principle circadian transmitter in *Drosophila* and is responsible for the coordination of

pacemaker interactions, similar to the mammalian VIP (Helfrich-Forster, 1995; Lin et al., 2004). PDF ablation results in elimination of behavioral rhythmicity (Renn et al., 1999) and is required for resetting the circadian period in DD (Guo et al., 2014). Excitability of the LN_vs is important for coordinating the oscillators. Regulation of electrical excitability of LN_v neurons is the basis for cycling PDF in the dorsalmedial terminals. When these neurons are made hyperexcitable, PDF accumulation in the terminals is disrupted and the flies become arrhythmic (Nitabach et al., 2006). The sLN_vs are particularly important in generating morning activity and maintaining rhythmicity in free-running conditions (Grima et al., 2004; Stoleru et al., 2004). The LN_ds and the PDF-negative sLN_vs are thought to promote activity in the evening (Grima et al., 2004; Stoleru et al., 2004). These two groups are loosely grouped as "M" cells and "E" cells, referring to morning or evening activity respectively. Unsurprisingly, these divisions are not binary as expression of the period-altering mutation DBT^s in the PDF-positive "M" cells does not change locomotor activity of phase in LD conditions (Guo et al., 2014). Contrary to that result, expression of DBT^s in a subset of "E" cells advanced evening peak phase (Guo et al., 2014). Inhibition of neurotransmitter release from a subset of "E" cells can also result in attenuation of both morning and evening anticipation in LD, thus giving these cells some influence over morning activity (Guo et al., 2014).

The DNs are not as well characterized in comparison to the LNs. The DN_1s are the most dorsal and have been implicated in maintaining locomotor rhythm in constant light conditions. Flies maintained in constant light are typically arrhythmic. Only flies with a mutant cry^b allele were known to be rhythmic in constant light (Emery et al., 2000). It was found that overexpression of PER in the DN_1s is sufficient to drive rhythmic

behavior and PDP1 abundance in constant light (Murad et al., 2007). More recent studies have identified a role for DN_1s in output pathways modulating sleep and locomotor activity (Cavanaugh et al., 2014; Kunst et al., 2014). Similar to the body temperature rhythm of mammals, *Drosophila* have a temperature preference rhythm (TPR) (Kaneko et al., 2012). This rhythm is under clock control and does not persist in *per* mutants. Adding PER back to the DN_2 neurons in a *per* mutant restores the TPR (but not the activity rhythm) (Kaneko et al., 2012).

Drosophila also have peripheral clocks including in the retina, fat body, prothoracic gland, digestive organs and reproductive organs (Ito and Tomioka, 2016). Unlike mammalian peripheral clocks, these have cell-autonomous oscillations, and most can respond to light through the blue-light photoreceptor CRY.

1.1.3 Clocks and diseases

Sleep and circadian disruption is comorbid with a large number of pathologies. Given the diverse functions governed by the master clocks, it is no surprise that malfunction of those genes can result in a variety of problems. Along with signals modulating sleep-wake activity, the SCN also sends signals influencing the rhythm of body temperature and the secretion of hormones. Common disorders that are the result of external forces include shift-work disorder and jet-lag (Sack et al., 2007a). Both are the result of the external entraining signals being misaligned with internal rhythms as a consequence of modern life.

For most of human existence, we did not have the capability of being exposed to light beyond the sun and later, with fire. With the advent of electric light, we began to routinely expose ourselves to more light. In the present day, we are inundated with light

due to the omnipresent-lighted screens of our phones, computers and televisions. Light has an important influence on melatonin, a sleep-promoting hormone produced by the pineal gland. Exposure to light resets the melatonin rhythm and inhibits its synthesis, resulting in a decreased duration of melatonin (Gooley et al., 2011; Lewy et al., 1980; Shanahan and Czeisler, 1991). The short wavelength blue-light of light emitting diodes (LEDs) is particularly potent in suppressing endogenous melatonin (Cajochen et al., 2011).

Two processes influence sleep: the sleep homeostat and the circadian clock. Circadian related sleep pathologies include advanced sleep phase disorder (ASPD), delayed sleep phase syndrome (DSPD), non-24 hour sleep-wake syndrome, and irregular sleep-wake rhythm (Sack et al., 2007b). These disorders are all likely to have intrinsic causes. Mutations in PER2 were implicated in one family with familial advanced sleep phase syndrome (FASPS) but genotyping of other families did not show mutations on PER2. Nevertheless, it appears FASP is autosomal dominant with high penetrance, suggesting other clock genes may be implicated. Those with non-24-hour sleep-wake disorder have circadian periods of ~24.5 hours or more. The majority of people with this disorder are blind and thus do not receive photic entrainment. By trying to live in a 24-hour society, these people will experience shifting amounts of misalignment that becomes extremely disruptive to their lives. Those with irregular sleep-wake rhythm have no circadian pattern to their sleep-wake cycle. Damage to the SCN is a likely contributor to this pathology and it is seen more often in older adults with dementia.

Circadian rhythms have also been implicated in mood disorders. A number of studies have identified polymorphisms in clock genes that are associated with psychiatric

diseases (Etain et al., 2011). A single-nucleotide polymorphism (SNP) in PER2 was found to associate with alcoholism in humans and mice with mutant PER2 have increased alcohol consumption (Spanagel et al., 2005). These mice have lower expression of the glutamate transporter EAAT1, resulting in decreased glutamate uptake by astrocytes. Mice with a mutation in *Clock* that produces a dominant-negative protein have phenotypes that resemble bipolar disorder in humans (Roybal et al., 2007).

Metabolism is also intricately linked to circadian rhythms. Clinical studies have shown correlation between late-eating and decreased weight-loss (Garaulet et al., 2013). Shift work is associated with higher rates of obesity and glucose intolerance (Karlsson et al., 2001). Homozygous *Clock* mutant mice are hyperphagic, obese and developed signs of metabolic disease (Turek et al., 2005). The mutants also had decreased abundance of *orexin* and *grehlin* mRNA, genes involved in appetite and energy regulation. Mice provided with a high-fat diet had lengthened free-running period and less robust feeding rhythm along with dampened amplitude of *Clock*, *Bmal1* and *per2* cycling in fat tissue (Kohsaka et al., 2007). The liver is a major target of the circadian clock. Deletion of *Bmal1* in the liver caused hypoglycemia during the normal fasting phase, exaggerated glucose clearance and loss of rhythms of glucose regulation genes (Lamia et al., 2008). A study in mouse liver found circadian phosphorylation of a quarter of over 20,000 phosphosites; overall, phosphoproteome oscillated in two major phases (Robles et al., 2017). Another group looked specifically at the nuclear proteome of the mouse liver and identified 522 proteins that rhythmically accumulate in the nucleus (Wang et al., 2017). The rhythmic expression of these proteins was mostly regulated at the post-transcriptional level.

1.2. Glia

My thesis is focused on roles for glial cells in rhythmicity, and therefore I provide a brief general background in the next two sections. In 1856, Rudolf Virchow first coined the term “nervenkitt” (neuroglia) or nerve putty in a footnote of a collection of published papers (Somjen, 1988). He considered neuroglia to be passive connected space-fillers without cellular components and used by neurons as architectural support. In adult mammals, there are three types of glia: oligodendrocytes, microglia and astrocytes. In 1893, the term ‘astrocyte’ was created by Michael von Lenhossek to describe the stellate-shaped cells (Verkhratsky and Butt, 2007). Astrocytes are the dominant type of glia in the nervous system. They are coupled to each other through gap-junctions and their processes can make contact with both vasculature and synapses. In this way, a “tripartite synapse” can form with the pre- and post-synaptic neuron being wrapped around by the astrocyte process (Araque et al., 1999). In this configuration, the astrocyte is in prime position to modulate interactions at the synapse. Any given astrocyte is estimated to be capable of contacting over 100,000 synapses (Bushong et al., 2002).

1.2.1 Astrocyte signaling

The explosion of research on glial signaling began nearly three decades ago with culture studies by Cornell-Bell and colleagues. They found that application of glutamate, the primary excitatory transmitter in the brain, was able to induce calcium waves in cultured astrocytes (Cornell-Bell et al., 1990). This type of coordination suggests that astrocytes are also functionally connected which dramatically increases their modulatory power. These calcium waves could also be induced by mechanical stimulation of astrocytes (Charles et al., 1991) and stimulation of glutamatergic afferents (Dani et al.,

1992). Using an eyecup preparation from rat, astrocytic calcium waves were also observed in the retina (Newman and Zahs, 1998). Studies in *situ* found that neuronal glutamate release stimulated metabotropic glutamate receptors on astrocytes to increase astrocyte calcium (Porter and McCarthy, 1996). In addition to responding to transmitters, astrocytes were found to be capable of eliciting responses from adjacent neurons through glutamate and ATP (Hassinger et al., 1995; Nedergaard, 1994; Parpura et al., 1994; Parpura and Haydon, 2000). Astrocytes were also found to mediate inhibitory signaling, as stimulation was capable of increasing the frequency of miniature inhibitory postsynaptic currents (mIPSCs) (Araque et al., 1998; Kang et al., 1998). In the other direction, interneurons were capable of elevating astrocytic calcium through $GABA_B$ receptors (Kang et al., 1998). Astrocytes have been shown to express a large number of receptors for many transmitters *in vitro* and *in vivo*, including receptors for glutamate, GABA, acetylcholine and ATP (Porter and McCarthy, 1997).

D-serine is a co-agonist for NMDA receptors and can bind to its glycine-binding site. Serine racemase, the enzyme that converts L-serine into D-serine, is expressed in astrocytes, and Ca^{2+} elevations in astrocytes can lead to the release of D-serine from astrocytes. Recent evidence has shown that D-serine from astrocytes is necessary for NMDAR-dependent plasticity, and that process is Ca^{2+} dependent. Depletion of D-serine or disruption of exocytosis blocked local long-term potentiation (LTP) (Henneberger et al., 2010).

Initially, some researchers hypothesized that calcium waves were propagated through gap junctions because application of gap junction inhibitors interfered with neuronal response to astrocyte stimulation (Nedergaard, 1994) and with maintenance of rhythmic

neuronal activity (Prosser et al., 1994). However, cell culture studies were able to demonstrate that calcium waves could propagate across physical gaps, suggesting the existence of a soluble signaling factor (Hassinger et al., 1996). Media collected from astrocyte cultures post-stimulation was also able to cause a calcium response in untreated astrocytes (Guthrie et al., 1999). ATP was identified as the primary signaling factor between astrocytes and mediates calcium wave propagation (Guthrie et al., 1999; Wang et al., 2000). Furthermore, ATP signaling is not dependent on calcium (Wang et al., 2000).

Work next focused on how transmitters were being released by astrocytes. Manipulations of astrocyte vesicle release components prevented glutamate release from astrocytes and the subsequent neuron response (Araque et al., 2000). Vesicle components such as VGLUT1/2, cellubrevin, synaptobrevin II, SNAP23, munc-18-1, and complexin 2 were also identified in astrocytes (Bezzi et al., 2004; Zhang et al., 2004).

To demonstrate astrocytic involvement at the synapse in *vivo*, Phil Haydon's group created a dominant-negative SNARE (dn-SNARE) mouse to block gliotransmission in astrocytes by using the cytosolic portion of the SNARE domain of synaptobrevin 2 (Pascual et al., 2005). The authors found that basal synaptic transmission was influenced by SNARE-dependent processes in astrocytes. Astrocytes controlled the strength of hippocampal synapses through adenosine by releasing ATP. ATP released from astrocytes is hydrolyzed and accumulates in the extracellular space as adenosine, which can then act on pre-synaptic adenosine receptors for tonic suppression of synaptic activity mediated by adenosine A1 receptors. As expected, the dn-SNARE mice also had reduced extracellular ATP.

Using Ca^{2+} dependent SNARE-vesicle release machinery, mammalian astrocytes produce and release gliotransmitters, such as ATP, adenosine, glutamate and D-serine, which act on both neurons and other glia (Halassa et al., 2007). Whisker stimulation response in layer 2 of the barrel cortex was used to assay an *in vivo* response. Astrocytes were loaded with fluorescent Ca^{2+} indicators and increases in Ca^{2+} were observed in response to whisker stimulation. Furthermore, when metabotropic glutamate receptors (mGluRs) were pharmacologically inhibited, the astrocytic Ca^{2+} response was reduced-suggesting a role for glutamate in modulating this response (Wang et al., 2006).

Drosophila also has different glial classes including perineurial glia, subperineurial glia, ensheathing glia, cortex glia and astrocytes. Fly astrocytes have strikingly similar morphology to mammalian astrocytes and are similarly spatially tiled. Their processes are associated with synapses in the neuropil, making them well situated to modulate synaptic transmission. These astrocytes express excitatory amino acid transporter 1 (EAAT1) (Freeman et al., 2003) and the GABA transporter GAT (Stork et al., 2014; Thimgan et al., 2006) for the clearance of glutamate and GABA. Astrocytes also contain glutamine synthetase (GS), the enzyme responsible for converting glutamate to glutamine in astrocytes (Freeman et al., 2003).

1.2.2 Glia and circadian rhythms

Circadian rhythms are a highly-regulated process and so it is not surprising that the ability of astrocytes to participate at the synapse would also provide a way to influence clock neurons and their downstream pathways. Evidence for an astrocytic role in circadian rhythms came from the finding that astrocyte cultures from rodent SCN could be entrained by VIP (Marpegan et al., 2009; Prolo et al., 2005). Rhythmic abundance of

adenosine, a gliotransmitter, was observed in SCN cultures and in astrocyte cultures (Womac et al., 2009).

The first evidence for glial involvement of circadian behavior in *vivo* came from studies in *Drosophila*. Protein and mRNA levels of the glial-specific gene *ebony* (N-b-alanyl-biogenic amine synthetase) cycle, and EBONY is required for rhythmic locomotor behavior (Suh and Jackson, 2007). Glia-specific manipulations of vesicle machinery in *Drosophila* resulted in behavioral arrhythmicity (Ng et al., 2011; Ng and Jackson, 2015). Of the glial classes, only astrocyte-specific genetic manipulations eliminated locomotor behavioral rhythmicity. Furthermore, disruption of vesicle recycling in glia resulted in altered PDF signaling, suggesting that signals released from glia are likely important for PDF neurons (Ng et al., 2011). Knockdown of Sarco-endoplasmic Reticulum Calcium ATPase (SERCA) in glia also led to arrhythmicity (Ng et al., 2011). Given calcium's role in astrocyte signaling, particularly in exocytosis, this result was further evidence for a role for released factors in regulating rhythmicity.

Recent studies have demonstrated the modulatory potential of mammalian astrocytes on circadian rhythms (Barca-Mayo et al., 2017; Brancaccio et al., 2017; Tso et al., 2017). Studies in SCN slices demonstrated calcium, glutamate and BMAL1 oscillations in astrocytes (Brancaccio et al., 2017; Tso et al., 2017). The calcium oscillations of astrocytes are anti-phasic to the calcium oscillations of neurons (Brancaccio et al., 2017). The glutamateric signal from astrocytes acts through pre-synaptic NMDAR subunit NR2C. Inhibition of GS increased extracellular glutamate and severely dampened PER2 rhythms. GABA has also been implicated in glia-neuron signaling, as inhibition of GABA-A-R prevents astrocyte entrainment of neuronal clock gene oscillations (Barca-

Mayo et al., 2017). Deletion of *Bmal1* from astrocytes lengthened circadian period and PER2 oscillations (Tso et al., 2017). Furthermore, the CK1ε mutant mouse has a period of ~22 hours due to a point mutation in exon 4. Deleting that exon in only astrocytes lengthens circadian period by about one hour. It is clear from these studies that astrocytes are important pieces of circadian circuits.

1.3. Identification of glial signaling components

It is of great interest to identify astrocyte components relevant for maintaining rhythms. As previously discussed, there is bidirectional communication between astrocytes and neurons. Manipulations in astrocytes in both flies and mammals can result in altered circadian behavior. Identification of components involved would provide greater understanding of how this communication happens and give us new therapeutic targets for circadian-related diseases. My thesis will describe two strategies: a microRNA-based approach and a gene profiling-based approach.

One growing area of research is on the role of miRNAs in circadian behavior. Victor Ambros and Gary Ruvkun characterized the first miRNA, *lin-4*, in *C. elegans* (Lee et al., 1993; Wightman et al., 1993) although earlier work had uncovered pathways important for miRNA processing (Fire et al., 1998). Mammalian miRNAs were first identified in HeLa cells; some of these miRNAs are highly conserved and have orthologs in both vertebrates and invertebrates, suggesting that this class of RNAs is evolutionary ancient (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). There are currently 466 mature miRNAs in *Drosophila*, 1915 in mouse and 2588 in humans listed in miRBase (Kozomara and Griffiths-Jones, 2014). miRNAs are small ~22 nucleotide

non-coding RNAs that most commonly bind to 3' untranslated region (3' UTR) of target RNAs to repress translation or degrade the transcript. At the 5' end of the miRNA lies the "seed" sequence at nucleotides 2-7. This sequence is particularly important for target recognition. The majority of transcripts in the mammalian genome are targeted by miRNAs (Friedman et al., 2009). Conversely, each miRNA can target many RNAs.

1.3.1 miRNA biogenesis

For the sake of brevity, I will broadly summarize the process of miRNA biogenesis. miRNAs are encoded in both introns and exons, occasionally sharing a promoter with its host gene. Like mRNAs, miRNAs are transcribed by RNA Polymerase II (RNA Pol II) in the nucleus (Cai et al., 2004; Lee et al., 2004). The resulting transcript is the primary miRNA (pri-miRNA), a long stem loop structure of about 1kb. It is next processed by the Microprocessor complex, which includes the RNase III Drosha and its cofactor DGCR8 (known as Pasha in *Drosophila*)(Denli et al., 2004; Gregory et al., 2004; Han, 2004; Landthaler et al., 2004; Lee et al., 2003). The pri-miRNA is cleaved into a smaller stem loop structure of ~65 bases termed the precursor miRNA (pre-miRNA) (Lee et al., 2002). It is next exported from the nucleus by forming a complex with exportin 5 and RAN-GTP (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). The hydrolysis of GTP disassembles the complex and releases the pre-miRNA into the cytosol. There, Dicer cleaves the loop off the pre-miRNA, turning it into a smaller double-stranded miRNA:miRNA* duplex (Bernstein et al., 2001; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). The cofactor Loquacious (Loq) is required in *Drosophila* for proper processing of the pre-miRNA (Förstemann et al., 2005; Saito et al., 2005). In mammals, Dicer interacts with TAR RNA-binding protein (TRBP)

or protein activator of PKR (PACT) to help process the pre-miRNA but it does not seem to be essential to the process (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006). The duplex is then loaded onto the protein Argonaut (AGO) to form the RNA-induced silencing complex (RISC) (Kawamata and Tomari, 2010). The passenger strand is then removed and the mature RISC complex can then bind to its target RNA.

1.3.2 miRNAs and circadian rhythms

While miRNAs are frequently studied in the context of development or disease, there is growing literature on their role in circadian behavior (summarized in Fig. 1.2). Early studies on miRNAs were primarily screens. In the retina, a number of miRNAs were identified as having diurnal oscillations. One cluster of these miRNAs, miR-183/96/182, was found to target Adenylyl cyclase VI (*Adcy6*) which modulates the synthesis of melatonin in the pineal gland (Xu et al., 2007). Microarray studies in *Drosophila* profiled 78 miRNAs at different time points and revealed rhythmic expression of miR-263a and miR-263b. Both miRNAs are under clock control as rhythmicity was eliminated in flies with disabled clocks (Yang et al., 2008).

To identify miRNAs that may target core clock genes, Na *et al*. profiled both miRNAs and mRNAs in the mouse liver over a 48-hour period (Na et al., 2009). By comparing the abundance of miRNAs at different times with the abundance of predicted mRNA targets, the authors compiled a list of 33 miRNA-mRNA pairs between 24 miRNAs and 10 clock genes (Na et al., 2009). miR-181d and miR-191 were found to target *Clock* and *Bmal1*, respectively and have an inverse correlation of abundance.

The first report of a miRNA modulating circadian behavior in *vivo* identified miR-219 as being targeted by the CLOCK:BMAL1 complex. In the SCN, miR-219 was found to

be rhythmic and its knockdown lengthened circadian period in mice (Cheng et al., 2007). In an analysis of night shift workers, the miR-219 promoter was found to have aberrant methylation. This miRNA is also associated with cancer (Shi et al., 2013). Cheng *et al.* also identified miR-132 as a rhythmic, light-induced miRNA (Cheng et al., 2007). Two cAMP response element motifs are located in the 5' promoter of the miR-132 gene, suggesting that it is regulated by CREB. Knockdown of miR-132 potentiated light-induced clock resetting (Cheng et al., 2007) while overexpression of miR-132 reduced this resetting (Alvarez-Saavedra et al., 2011). A robust response to the light-induced increase in miR-132 required the activation of ERK/MAPK signaling pathways (Cheng et al., 2007). miR-132 was found to target multiple chromatin remodeling factors and translational control regulators, suggesting that light may regulate entrainment through these mechanisms (Alvarez-Saavedra et al., 2011).

Directly investigating components of miRNA biogenesis makes clear that miRNAs are important regulators of circadian rhythms. Immunoprecipitation of AGO1, a member of the silencing complex, revealed interactions between AGO1 and core clock members *Clk*, *vri* and *cwo* (Kadener et al., 2009). Cells deficient in the pre-miRNA processor *Dicer*, have shortened circadian periods due to faster translation and accumulation of PER1 and PER2 (Chen et al., 2013).

Profiling of clock cells in *Drosophila* identified a number of miRNAs, including *bantam*, that target *Clk* (Kadener et al., 2009). Overexpression of *bantam* in clock cells lengthened circadian period by nearly three hours (Kadener et al., 2009). Overexpression of let-7 in clock cells also resulted in period lengthening through let-7 targeting *cwo* (W. Chen et al., 2014). Conversely, knockout of let-7 eliminates anticipation in LD and

decreases rhythmicity in DD (W. Chen et al., 2014). miR-276a is light-regulated, oscillates in LD and targets *tim* (Chen and Rosbash, 2016). Manipulation of miR-276a abundance in either direction results in decreased rhythmicity. Presumably, this result is due to its regulation of *tim*.

In mammalian models, the cluster miR-192/194 was found to target all three mammalian *per* genes and overexpression of these miRNAs shortened circadian period (Nagel et al., 2009). miR-494 and miR-142-3p were found to target *Bmal1* (Shende et al., 2011). Interestingly, only miR-494 had diurnal oscillations. miR-17 oscillates in LD and DD, and targets *Clock*. In turn, CLOCK can bind to the miR-17 promoter and induce its production (Gao et al., 2016).

Chicken miR-26a targets the L-type voltage gated calcium channel (L-VGCC) subunit α1c in the retina (Shi et al., 2009). The L-VGCC currents are under circadian control and expression of miR-26a was sufficient to decrease these currents when they should be highest. Inhibition of miR-26a increased the currents when they should be lowest. Furthermore, CLOCK was able to induce miR-26a expression. In the liver, the non-rhythmic miR-122 targets the circadian deadenylase nocturnin (Kojima et al., 2010)-an important demonstration that miRNAs do not need to be rhythmic in order to regulate rhythms. An investigation of clock output revealed that manipulation of miR-279 in *Drosophila* disrupts rhythmicity by targeting *Upd*, a ligand for the JAK/STAT pathway (Luo and Sehgal, 2012). The miRNAs 959-964 were identified as cycling and found to be involved in feeding behavior (Vodala et al., 2012). miR-124 regulates the phase of activity without affecting core clock mechanisms, suggesting an output pathway as a target (Zhang et al., 2016). More generally, GW182, via its AGO1 interaction domain, is

a key regulator of PDF-R signaling (Zhang and Emery, 2013). Knockdown of GW182 in clock cells results in arrhythmic flies.

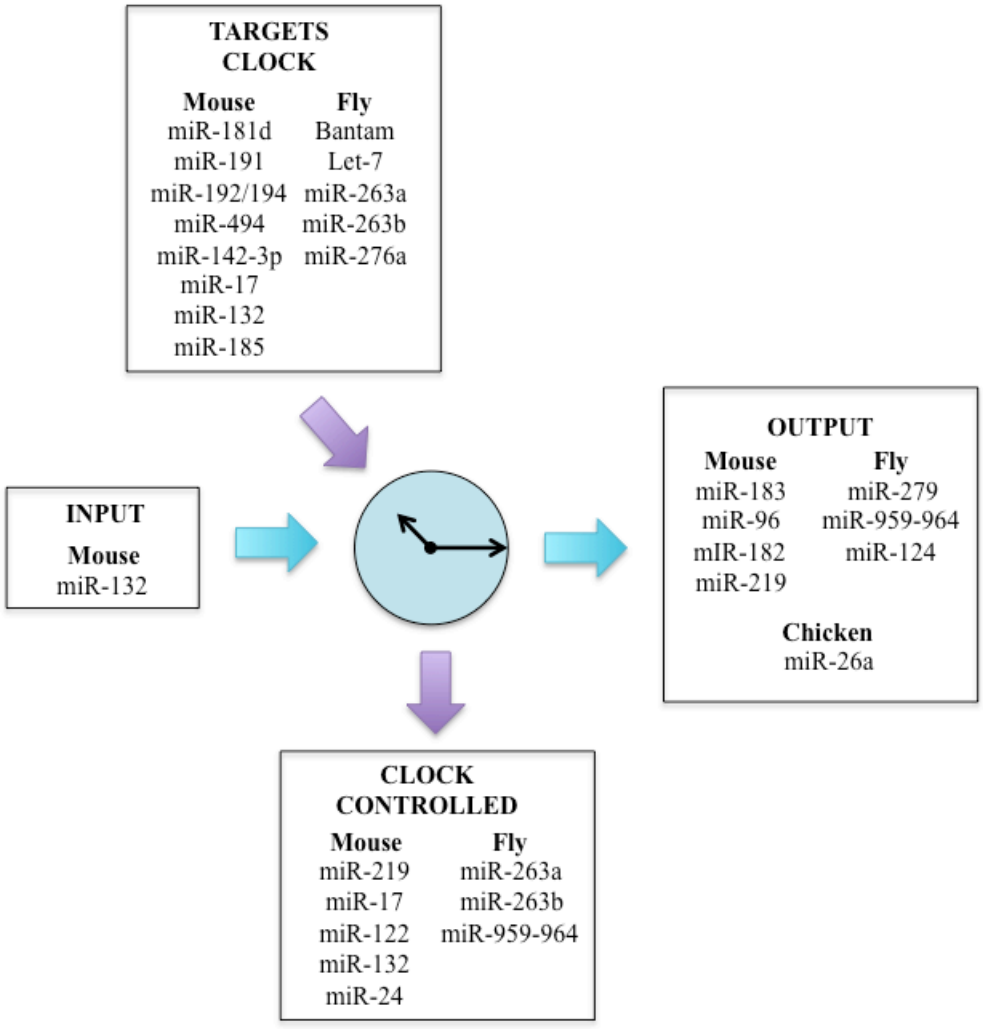


Figure 1.2: Summary of miRNAs with circadian functions.
As discussed in Chapter 1.3.2, the miRNAs identified as relevant for circadian research can be split into four groups: input, clock controlled, clock targeting and output.

1.3.3 Studying miRNAs in vivo

Tools for systematically interrogating the role of specific miRNAs in *Drosophila* have emerged in recent years. Multiple libraries of miRNA overexpression strains have been constructed, allowing for control over the spatial and temporal expression of these miRNAs using the Gal4/UAS expression system (Bejarano et al., 2012; Schertel et al., 2012; Suh et al., 2015). Studies using these strains are informative but risk creating circumstances that do not occur in nature due to temporal or spatial restrictions. Genetic screens using these UAS-miRNA strains have identified miRNAs with roles in wing phenotypes (Bejarano et al., 2012; Schertel et al., 2012) and body growth(Suh et al., 2015). A library of knockout strains has also been created (Y.-W. Chen et al., 2014). As many miRNAs have functions in development, a number of these strains are homozygous lethal, limiting their utility. Nevertheless, studies of these mutants have described phenotypes in survival, life span and self-righting behavior (Y.-W. Chen et al., 2014; Picao-Osorio et al., 2017). Additionally, these strains preclude the study of physiological functions in adulthood and do not allow for cell-type specific studies of miRNAs. For example, miR-9a mutants have decreased body length but inhibition of miR-9a specifically in insulin-producing cells (IPCs), results in increased body length (Suh et al., 2015). Inhibitors of miRNAs (i.e. miR-sponges, tough decoys) have been created as a way of manipulating endogenous levels of miRNAs in a cell type-specific manner (Fulga et al., 2015; Haraguchi et al., 2009; Loya et al., 2009). Genetic screens using miR-sponges recently identified miRNAs that regulate muscle formation (Fulga et al., 2015) and learning/memory (Busto et al., 2015).

I utilized the library of miR-sponges to screen for miRNAs in glia with functions in maintaining normal circadian behavior. By manipulating endogenous miRNAs, I aimed to identify miRNAs and their RNA targets that have normal physiological functions in glial cells.

1.3.4 Gene expression profiling and circadian rhythms

The advent of molecular biology tools such as microarrays and sequencing has accelerated work in uncovering components of circadian oscillations. The early circadian profiling studies used microarrays to identify rhythmically expressed genes (Ceriani et al., 2002; Claridge-Chang et al., 2001; Y. Lin et al., 2002; McDonald and Rosbash, 2001; Ueda et al., 2002). While informative, there was little overlap among the lists of cycling genes from different labs.

Most profiling studies do not differentiate between cell-types. While whole head experiments can provide important information, the brain is heterogeneous. Both neurons and glia have diverse molecular components. In circadian research, most cell-type specific profiling has been performed on clock-cells. These profiling approaches generally included the use of hand dissection or fluorescent activated cell sorting (FACS) (Kula-Eversole et al., 2010; Nagoshi et al., 2010; Ruben et al., 2012; Xu et al., 2011). To obtain the amount of cells required for profiling by hand-dissection is laborious and time-consuming. FACS isolates transcripts after dissociation, which is less likely to provide an accurate snapshot of the cells in *vivo*.

Translating ribosome affinity purification (TRAP) was developed as a method of profiling polysomal RNAs in a cell-type specific manner (Doyle et al., 2008; Heiman et al., 2008). To isolate RNAs in specific cell-types, a ribosomal subunit (L10a) is tagged

with enhanced green fluorescent protein (EGFP) and expressed genetically. RNAs can then be immunoprecipitated using a high-affinity EGFP antibody. Thus, ribosome association is used as a proxy for translation. The Jackson Lab adapted this technology for use in *Drosophila* to take advantage of the abundant number of available GAL4 drivers. TRAP allows for relatively simple isolation of RNAs without the dissociation process, thereby providing a more accurate molecular image of the profiled cell-type.

By using the TRAP followed by RNA-seq, previous studies in the lab constructed circadian translational profiles of clock cells (Huang et al., 2013) and fat body (Yu and Jackson, not published). Additionally, previous studies profiled both larval and adult astrocytes in *Drosophila* (Huang et al., 2015; Ng et al., 2016). Little is known about the identity of cycling genes in astrocytes. By identifying ribosome-bound RNAs with cyclic changes in abundance in astrocytes, I aim to identify important components of astrocyte circadian regulation.

1.3.5 Summary

Circadian rhythms influence nearly every facet of life on earth. Understanding how these rhythms adapt and persist is essential for understanding the pathologies that occur when the rhythms break down. Astrocytes are now known to be capable of modulating circadian behavior by interacting with relevant neurons. What is not known is how these cells interact with neurons in this context. This thesis describes two approaches for uncovering components important for astrocyte regulation of circadian behavior. The astrocyte specific studies of miRNAs with circadian functions and of RNAs that cycle in abundance are important contributions to our understanding of astrocytes in behavior.

Chapter 2: Materials and Methods

2.1. miRNA Experiments

2.1.1 Fly Strains and Maintenance

Drosophila cultures were reared on standard cornmeal/sugar/wheat germ medium in an environmentally controlled incubator set at 25°C on a 12 hr:12 hr light-dark (LD) schedule. For the primary miR-SP screen, experimental flies were generated by crossing virgin female repo-Gal4 (w; Sp/SM1; repo-Gal4) with male 2X UAS-miR-SP (w^{1118}; UAS-miR-SP; UAS-miR-SP). As controls, both the driver and UAS strains were crossed to w^{1118}. Similar crosses were generated for miR-SP expression in other cell-types including alrm-Gal4, elav-Gal80, eaat1-Gal4, and nSyb-Gal4. For all constitutive miR-SP expression experiments, crosses were reared at 25°C. To temporally restrict expression of the miR-SP to astrocytes, tub-$Gal80^{ts}$, alrm-Gal4 was used to conditionally activate the UAS-miR-SP. Temperatures of 23°C and 30°C, respectively, were used to inhibit or activate Gal4. For the miRNA target screening, virgin female repo-Gal4 flies were crossed to male UAS-RNAi flies. For the miRNA mutant strains, the experimental flies were homozygous for the mutation and control flies were generated by crossing each strain to w^{1118} to obtain heterozygous flies without any chromosome balancers. All strains were obtained from the Bloomington Stock or Vienna *Drosophila* Resource Centers.

2.1.2 Collection of locomotor activity and data analysis

Flies (<1 week old) were placed in *Drosophila* Activity Monitors (DAM; Trikinetics, Inc.) housed in environmentally controlled incubators. For each experiment, flies were entrained for 4 days (LD) followed by 8-10 days in constant darkness (DD) to assess free running behavior. For experiments assessing behavior with constitutive expression of

UAS-miR-SP transgenes, the behavior assay was run at 25°C. For conditional expression experiments, flies were entrained at 23°C and free running behavior was assessed at different temperatures depending on the experiment. Behavioral data were collected as beam breaks in 30 minute bins and analyzed using the MATLAB-based signal processing fly toolbox (Levine et al., 2002). The fly toolbox provided information on activity level (beam breaks) and calculated overall periodicity. Entrainment and rhythmicity was assessed using the rhythmicity index (RI) and visual examination of actograms.

To assess statistical significance of circadian data, I determined normality using the D'Agostino & Pearson omnibus normality test. If the data did not pass a normality test, I then used the Kruskal-Wallis test (nonparametric ANOVA) with Dunn's Multiple Comparison test. A one-way ANOVA with the Tukey-Kramer Multiple Comparisons test was used if the data passed a normality test. Values listed for both Dunn's Multiple Comparison and Tukey-Kramer Multiple Comparisons tests are multiplicity adjusted p values. For experiments with comparisons of two groups, a two-tailed, two-sample Student's *t*-test was used. For behavioral studies, separate experiments employing flies from independent genetic crosses are considered biological replicates.

2.1.3 Immunohistochemistry and image analysis

For most experiments, four to eight individual males of experimental and control strains (technical replicates) were fixed in 4% paraformaldehyde (PFA) in PBST (1X PBS, 0.5% Triton X-100) on ice for 30 minutes. Brains were hand-dissected and then fixed in 4% PFA for 20 minutes. Fixed brains were then washed with PBST three times, for 10 minutes each, followed by blocking in 5% normal goat serum (in PBST) for three hours. Brains were incubated with the primary antibody overnight at 4°C, washed with

PBST, three times quickly and three times for 25 minutes each, and then incubated with the secondary antibody overnight at 4°C. Brains were washed again three times quickly and three times for 25 minutes each, before mounting with VectaShield (Vector laboratories) to preserve fluorescence. For the GAT staining, I followed the general procedure with the following exceptions: pre-dissection flies were fixed for 17 minutes, post-dissection flies were fixed for 7 minutes, and blocking was restricted to one hour. Additionally, primary antibody incubation occurred over 2 days in 4°C. I employed the following primary antibodies: mouse anti-REPO (1:500, Developmental Studies Hybridoma Bank, DSHB), rabbit anti-PDF (1:100, DSHB), rabbit anti-PER (1:10,000, a gift of R. Stanewsky), and rabbit anti-GAT (1:500, a gift of M. Freeman). Relevant secondary antibodies were employed at (1:500 or 1:1000) including Alexa Fluor 488 goat anti-rabbit or Alexa Fluor 488 goat anti-mouse (both from Invitrogen).

Brain images were acquired using a Nikon A1R confocal microscope. For all experiments, experimental and control brains were imaged on the same day with the same acquisition parameters. Fiji ImageJ was used to generate projected images from the optical sections. PER staining was quantified by measuring the mean fluorescence signal in different groups of clock cells. I quantified glial cell number for both experimental and control brains using a region of interest (ROI) around the inner chiasm giant glial cells. A z-projection substack was employed for these measurements. Images were thresholded and cells within the ROI were counted by Fiji. For the GAT staining quantification, an ROI was drawn on an optical section at approximately the same depth (the point at which glial cells in the optic lobe become a single row); mean pixel intensity was determined by

Fiji. A two-tailed Student's *t*-test was used to assess significance between the experimental and control brains.

2.1.4 Quantification of MESK2 RNA abundance

RNA was extracted from samples containing 5-10 pupae (pharate adults) and converted to cDNA using SuperScript II reverse transcriptase (Invitrogen) and random hexamers. The primers employed for MESK2 were CTACCACGATTTGGGCCTCAA (Forward) and CAGCAGACCTCGCATCACTG (Reverse). A Stratagene real-time cycler was employed for cDNA amplification with SYBR green as a reporter. RNA abundance was analyzed using *Drosophila rp49* as an internal control.

2.2. TRAP experiments

2.2.1 Fly strains and maintenance

Drosophila cultures were reared on standard cornmeal/sugar/wheat germ medium in an environmentally controlled incubator set at 25°C on a 12 hr:12 hr light-dark (LD) schedule. Virgin female flies carrying elav-Gal80, eaat1-Gal4 were crossed with male elav-Gal80, UAS-EGFP-mL10a flies to generate F1 flies that express EGFP-mL10a in astrocytes.

2.2.2 Purification and isolation of ribosome-bound RNAs

Flies were collected at 12 times points across a 48 hour period into 15 mL conical tubes before flash-freezing in liquid nitrogen. Flies were then stored at -80°C until further processing. Heads were separated by quickly vortexing the tubes and then using sieves to collect heads. There were ~200 heads per TRAP experiment sample. Frozen heads were quickly homogenized in buffer containing 20 mM HEPES-KOH (pH 7.4), 150 mM KCl,

5 mM $MgCl_2$, 0.5 mM DTT, 100 µg/ml Cycloheximide and 2 U/mL SUPERase. Samples were then centrifuged at 20,000xg for 20 minutes. The clear lysate was separated from the debris into a new tube and DHPC and Igepal CA-630 was added to the solution at a concentration of 30mM and 1%, respectively. Samples incubated on ice for 5 minutes and were then centrifuged again at 20,000xg for 20 minutes. After centrifugation, the supernatant was combined with magnetic beads coated with purified anti-EGFP antibody (HtzGFP-19C8, Sloan Kettering Antibody & Bioresource Core Facility; Dynabeads Antibody Coupling Kit, Invitrogen) and incubated at 4°C for 1 hour with end over end rotation. Samples were then washed 6 times with 20 mM HEPES-KOH (pH 7.4), 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 100 µg/ml Cycloheximide. RNA was extracted from the magnetic beads using TRIzol. RNA quality and quantity were analyzed using a Bioanalyzer (Agilent).

2.2.3 RNA-Seq Library Construction and Sequencing

I used~400 ng of RNA from each time point (combining samples if necessary) and constructed libraries for RNA-Seq by following standard Illumina protocol from the TruSeq RNA sample preparation kit. mRNAs were isolated from ribosomal and other small RNAs using poly-dT coupled magnetic beads and fragmented by addition of divalent cations at 94°C. Cleaved mRNAs were then reverse transcribed into cDNA using random primers, and cDNA was subjected to second strand synthesis using DNA polymerase I and RNaseH. DNAs were end repaired, ‘‘A’’ tailed, and then ligated to Illumina sequencing adaptors prior to enrichment by PCR to create a library. Quality and quantity of each library was assessed using a Bioanalyzer. Sequencing of libraries was performed by the Tufts Medical School Molecular Core Facility using the Illumina HiSeq

2000. Sequence reads were obtained and their quality analyzed by Dr. Amy Yu, using the quality control metrics provided by the FastQC pipeline. I obtained, on average, about >20 million high quality reads per sample after removing low quality reads.

2.2.4 Analysis of RNA-seq Data

Initial processing of raw RNA-seq data was performed by Dr. Amy Yu. Sequencing reads were mapped to the *Drosophila* genome (BDGP realease 6) using Tophat v.2.0.9. Low quality and short reads were trimmed using Fastx Clipper. Reads were processed through HTSeq for gene IDs and read counts. IDs were reconciled to Flybase version FB2017_1. Genes with average read counts of less than 20 across all time points were removed. Read counts were then quantile normalized to control for variation among experiments. Cycling analysis was performed using JTK_Cycle (Hughes et al., 2010; Miyazaki et al., 2011). At this stage, all genes with cycling amplitudes of less then 0.5 were removed. Genes were then associated with gene names. Genes with adjusted p-values of <0.05 were considered cycling.

I performed gene ontology (GO) analyses using the Database for Annotation, Visualization and Integrated Discover (DAVID, v. 6.8) annotation tools to determine overrepresented categories of GO biological processes and cellular components(Huang et al., 2009). Genes were also manually annotated using FlyBase (FB2017_6) (Gramates et al., 2017).

Chapter 3: Results

3.1 Screening for miRNAs with circadian functions in glia

I performed a genome-wide, glial-specific screen using a collection of Gal4-regulated miRNA sponges (miR-SPs). Each strain carries two Gal4-regulated UAS-miR-SP transgenes expressing a sponge specific to a miRNA; sponges contain twenty copies of a sequence complementary to a specific miRNA with deliberate mismatches at nucleotides 9-12 (Fulga et al., 2015). Each sponge also contains a mCherry tag that can be used to visualize expression. As a miR-SP binds to and inhibits a particular miRNA, the expectation is that translation for its target will be increased and result in increased protein product. For my initial screen, I decided to look for phenotypes in entrainment, rhythmicity, circadian period and overall activity level (Fig. 3.1, Table 5.1).

Sponge-expressing flies were generated by crossing UAS-miR-SP strains to those carrying the glial-specific repo-Gal4 driver (Fig. 3.2 A). Background controls arose from a cross of the UAS-miR-SP strain to w^{1118} flies. I also examined flies from a cross of repo-Gal4 to w^{1118} and did not observe any effects on behavior. Male F1 flies from experimental and control groups (repo-Gal4>UAS-miR-SP and UAS-miR-SP) were collected for analysis of locomotor activity. Both types of progeny carried two copies of a specific miR-SP transgene, although it was not expressed in control flies lacking repo-Gal4. The *Drosophila* Activity Monitor (DAM) system was employed to monitor locomotor activity (see Material and Methods 2.1.2).

3.1.1 Circadian Period

Previous studies in *Drosophila* showed RNAi knockdown of PER in glia does not result in a change in circadian period (Ng et al., 2011). However, knockdown of *Bmal1* in

mammalian astrocytes did result in a period phenotype (Tso et al., 2017). To look for miRNAs with functions relating to circadian period, I used two criteria: the difference between the experimental group and the UAS-miR-SP control group must be statistically significant, and at least 30 minutes. Since flies without rhythms do not have periodicity, arrhythmic flies were excluded from my analysis. My screen identified four miRNAs whose inhibition in glia decreased circadian period in DD: miR-980, miR-981, miR-998 and miR-1012 (Fig. 3.1 A). Glial expression of each miR-SP resulted in significantly decreased period of at least 30 minutes ($p<0.001$). miR-980 was recently identified as a memory suppressor, as inhibition of miR-980 improved memory acquisition and stability (Guven-Ozkan et al., 2016). One potential target of miR-980 is the clock gene *Pdp1*, a component of the second feedback loop for the *Drosophila* clock (TargetScan and DIANA microT-CDS). *cwo* is another clock component that miR-980 is predicted to target (DIANA microT-CDS). The other identified miRNAs do not appear to target clock genes but are predicted to target transporters with miR-981 predicted to target the glutamate transporter, *Eaat1* (TargetScan and DIANA microT-CDS) and miR-998 predicted to target the GABA transporter, *Gat* (DIANA microT-CDS).

3.1.2 Entrainment

Only one miRNA has been implicated in regulating entrainment, miR-132 (Cheng et al., 2007). My initial screen identified miR-184 as another candidate for regulating entrainment. To assess which flies have defects in entrainment, I analyzed 4 days of activity in LD 12:12. I used a cutoff of a 30% decrease in RI value and percent entrainment – compared to the UAS-miR-SP control – to identify interesting candidates. Only glial inhibition of miR-184 resulted in decreased entrainment (Fig. 3.1 B). Profiling

studies comparing wild-type and *cyc* mutant flies found that miR-184 abundance is significantly increased in *cyc* mutant flies (Yang et al., 2008), indicative of possible clock regulation of the miRNA.

3.1.3 Activity

Changes in activity level may be associated with sleep phenotypes the behavioral assay is not sensitive enough to detect. They may also be associated with the health of the fly. The dopamine transporter mutant *fumin* (*fmn*) is hyperactive due to increased synaptic dopamine (Kume et al., 2005). The combination of the glial specific *ebony (e)* mutation with *fmn* rescues the increased activity of the *fmn* mutants (Suh and Jackson, 2007). This result suggests that glia can regulate activity level. Indeed, the Jackson lab previously found that inhibition of CG14141 in glial cells significantly increased activity level (Ng et al., 2016). CG14141 is a predicted secreted protein and thus a good candidate as a signal for glia-neuron communication.

To assess changes in activity level, I used the average beam crossings from the DD portion of the behavior run. This resulted in the identification of 22 miR-SPs that decreased activity and one that increased activity (Fig. 3.1 C). Previous studies identified the enzyme *Trehalase* (*Treh)* as an astrocyte-enriched gene with involvement in locomotor activity (see Chapter 4.3.3 for further discussion) (Ng et al., 2016). Glial expression of let-7-SP and miR-79-SP, two miRNAs predicted to target *Treh*, resulted in decreased in activity level (DIANA microT-CDS) (Fig. 3.1 C). The miR-7-SP was the only sponge to increase activity, relative to its control, but the experimental flies (repo>miR-7-SP) did not have activity higher than several other controls.

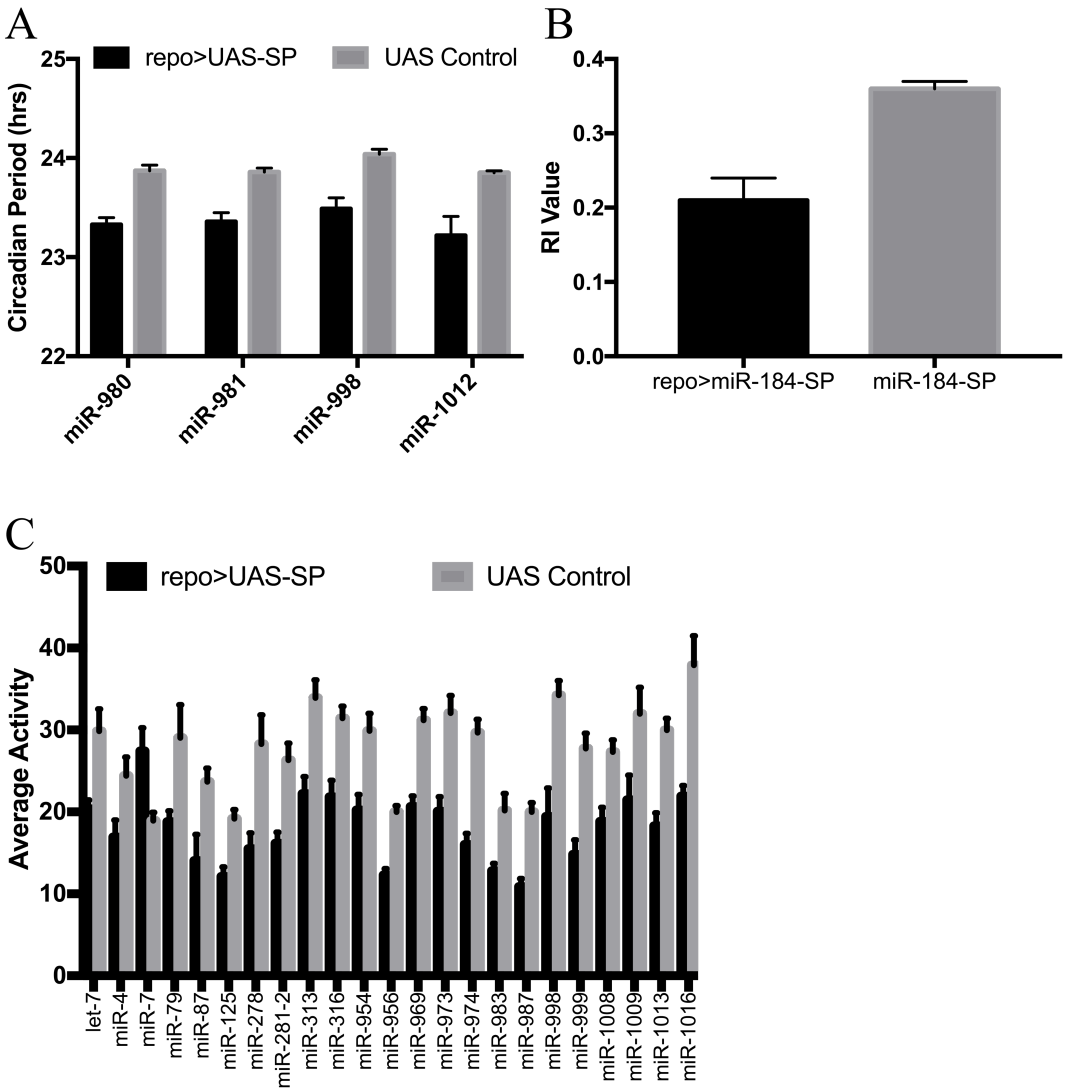


Figure 3.1: Inhibition of miRNAs in glia generates behavioral phenotypes in circadian period, entrainment and activity level.
A) Glial expression of four miR-SPs resulted in significantly decreased period of at least 30 minutes compared to UAS control. B) Glial expression of miR-184-SP significantly impaired entrainment. C) Glial expression of number of miR-SPs decreased activity level and one miR-SP increased activity level (miR-7-SP). Results are mean ± SEM, two-tailed Student's *t*-test, $p < 0.05$ for all experiments shown.

3.1.4 Rhythmicity

Rhythmicity in DD was assessed using two measures: the rhythmicity index (RI), a statistic which indicates the robustness of rhythms (Levine et al., 2002), and percent rhythmicity (calculated from the RI and manual scoring of actograms). Values for RI and percent rhythmicity were normalized by comparison of each experimental cross to its UAS-miR-SP control. To identify candidate miRNAs, I used a cut-off of 30% decreased RI and percent rhythmicity relative to controls (Fig. 3.2 B, candidates in red). This cutoff corresponds to statistical significance at $p<0.0001$ for almost all hits compared to background controls (Table 3.1). Distributions of normalized RI and percent rhythmicity values (see Methods) are shown for all tested miR-SPs in Fig. 3.2 C-D. Altogether, I identified 20 strains having significantly reduced rhythmicity (Table 3.1), a hit rate of ~14%. This percentage is similar to those found for certain phenotypes previously studied using these strains (Fulga et al., 2015). A subset of the interesting miR-SPs is shown in Fig. 3.2 E. I also included behavioral data for miR-33-SP, miR-315-SP and a strain containing a scrambled sequence to serve as controls for sponge-based manipulation (Fig. 3.2 E, Table 3.1). Glial expression of miR-33-SP and miR-315-SP did not decrease RI value or percent rhythmicity compared to the control. Glial expression of the scrambled-SP did result in a modest but significant decrease in RI compared to UAS control flies. However, all flies in the experimental population were statistically rhythmic. In addition, the average RI for scrambled-SP expressing flies was equal to or greater than the average RI value of for several other UAS-miR-SP controls. Given these results, I conclude that the observed behavioral phenotypes for the 20 "hits" are not the result of non-specific miR-SP expression in glial cells. Of the "hits", two – miR-263b and miR-963 – were

previously described as having circadian-relevant functions (Vodala et al., 2012; Yang et al., 2008).

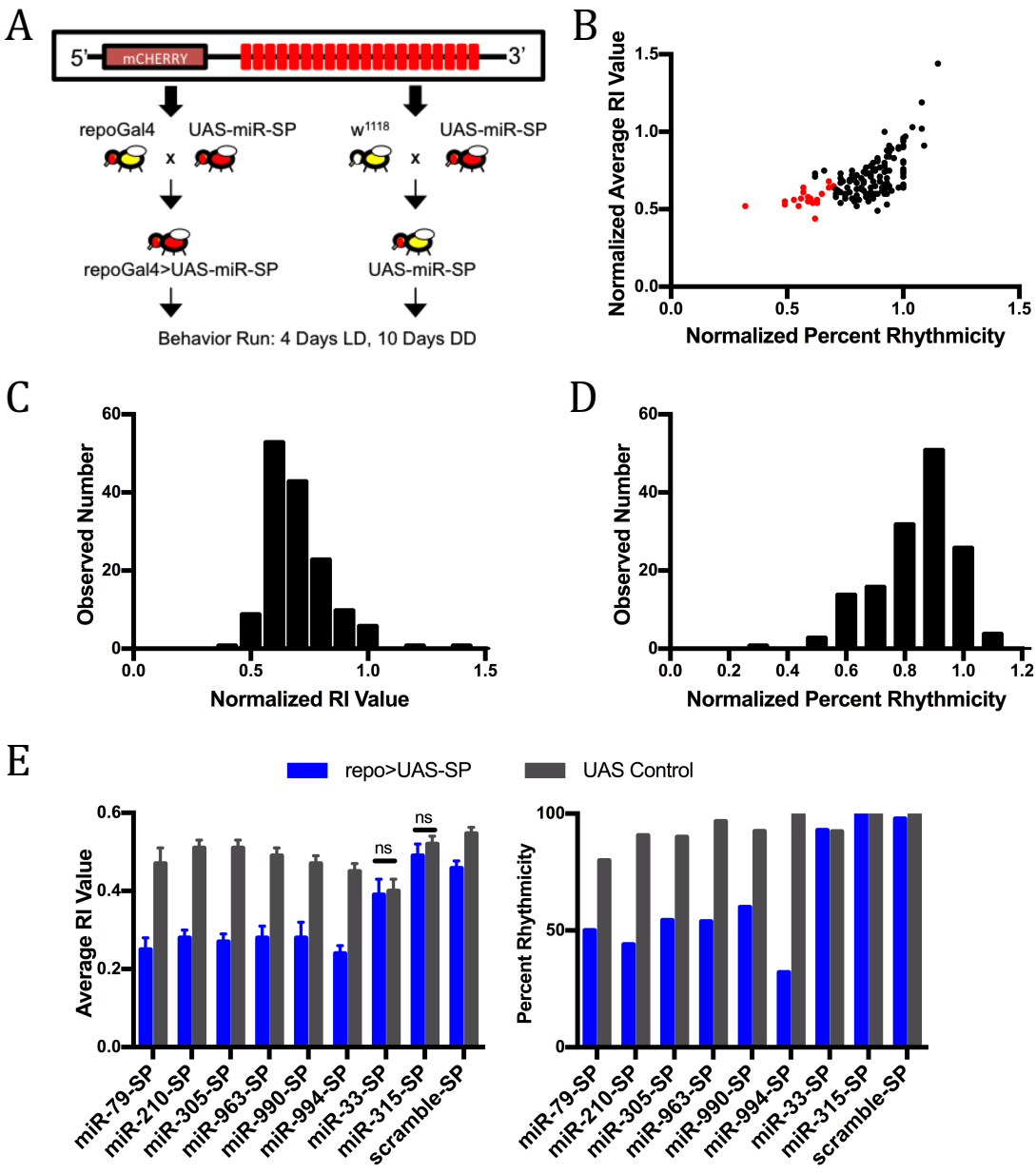

A
5'
mCHERRY
3'
repoGal4
UAS-miR-SP
x
w1118
UAS-miR-SP
x
repoGal4>UAS-miR-SP
UAS-miR-SP
Behavior Run: 4 Days LD, 10 Days DD
B
Normalized Average RI Value
Normalized Percent Rhythmicity
C
Observed Number
Normalized RI Value
D
Observed Number
Normalized Percent Rhythmicity
E
repo>UAS-SP
UAS Control
Average RI Value
Percent Rhythmicity
ns
ns
miR-79-SP
miR-210-SP
miR-305-SP
miR-963-SP
miR-990-SP
miR-994-SP
miR-33-SP
miR-315-SP
scramble-SP

Figure 3.2: miR-SP rhythmicity screen for decreased rhythmicity.
A) Schematic of a miR-SP construct and genetic crosses to generate flies expressing it. Each miR-SP strain carries two copies of the miR-SP construct, which contains an mCHERRY tag followed by twenty oligomer repeats complementary to the miRNA with the exception of mismatches in nucleotides 9-12. For each experiment, the repo-Gal4 pan-glial driver strain or w^{1118} were crossed to the UAS-miR-SP strain (experimental and control groups, respectively). Flies were entrained for 4 days to LD 12:12, then transferred to constant darkness (DD) for 10 days. For each experimental and control group $n \geq 8$. B) Normalized percent rhythmicity versus normalized RI values for all tested miR-SP strains (obtained by dividing experimental by control values). miR-SPs resulting in a decrease of 30% for both measures are denoted in red. Histograms showing the distribution of normalized RI value (C) and normalized percent rhythmicity (D) for all miR-SPs tested. E) Actual RI and percent rhythmicity values with glial expression of several selected miR-SP strains (See Table 3.1 for full list). Results are mean RI ± SEM ($n=13-44$; two-tailed Student's *t*-test, $p<0.05$ for all comparisons of experimental to control populations).

Genotype	Experimental			Control				
	RI ± SEM	N	Percent Rhythmicity	RI ± SEM	N	Percent Rhythmicity	p-value	Biological Replicates
miR-79-SP	0.25 ± 0.03	18	50.00	0.47 ± 0.04	20	80.00	<0.0001	2
miR-79-SP (2)	0.25 ± 0.02	16	50.00	0.44 ± 0.03	26	84.62	0.0002	2
miR-210-SP	0.28 ± 0.02	25	44.00	0.51 ± 0.02	32	90.63	<0.0001	2
miR-263b-SP	0.24 ± 0.03	20	60.00	0.55 ± 0.02	31	96.77	<0.0001	2
miR-274-SP	0.31 ± 0.03	20	65.00	0.48 ± 0.02	25	96.00	<0.0001	2
miR-281-1-SP	0.30 ± 0.03	23	60.87	0.53 ± 0.03	30	96.67	<0.0001	2
miR-285-SP	0.32 ± 0.02	21	52.38	0.49 ± 0.03	27	92.59	<0.0001	2
miR-304-SP	0.28 ± 0.02	31	51.61	0.50 ± 0.02	30	96.67	<0.0001	2
miR-305-SP	0.27 ± 0.02	44	54.41	0.51 ± 0.02	44	90.00	<0.0001	4
miR-309-SP	0.28 ± 0.02	35	57.14	0.51 ± 0.02	44	97.73	<0.0001	2
miR-310-SP	0.26 ± 0.03	34	57.14	0.50 ± 0.02	39	94.74	<0.0001	4
miR-317-SP	0.29 ± 0.03	22	57.14	0.51 ± 0.02	37	100.00	<0.0001	4
miR-927-SP	0.27 ± 0.03	28	53.57	0.51 ± 0.02	36	97.22	<0.0001	2
miR-963-SP	0.28 ± 0.03	26	53.85	0.49 ± 0.02	30	96.67	<0.0001	2
miR-967-SP	0.25 ± 0.03	25	56.00	0.45 ± 0.02	45	93.33	<0.0001	3
miR-971-SP	0.28 ± 0.04	23	60.87	0.51 ± 0.02	31	100.00	<0.0001	2
miR-981-SP	0.32 ± 0.02	41	67.39	0.50 ± 0.02	41	95.65	<0.0001	3
miR-990-SP	0.28 ± 0.04	13	60.00	0.47 ± 0.02	29	92.50	<0.0001	3
miR-992-SP	0.34 ± 0.02	67	67.16	0.52 ± 0.01	75	96.00	<0.0001	4
miR-994-SP	0.24 ± 0.02	28	32.14	0.45 ± 0.02	30	100.00	<0.0001	2
miR-iab-4-3p-SP	0.31 ± 0.03	26	65.38	0.45 ± 0.02	30	96.67	0.0002	2

Genotype	Experimental			Control				
	RI ± SEM	N	Percent Rhythmicity	RI ± SEM	N	Percent Rhythmicity	p-value	Biological Replicates
miR-33-SP	0.39 ± 0.04	14	92.86	0.40 ± 0.03	13	92.31	NS	1
miR-315-SP	0.49 ± 0.03	14	100.00	0.52 ± 0.02	16	100.00	NS	1
Scramble-SP	0.49 ± 0.02	45	97.78	0.54 ± 0.02	32	100.00	0.0009	2
repoGal4*	0.52 ± 0.03	14	100.00					

Table 3.1: Expression of 20 miR-SPs in glia results in decreased rhythmicity.
Twenty miRNAs for which glial miR-SP-mediated inhibition resulted in decreased rhythmicity. miR-33-SP, miR-315-SP and scramble-SP were included as examples of miR-SPs with no effect on rhythmicity. Significance was determined using a two-tailed Student's *t*-test; p-values for each set of experiments are listed. *repoGal4 was included as a control in every behavioral experiment. Listed is a representative result.

My interest in astrocytes stems from previous work that determined manipulation of vesicle recycling in astrocytes and not other glial subtypes disrupted behavioral rhythms (Ng et al., 2011). However, this does not preclude the other glial subclasses from functions relating to locomotor rhythmicity. Ensheathing glia enwrap major brain structures, and are neuropil associated, although not closely associated with synapses (Doherty et al., 2009). Cortex glia surround neuronal cell bodies and thus are in prime location to modulate neuronal cell body function (Awasaki et al., 2008).

I performed a secondary screen of these candidate miRNAs to assess whether any of them function specifically in astrocytes. I used the astrocyte-specific drivers alrm-Gal4 and eaat1-Gal4 (combined with elav-Gal80 to inhibit any neuronal Gal4 expression) to express miR-SPs in astrocytes (Table 3.2). A subset of experiments is shown in Fig. 3.3, showing astrocyte expression of some but not all of the candidate miR-SPs also decreased rhythmicity. Astrocyte inhibition of miR-990 significantly decreased rhythmicity (Fig. 3.3, Table 3.2). miR-990 is predicted to target *complexin* (*cpx*), a SNARE-binding protein that functions like a fusion clamp to regulate vesicle release (TargetScan) (Huntwork and Littleton, 2007). It is possible that increased abundance of CPX may interfere with signals released by glia to communicate with neurons.

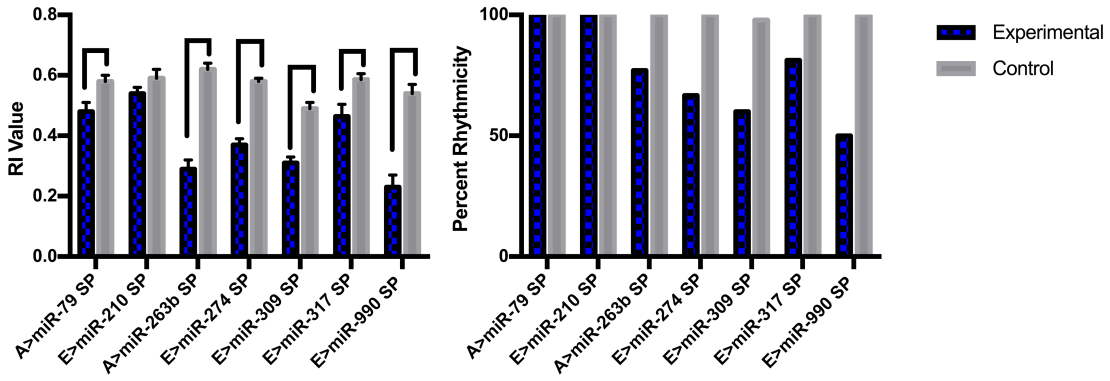


Figure 3.3: Astrocyte specific inhibition of candidate miRNAs.
Candidate miR-SPs were screened for effects in astrocytes using alrm-Gal4 (A) or elav-Gal80, eaat1-Gal4 (E). Results are mean ± SEM, two-tailed Student's *t*-test, $p<0.05$.

	Experimental			Control			
Genotype	RI ± SEM	N	%R	RI ± SEM	N	%R	p-value
E>UAS-miR-79 SP	0.46 ± 0.04	15	86.67	0.55 ± 0.02	29	100.00	0.0418
A>UAS-miR-79 (2) SP	0.48 ± 0.03	16	100.00	0.58 ± 0.02	16	100.00	0.0095
E>UAS-miR-210 SP	0.54 ± 0.02	15	100.00	0.59 ± 0.03	15	92.31	0.0296
A>UAS-miR-263b SP	0.29 ±0.03	26	76.92	0.62 ± 0.02	14	100.00	<0.0001
E>UAS-miR-274 SP	0.37 ± 0.02	54	66.67	0.58 ± 0.01	63	100.00	<0.0001
A>UAS-miR-281-1 SP	0.38 ± 0.04	13	84.62	0.58 ± 0.03	16	100.00	0.0007
E>UAS-miR-285 SP	0.35 ± 0.03	19	68.42	0.55 ± 0.01	42	97.62	<0.0001
E>UAS-miR-304 SP	0.39 ± 0.04	10	92.86	0.42 ± 0.03	11	83.33	NS
E>UAS-miR-305 SP	0.45 ± 0.02	42	88.10	0.56 ± 0.02	45	93.33	0.0104
E>UAS-miR-309 SP	0.31 ± 0.02	40	60.00	0.49 ± 0.02	47	97.87	<0.0001
E>UAS-miR-310 SP	0.38 ± 0.04	14	85.71	0.43 ± 0.03	16	100.00	NS
E>UAS-miR-317 SP	0.47 ± 0.04	16	81.25	0.59 ± 0.02	28	100.00	0.0170
E>UAS-miR-927 SP	0.45 ± 0.05	12	91.67	0.52 ± 0.04	11	100.00	NS
A>UAS-miR-963 SP	0.26 ± 0.03	15	66.67	0.37 ± 0.04	14	92.86	0.0357
E>UAS-miR-967 SP	0.34 ± 0.03	16	81.25	0.51 ± 0.03	12	91.67	0.0008
E>UAS-miR-981 SP	0.34 ± 0.02	44	70.45	0.50 ± 0.02	44	100.00	<0.0001
E>UAS-miR-990 SP	0.23 ± 0.04	12	50.00	0.54 ± 0.03	12	100.00	<0.0001
E>UAS-miR-992 SP	0.40 ± 0.03	28	75.00	0.56 ± 0.02	30	96.67	0.0003
E>UAS-miR-994 SP	0.34 ± 0.03	28	82.14	0.46 ± 0.03	30	96.67	0.0017
E>UAS-miR-iab-4-3p SP	0.38 ± 0.04	15	80.00	0.44 ± 0.04	11	90.91	NS

Table 3.2: Astrocyte-specific inhibition of candidate miRNAs
Candidates from the preliminary screen were tested for astrocyte-specific effects using alrm-Gal4 (A) or elav-Gal80, eaat1-Gal4 (E).

3.2 miR-263b and miR-274 regulate circadian behavior in adult astrocytes

3.2.1 Glial cell-specific inhibition of miR-263b or miR-274 results in decreased rhythmicity

Earlier studies identified miR-263b as a cycling miRNA under clock control (Yang et al., 2008). I found that inhibition of miR-263b specifically in glial cells significantly decreased rhythmicity compared to both the UAS-miR-SP control and the repo-Gal4 control (Kruskal-Wallis test; $p<0.0001$; Fig. 3.4 A-B). In unpublished studies, the Jackson lab previously found that a second candidate, miR-274, exhibited circadian rhythms in abundance in pharate adult flies (Y. Huang and F.R. Jackson, unpublished results), indicative of a role in circadian behavior. miR-274 was also previously identified as present in TIM positive clock cells (Kadener et al., 2009). Similar to results with miR-263b, glial inhibition of miR-274 resulted in significantly decreased rhythmicity compared to UAS and Gal4 controls (Kruskal-Wallis test; $p<0.0001$; Fig. 3.4 A). To track miR-SP expression, experimental and control brains were dissected and imaged for mCHERRY expression (Fig. 3.4 C).

As miR-SP inhibition of miRNA function is predicted to result in increased expression of target RNAs, I wondered if overexpression of miRNAs – which should decrease target abundance – would also result in altered behavioral rhythmicity. Indeed, recent studies on miR-276a demonstrated that either increased or decreased miR-276a abundance altered behavioral rhythmicity (Chen and Rosbash, 2016). MicroRNA overexpression might result in enhanced or decreased rhythmicity depending on the function of the particular target RNA. Certain targets might be required in optimal amounts, for example, resulting

in arrhythmicity with either increased or decreased function. Using the same pan-glial driver (repo-Gal4), I found that overexpression of miR-263b (using a UAS-miR-263b transgene) significantly decreased RI and percent rhythmicity (Student's *t*-test; $p<0.0001$; Fig. 3.4 D). This suggests that expression of at least one miR-263b target is required at optimal levels for normal circadian behavioral rhythms. Unfortunately, glial overexpression of miR-274 throughout development was lethal (data not shown, see Fig. 3.11 for adult overexpression), suggesting that the function of at least one of its targets is essential in glia for survival.

I also asked if the observed behavioral phenotype was a glia-specific effect. To answer this question, I used a neuron-specific Gal4 (nSyb-Gal4) to inhibit miR-263b or miR-274 in neurons. Inhibition of either miRNA in neurons did not result in significantly decreased rhythmicity when compared to the corresponding UAS-miR-SP control (Fig. 3.5). These results indicate a glial-specific function for these miRNAs in the context of circadian behavior.

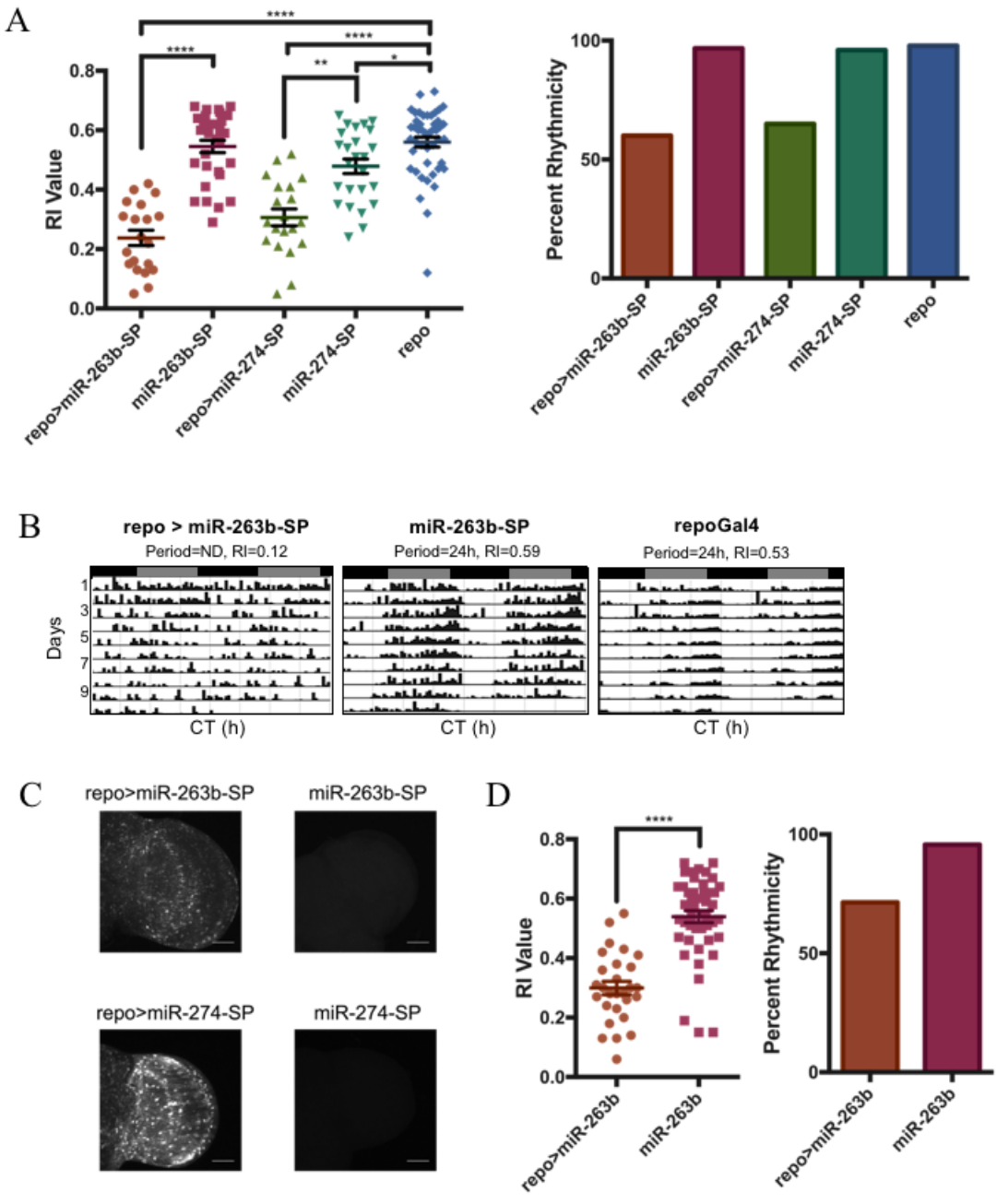

A
****
****
****
**
*
RI Value
0.8
0.6
0.4
0.2
0.0
repo>miR-263b-SP
miR-263b-SP
repo>miR-274-SP
miR-274-SP
repo
Percent Rhythmicity
100
50
0
repo>miR-263b-SP
miR-263b-SP
repo>miR-274-SP
miR-274-SP
repo
B
repo > miR-263b-SP
Period=ND, RI=0.12
miR-263b-SP
Period=24h, RI=0.59
repoGal4
Period=24h, RI=0.53
Days
1
3
5
7
9
CT (h)
CT (h)
CT (h)
C
repo>miR-263b-SP
miR-263b-SP
repo>miR-274-SP
miR-274-SP
D
****
RI Value
0.8
0.6
0.4
0.2
0.0
repo>miR-263b
miR-263b
Percent Rhythmicity
100
50
0
repo>miR-263b
miR-263b

Figure 3.4: Glial manipulation of miR-263b or miR-274 alters rhythmicity.
A) Pan-glial expression of miR-263b-SP or miR-274-SP decreased rhythmicity compared to UAS-miR-SP and repoGal4 controls. RI value (Left) results shown are individual flies with mean ± SEM, n=20-46; Kruskal-Wallis Test ($p<0.0001$) with Dunn's Multiple Comparison Test, *$p<0.05$; **$p<0.01$; ****$p<0.0001$. Percent rhythmicity for the population is depicted on the right. B) Representative DD activity plots (actograms) for individual flies of each genotype in miR-263b-SP experiments. ND refers to not determinable. C) Representative images of mCHERRY expression of repo>miR-263b-SP and repo>miR-274-SP and their respective controls. D) Glial overexpression of miR-263b decreased rhythmicity relative to the UAS-miR-263b control. Results are individual flies with mean ± SEM, n=28-46 (2 biological replicates), two-tailed Student's *t*-test, ****$p<0.0001$.

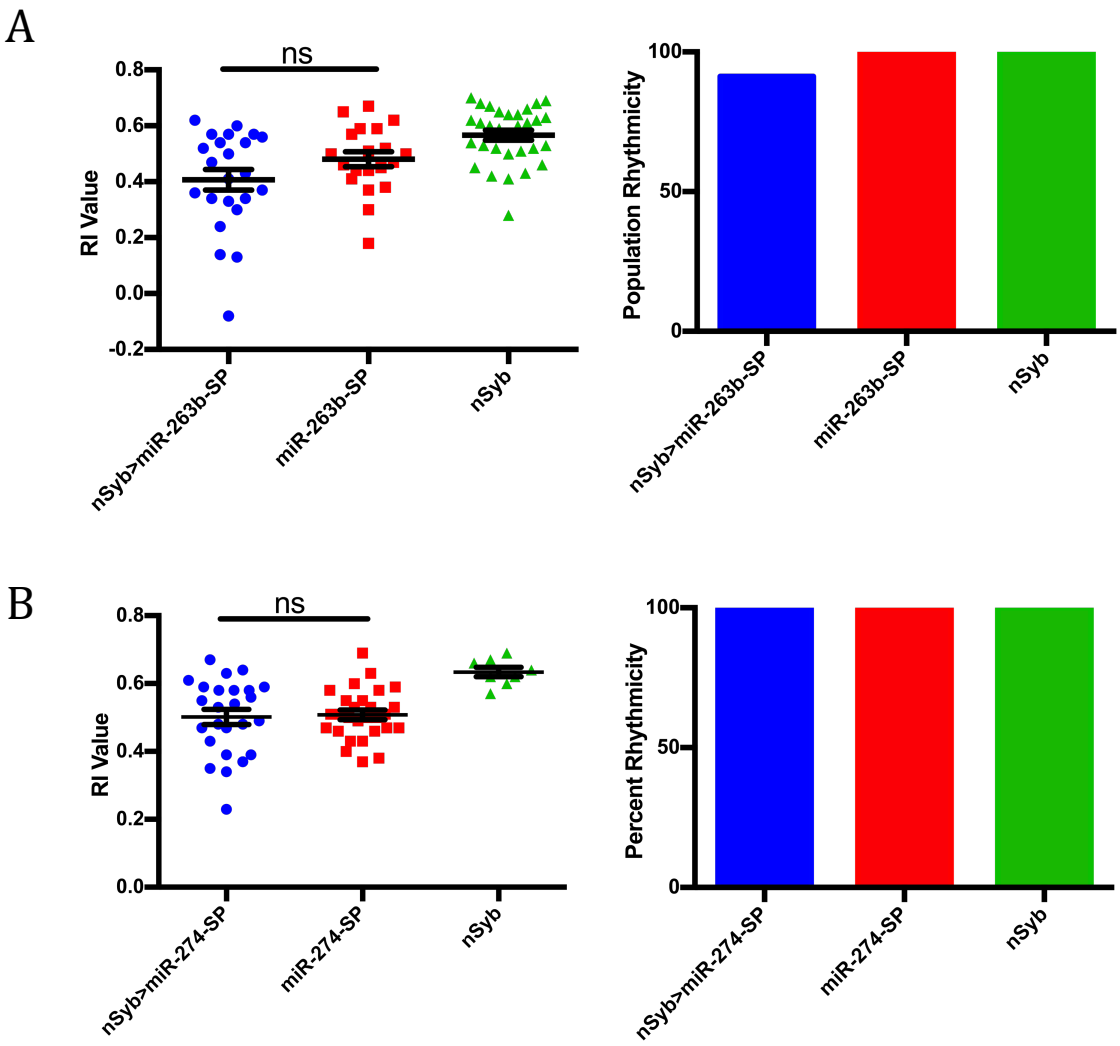


Figure 3.5: Neuronal expression of miR-263b-SP or miR-274-SP does not significantly affect circadian behavior.
nSyb-Gal4 was used to express miR-263b-SP (A) or miR-274 SP (B) in neurons. The mean RI for flies carrying only the nSyb-Gal4 driver differs from RIs for nSyb>miR-SP and miR-SP (UAS only) populations but there is no significant difference between nSyb>miR-SP and miR-SP populations. Results for miR-263b-SP are individual flies with mean ± SEM with n=20-31, Kruskal-Wallis test (p=0.0004) with Dunn's multiple comparison test, *p=0.036, *** p=0.004 (nSyb>miR-263b-SP vs. miR-263b-SP, p=0.822). Results for miR-274-SP are individual flies with mean ± SEM with n=8-26, One-way ANOVA (p=0.001) with Tukey's multiple comparison test, **p<0.01 (nSyb>miR-274-SP vs. miR-274-SP, p=0.964; nSyb>miR-274-SP vs.nSyb, p=0.001; miR-274-SP vs. nSyb, p=0.002).

3.2.2 Global knockout of miR-263b and miR-274 results in decreased rhythmicity

To assess the specificity of the miR-263b- and miR-274-SPs, I utilized recently described knockout strains for both miRNAs (Y.-W. Chen et al., 2014). For either miRNA knockout, mean RI was significantly decreased compared to controls (Student's *t*-test*;* $p<0.0001$; Fig. 3.6 A-B), and the distributions of RI values were visually different in knockouts versus controls. Percent rhythmicity was decreased only slightly for the mutants (for miR-263b, 82% for knockout versus 98% for control; for miR-274, 78% for knockout and 99% for control); however, many "rhythmic" knockout flies had significantly reduced RI indicative of weak rhythmicity. This seemingly weaker effect of a knockout, relative to sponge expression, may be due to compensation that occurs as a consequence of miRNA loss-of-function throughout development. I also performed experiments using mutant strains outcrossed for 4 generations and obtained similar albeit less severe effects (Fig. 3.6 C). Thus, phenotypes for a miRNA knockout are similar to those observed with miR-SP expression, indicative of specificity. While I did not anticipate the less severe effects of the outcrossed strains, it is possible these strains may have acquired suppressors to adapt to the mutation.

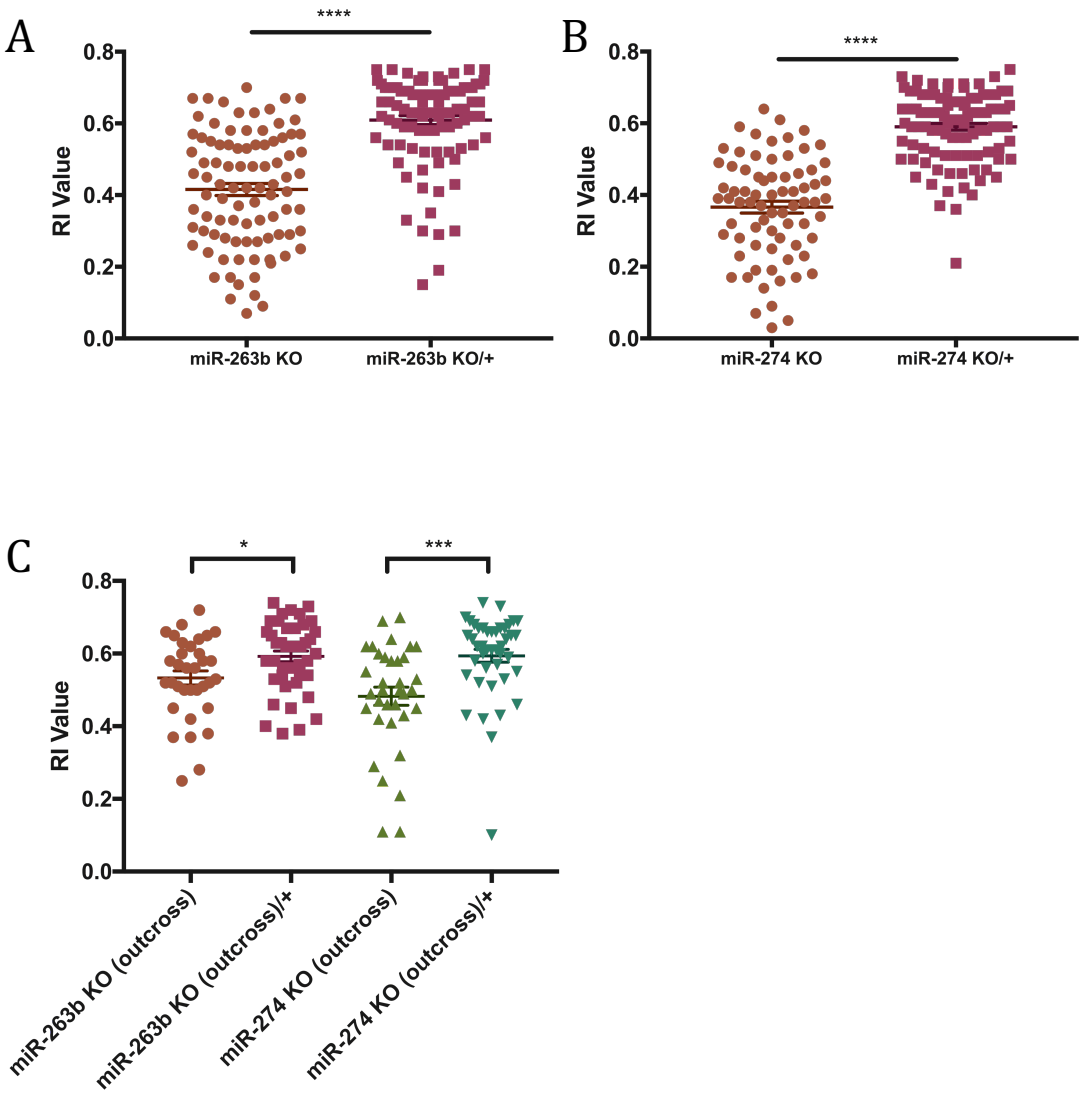


Figure 3.6: Global knockout of miR-263b and miR-274 results in decreased rhythmicity. Both miR-263b (A) and miR-274 (B) knockout flies had decreased robustness of rhythmicity when compared to heterozygous controls. Controls were generated by crossing mutant strains to w^{1118} flies. Results are individual flies with mean ± SEM, n=50-69, two-tailed Student's *t*-test, ****p<0.0001. C) Experiments for both miRNA knockouts were repeated with strains outcrossed for four generations. Results are individual flies with ± SEM, n=34-43, two-tailed Student's t-test, *p<0.05, ***p<0.001.

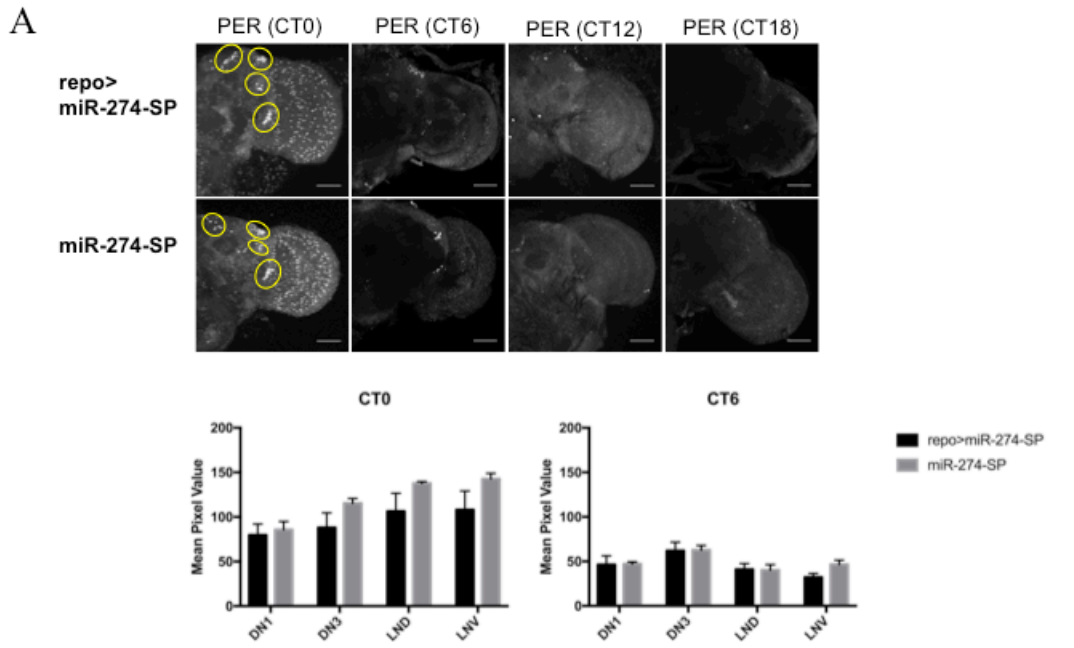
3.2.3 Inhibition of miR-263b or miR-274 does not result in altered clock or glial cell development

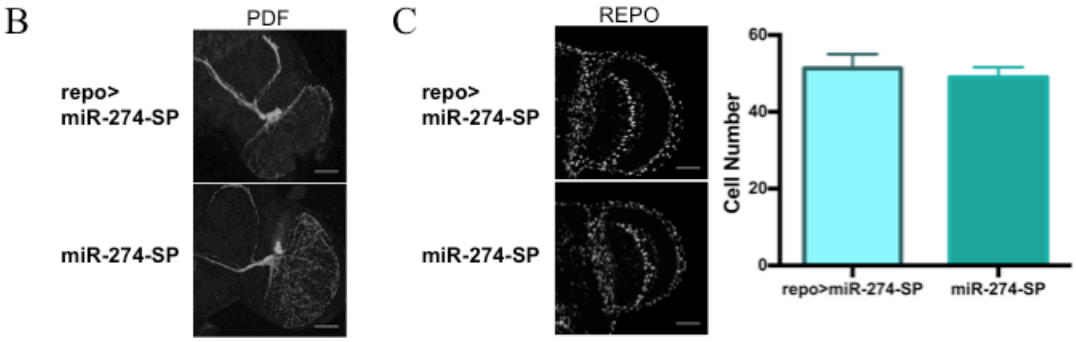
As miRNAs often function in the context of development, I next asked if the observed behavioral phenotype was the result of altered clock cell development. To examine the entire circadian circuit, I stained for the core clock protein PERIOD (PER) at four different time points to determine whether cells expressing PER were present and whether there was altered cyclic expression of PER. As shown in Fig. 3.7 A, repo-Gal4>UAS-miR-274-SP (experimental) and UAS-miR-274-SP (control) brains exhibited similar PER immunoreactivity for the LN_VS, dorsal lateral neurons (LN_DS) and dorsal neurons (DNs) at CT0, the high point of PER abundance. Both showed similar reductions in immunosignal at CT6 relative to CT0, indicative of normal cycling in both genotypes (Fig. 3.7 A; n=6-8 hemispheres). At CT12 and CT18, I observed low or absent immunosignal in both experimental and control genotypes (Fig. 3.7 A), and did not see detectable signal in all PER-containing cells. Therefore, I was not able to quantify abundance of the protein at its nadir. Nonetheless, these results indicate that PER abundance and cycling are normal in miR-274-SP-expressing flies. Similar results were obtained with pan-glial expression of miR-263b-SP (Fig. 3.8 A).

I next examined PDF, as it is the principle circadian transmitter in *Drosophila*, and its rhythmic secretion from the ventral lateral neurons (LN_vS) is important for maintenance of rhythmicity (Klose et al., 2016; Shafer and Yao, 2014). However, comparison of PDF staining between experimental and control brains did not reveal any gross differences in peptide immunoreactivity or s-LNv projection morphology (Fig. 3.7 B, Fig. 3.8 B n=3).

Given these results, it is unlikely that the observed decreased rhythmicity is a result of altered clock cell development.

I also asked whether glial expression of these miR-SPs altered glial cell development. To assess this, I examined glia of experimental and control brains using a REPO antibody, which stains all glia of the adult brain. I detected no gross differences between brain types. In addition, I counted large optic lobe glial cells of both experimental and control brains and found no significant differences in the number of REPO positive cells with either miR-274-SP or miR-263b-SP expression (Student's *t*-test; p=0.636, Fig. 3.7 C; p=0.109, Fig. 3.8 C). I also examined synaptic neuropil regions – which contain astrocytic processes – of miR-263b-SP- and miR-274-SP-expressing brains. To accomplish that, I performed antibody staining against the GABA Transporter (GAT), which is specific for astrocytes (Stork et al., 2014). For each brain, I selected an ROI at approximately equivalent depth through the brain and measured pixel intensity. There was no significant difference in staining intensity between experimental and control brains (Student's *t*-test; p=0.754, Fig. 3.7 D; p=0.908, Fig. 3.8 D). From these experiments I conclude that glial expression of miR-274-SP or miR-263b-SP does not result in obvious changes in clock neuron or glial cell development. Furthermore, flies expressing the miR-274-SP or miR-263b-SP had seemingly normal viability and lived for several weeks (the course of an activity experiment). As I previously showed that severe perturbations of glial cells result in lethality within four days (Ng et al., 2011, 2016), it seems unlikely that glial cell physiology is severely impacted in sponge-expressing animals.


A
PER (CT0)
PER (CT6)
PER (CT12)
PER (CT18)
repo>
miR-274-SP
miR-274-SP
CT0
CT6
Mean Pixel Value
200
150
100
50
0
DN1
DN3
LND
LNV
repo>miR-274-SP
miR-274-SP


B
PDF
repo>
miR-274-SP
miR-274-SP
C
REPO
repo>
miR-274-SP
miR-274-SP
Cell Number
60
40
20
0
repo>miR-274-SP
miR-274-SP

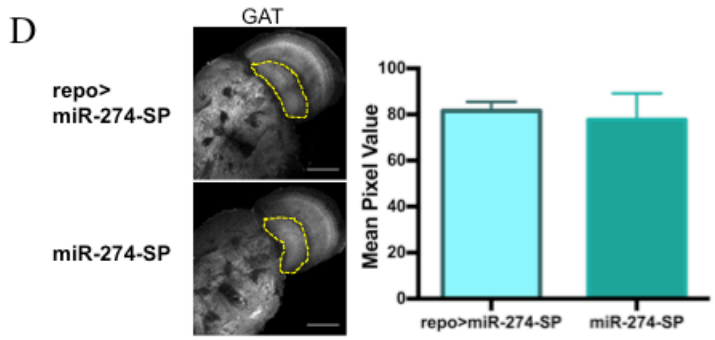

D
GAT
repo>
miR-274-SP
miR-274-SP
Mean Pixel Value
100
80
60
40
20
0
repo>miR-274-SP
miR-274-SP

Figure 3.7: Glial manipulation of miR-274 does not alter clock neuronal morphology, glial cell number or GAT immunostaining intensity
A) Maximum z-projections for experimental (repo>miR-274-SP) and control (miR-274-SP) brains stained for PER at CT0, CT6 CT12 and CT18. Major groups of clock neurons cells are circled in the CT0 images including the DNs (top 2 circles), the LN_ds (middle) and LN_vs (bottom). Graphs depict signal intensity for each group of clock neurons at CT0 and CT6 time points. Results are mean pixel intensity ± SEM, n=6-8 hemispheres for each group. B) Maximum z-projections of confocal images for experimental or control brains stained for PDF. n=3 brains for each group. C) Confocal images depicting REPO staining. Images were acquired for quantification of REPO positive glial cells in experimental and control flies. A maximum z-projection of a substack spanning the depth of the giant glial cells of the optic lobe was created to clearly visualize those cells. For both experimental and control brains, the giant glia cells of the optic lobe were counted (graph). Values in histograms represent mean ± SEM; n=3 brains for each group (two-tailed Student's *t*-test, p=0.636). D) Confocal images depicting GAT staining. Optical sections in approximately equivalent anatomical depth (the point at which the glial cells of the optic lobe form a single row) were selected for GAT staining quantification. For each section, an ROI (outlined in yellow) was drawn around the inner optic lobe for fluorescence quantification. Values in histograms represent mean pixel intensity ± SEM; n=5 brains for each group (two-tailed Student's *t*-test, p=0.755). All mages were acquired with a 40X objective with 2 μm optical z-steps. Scale bar = 50 μm.

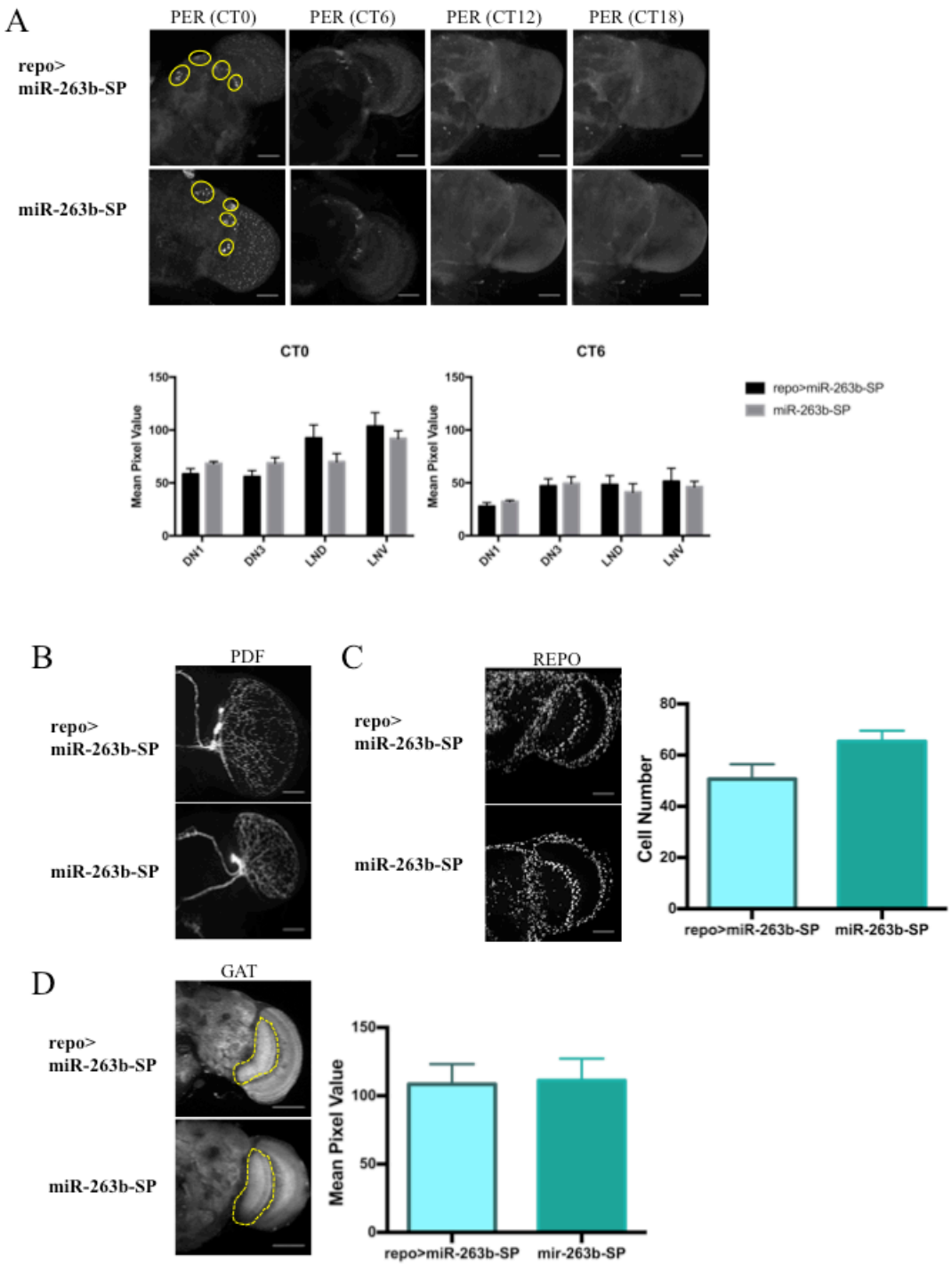

A
PER (CT0)
PER (CT6)
PER (CT12)
PER (CT18)
repo>
miR-263b-SP
miR-263b-SP
CT0
CT6
Mean Pixel Value
150
100
50
0
DN1
DN3
LND
LNV
repo>miR-263b-SP
miR-263b-SP
B
PDF
repo>
miR-263b-SP
miR-263b-SP
C
REPO
repo>
miR-263b-SP
miR-263b-SP
Cell Number
80
60
40
20
0
repo>miR-263b-SP
miR-263b-SP
D
GAT
repo>
miR-263b-SP
miR-263b-SP
Mean Pixel Value
150
100
50
0
repo>miR-263b-SP
mir-263b-SP

Figure 3.8: Glial manipulation of miR-263b does not significantly alter clock neuronal morphology, glial cell number or GAT immunostaining intensity.
A) Maximum z-projections for experimental (repo>miR-263b-SP) and control (miR-263b-SP) brains stained for PER at CT0, CT6 CT12 and CT18. Major groups of clock neurons cells are circled in the CT0 images including the DNs (top 2 circles), the LN_ds (middle) and LN_vs (bottom). Graphs depict signal intensity for each group of clock neurons at CT0 and CT6 time points. Results are mean pixel intensity ± SEM, n=3-9 hemispheres for each group. B) Maximum z-projections of experimental and control brains stained for PDF. n=3 for each group. C) Confocal images depicting REPO staining. Images were acquired for quantification of REPO positive glial cells in experimental and control flies. A maximum z-projection of a substack spanning the depth of the giant glial cells of the optic lobe was created to clearly visualize those cells. For both experimental and control brains, the giant glia cells of the optic lobe were counted (graph). Values in histograms represent mean ± SEM; n=3 brains for each group (two-tailed Student's *t*-test, p=0.109). D) Confocal images depicting GAT staining. Optical sections in approximately equivalent anatomical depth (where the glial cells of the optic lobe form a single row) were selected for GAT staining quantification. For each section, an ROI was drawn around the inner optic lobe for fluorescence quantification. Values in histograms represent mean pixel intensity ± SEM; n=5-6 brains for each group (two-tailed Student's *t*-test, p=0.908). All images were acquired with a 40X objective with 2 μm optical z-steps. Scale bar =50 μm.

3.2.4 miR-263b and miR-274 have circadian functions in adult astrocytes

In previous studies, the Jackson lab found that the astrocyte class of glial cells is important for regulation of the circadian circuit (Ng et al., 2011). Given that finding, I wished to determine if any of my candidate miRNAs were required in astrocytes for circadian behavior. Using astrocyte-specific drivers, I identified a number of miR-SPs that resulted in significantly decreased rhythmicity (≥30%) as assessed by RI value (Fig. 3.3, Table 3.2). For several other miRNAs, there was no effect of sponge expression in astrocytes, suggesting they might be required in other glial classes (i.e., cortex, ensheathing or surface glia).

Two different miR-SPs – miR-263b and miR-274 – had robust and reproducible effects on rhythmicity when expressed in glial astrocytes. To ask whether miR-263b or miR-274 was required in mature astrocytes, I utilized the temporal and regional gene expression targeting (TARGET) system (McGuire et al., 2004) to temporally restrict miR-SP expression to development or adulthood. This system employs a temperature sensitive Gal4 inhibitor, $Gal80^{ts}$, to restrict Gal4 activity at low temperature but allow activity at high temperature. In my studies, I utilized the alrm-Gal4 driver, which is specific for astrocytes. In a first set of experiments, tubulin-$Gal80^{ts}$, alrm-Gal4>2X UAS-miR-263b-SP (experimental) and UAS-miR-263b-SP (control) flies were reared at 23°C to inhibit Gal4 activity. The behavior of experimental and control flies was then assessed at 23°C to determine if Gal4 activity was sufficiently inhibited. I found no significant difference between experimental and control groups (left bars, Student's *t*-test; p=0.215; Fig. 3.9 A; Table 3.3). I next assessed rhythmicity in both groups when flies were reared and entrained to LD at 23°C, but then monitored in DD at 30°C. In those conditions,

there was significantly decreased rhythmicity in the miR-263b-SP-expressing flies compared to UAS-miR-263b-SP controls (middle bars, Student's *t*-test; $p<0.0001$; Fig. 3.9 A; Table 3.3). Finally, I performed the reverse experiment, rearing flies at 30°C and then monitoring activity at 23°C, so that miR-263b-SP was expressed throughout development but not in adulthood. After rearing, activity was monitored during entrainment and in DD at 23°C, conditions in which miR-263b-SP expression ought to be repressed. In this case, both experimental and control flies were rhythmic in DD, indicating that miR-263b is not required during development but rather functions in adult astrocytes (right bars, Student's *t*-test; $p=0.132$; Fig. 3.9 A, Fig. 3.10 C, Table 3.3). I performed an identical set of studies using the miR-274-SP strain, with similar results (Fig. 3.9 B; Table 3.3). I note that conditional astrocyte expression of several other miR-SPs identified in the primary screen did not affect rhythmicity, suggesting a specific requirement for miR-263b and miR-274 in adult astrocytes.

To ensure that these acute manipulations did not result in glial cell death, I counted the number of REPO-positive cells in the optic lobe of flies kept at 30°C (experimental) and at 23°C (control). There were no significant differences in the number of cells between experimental and control groups with expression of either miR-263b-SP (Student's *t*-test; $p=0.154$; Fig. 3.10 A) or miR-274-SP (Student's *t*-test; $p=0.546$; Fig. 3.10 B). These results strongly support the idea that miR-263b and miR-274 have physiological functions relevant for rhythmicity in adult astrocytes.

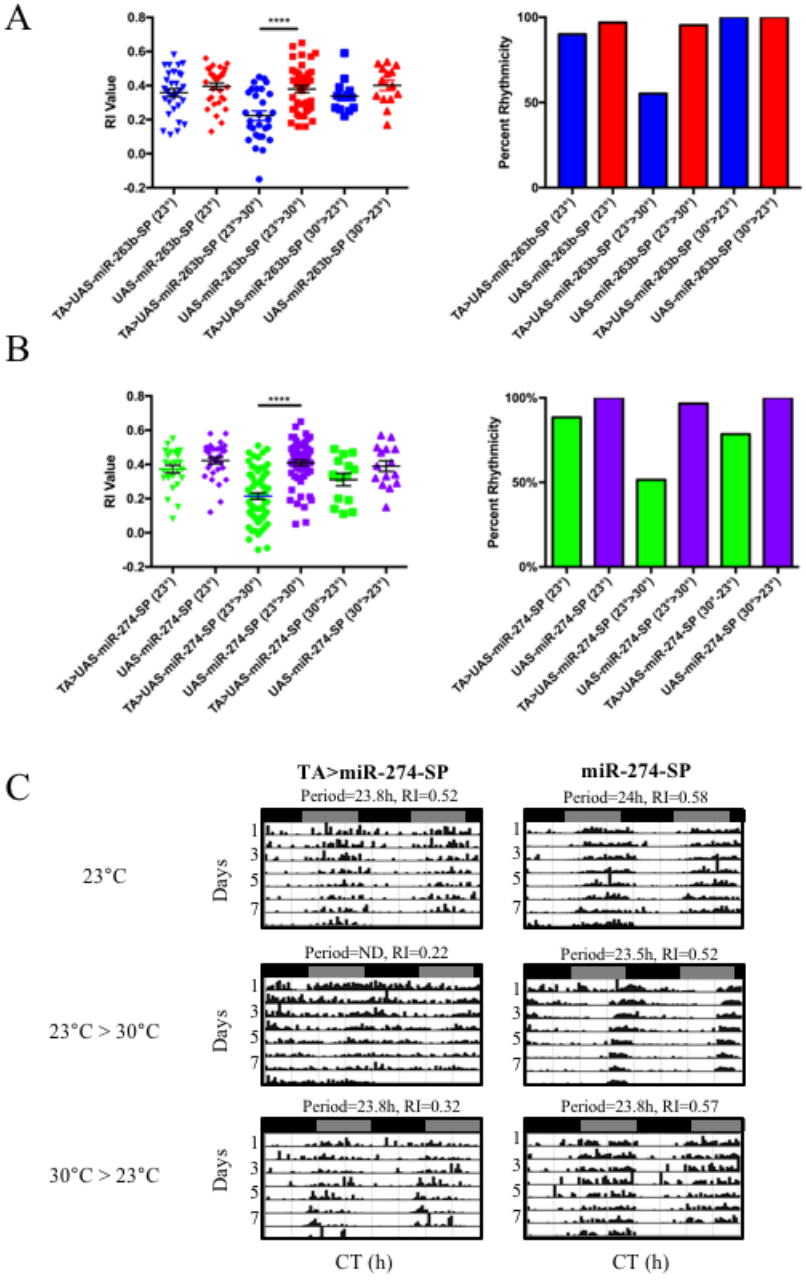

A
RI Value
****
Percent Rhythmicity
TA>UAS-miR-263b-SP (23°)
UAS-miR-263b-SP (23°)
TA>UAS-miR-263b-SP (23°>30°)
UAS-miR-263b-SP (23°>30°)
TA>UAS-miR-263b-SP (30°>23°)
UAS-miR-263b-SP (30°>23°)
B
RI Value
****
Percent Rhythmicity
TA>UAS-miR-274-SP (23°)
UAS-miR-274-SP (23°)
TA>UAS-miR-274-SP (23°>30°)
UAS-miR-274-SP (23°>30°)
TA>UAS-miR-274-SP (30°>23°)
UAS-miR-274-SP (30°>23°)
C
TA>miR-274-SP
miR-274-SP
23°C
Period=23.8h, RI=0.52
Period=24h, RI=0.58
23°C > 30°C
Period=ND, RI=0.22
Period=23.5h, RI=0.52
30°C > 23°C
Period=23.8h, RI=0.32
Period=23.8h, RI=0.57
Days
CT (h)

Figure 3.9: Conditional, adult inhibition of miR-263b or miR-274 in astrocytes results in decreased rhythmicity.
Adult astrocyte expression of the miR-SP transgene was accomplished using tubulin-Gal80^{ts}, alrm-Gal4>miR-SP (TA) flies. In one set of experiments (23°C > 30°C, 3rd and 4th columns), the expression of miR-263b-SP (A) or miR-274-SP (B) was inhibited throughout development and during LD. Temperature was raised to 30°C during DD to effect miR-SP expression. In an independent set of experiments (30°C > 23°C, 5th and 6th columns), crosses were reared at a temperature permitting expression of miR-SPs, but then temperature was reduced to inhibit Gal4 activity. To control for temperature, 3rd set of experiments were performed with crosses reared and kept at 23°C to inhibit miR-SP expression during development and the behavior run (23°C, 1st and 2nd columns). Results are individual flies with mean ± SEM, two-tailed Student's *t*-test, ****p<0.0001. C) Representative actograms for each set of experiments summarized in (B). ND refers to not determinable.

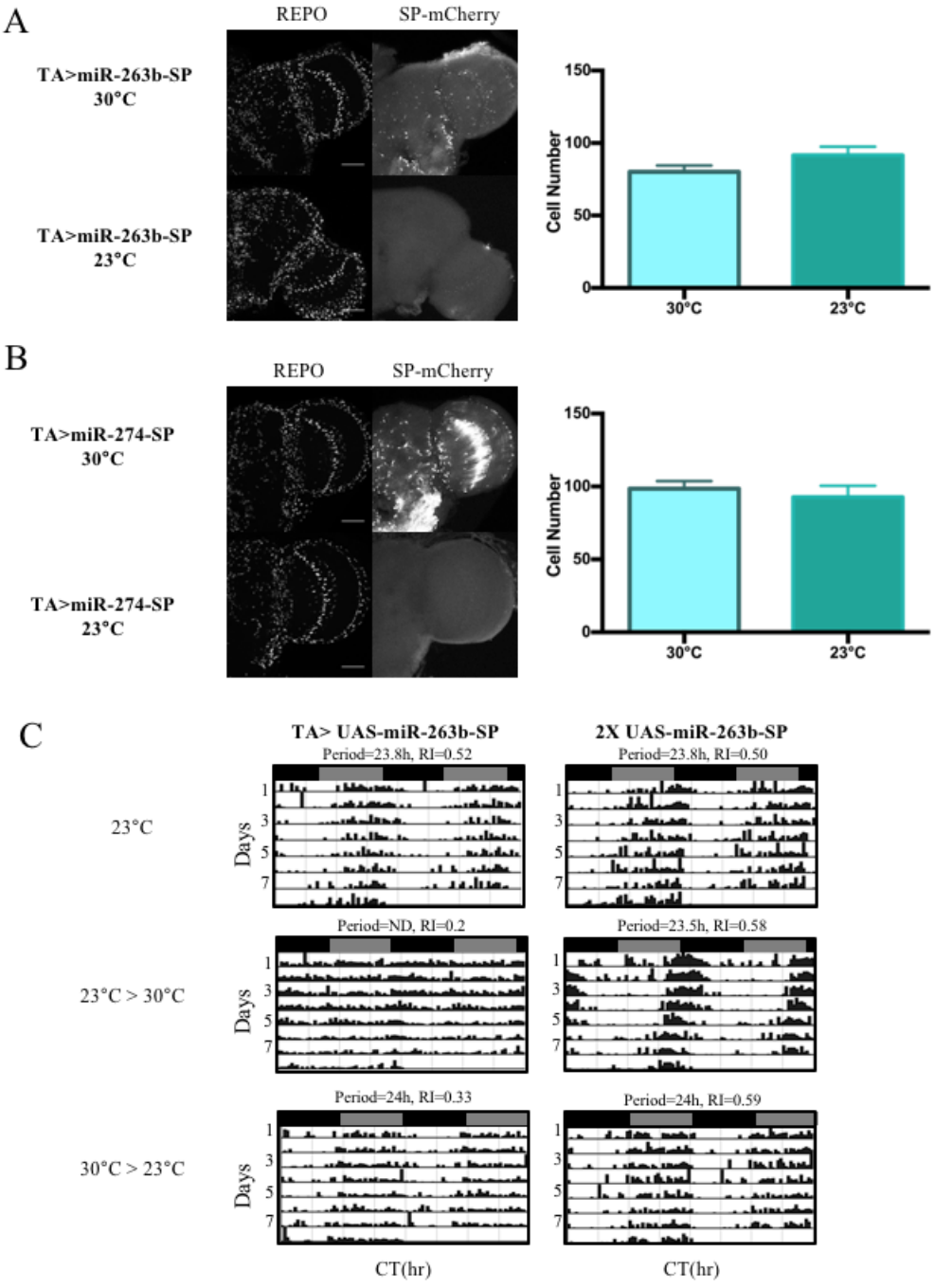

A
REPO
SP-mCherry
TA>miR-263b-SP
30°C
TA>miR-263b-SP
23°C
Cell Number
150
100
50
0
30°C
23°C
B
REPO
SP-mCherry
TA>miR-274-SP
30°C
TA>miR-274-SP
23°C
Cell Number
150
100
50
0
30°C
23°C
C
TA> UAS-miR-263b-SP
2X UAS-miR-263b-SP
23°C
Period=23.8h, RI=0.52
Period=23.8h, RI=0.50
23°C > 30°C
Period=ND, RI=0.2
Period=23.5h, RI=0.58
30°C > 23°C
Period=24h, RI=0.33
Period=24h, RI=0.59
Days
1
3
5
7
CT(hr)
CT(hr)

Figure 3.10: Neither miR-263b-SP nor miR-274-SP conditional adult expression affects glial cell number. Conditional adult glial inhibition of miR-263b alters rhythms. Images and histograms show comparisons of fly brains of the same genotype (TA>miR-SP) at 23°C or 30°C. Quantification of REPO-positive cells was accomplished as described in Fig. 3.7. Adult inhibition of miR-263b (A) or miR-274 (B) did not result in significantly different glial cell number. n=6 for TA>miR-263b-SP brains at both temperatures; two-tailed Student's t-test, p=0.154; n=4 for TA>miR-274-SP brains at both temperatures; two-tailed Student's t-test, p=0.546. C) Representative actograms for experiments described in Fig. 3.9 A. ND refers to not determinable.

DD (8 Days)						
Genotype	Temperature	N	RI ± SEM	Percent Rhythmicity	p-value	Replicates
tubulin-Gal80^{ts}, alrm-Gal4> 2X UAS-miR-263b-SP	23°C	30	0.36 ± 0.02	90	0.215	2
2X UAS-miR-263b-SP	23°C	45	0.40 ± 0.02	96.88		
tubulin-Gal80^{ts}, alrm-Gal4> 2X UAS-miR-263b-SP	23°C-30°C	29	0.22 ± 0.03	55.17	<0.0001	3
2X UAS-miR-263b-SP	23°C-30°C	43	0.38 ± 0.02	95.35		
tubulin-Gal80^{ts}, alrm-Gal4> 2X UAS-miR-263b-SP	30°C-23°C	13	0.33 ± 0.03	100	0.132	1
2X UAS-miR-263b-SP	30°C-23°C	14	0.40 ± 0.03	100		
tubulin-Gal80^{ts}, alrm-Gal4> 2X UAS-miR-274-SP	23°C	26	0.37 ± 0.02	88.46	0.091	2
2X UAS-miR-274-SP	23°C	31	0.42 ± 0.02	100		
tubulin-Gal80^{ts}, alrm-Gal4> 2X UAS-miR-274-SP	23°C-30°C	64	0.21 ± 0.02	51.56	<0.0001	3
2X UAS-miR-274-SP	23°C-30°C	60	0.41 ± 0.02	96.61		
tubulin-Gal80^{ts}, alrm-Gal4> 2X UAS-miR-274-SP	30°C-23°C	14	0.34 ± 0.01	78.57	0.108	1
2X UAS-miR-274-SP	30°C-23°C	15	0.41 ± 0.02	100		

Table 3.3: Conditional astrocyte expression of miR-SPs
Conditional astrocyte expression of miR-263b-SP or miR-274-SP alters activity rhythms. A two-tailed Student's t-test was employed to determined significance; p-values for each set of experiments are listed.

3.2.5 Adult-specific overexpression of miR-274 results in decreased rhythmicity

Since glial overexpression of miR-274 throughout development resulted in lethality, I utilized the aforementioned TARGET system to manipulate astrocyte miR-274 abundance in adult flies. Flies were reared and entrained at 23°C prior to transfer to 30°C for assessment of behavior in constant dark conditions. Adult, astrocyte-specific overexpression of miR-274 resulted in significantly decreased rhythmicity compared to the UAS control (Student's *t*-test; $p<0.01$; Fig 3.11). Thus, overexpression of either miR-263b (Fig. 3.5 C) or miR-274 results in arrhythmicity, and this predicts that a knockdown of a target RNA will also result in arrhythmic behavior.

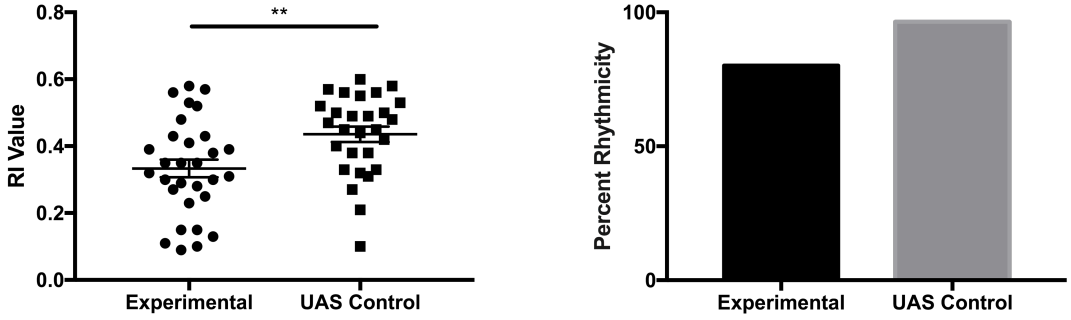


Figure 3.11: Conditional overexpression of miR-274 in astrocytes results in decreased rhythmicity.
Overexpression of miR-274 was done using alrm-Gal4, tub-Gal80^{ts}>miR-SP (TA). Flies were raised at 23°C and entrained at 23°C before transfer to 30°C for assessment of DD behavior. Results are individual flies with mean ± SEM, n=28-30, two-tailed Student's *t*-test, $p<0.01$.

3.2.6 Identification of putative miRNA targets

To search for RNA targets of miR-263b and miR-274, I first utilized target prediction algorithms (TargetScan-FLY and DIANA microT-CDS) (Paraskevopoulou et al., 2013; Reczko et al., 2012; Ruby et al., 2007). For both miRNAs, I compiled a list of potential targets from the combined predictions of both programs. I then compared that list to one derived from an earlier cell type-specific profiling studies that defined transcripts with expression enriched in astrocytes (Huang et al., 2015). I identified RNAs in astrocytes that were predicted to be targets of either miRNA, based on the analyses by TargetScan or DIANA.

Based on my results with miRNA overexpression, I predicted that decreased RNA target abundance would perturb rhythmicity. Therefore, I performed RNA interference (RNAi)-based screens to identify predicted astrocyte target RNAs that function in circadian behavior. Altogether, I examined 12 and 20 putative targets for miR-263b and miR-274, respectively and one putative target of both miRNAs (Table 4.2). For these screens, I utilized existing collections of UAS-RNAi strains that represent nearly all genes of the fly genome. When possible, I screened each of my putative targets using multiple RNAi lines. Similar to the miR-SP screen, I crossed each UAS-RNAi strain to w^{1118} flies (control) or to those carrying the pan-glial repo-Gal4 driver (experimental). Activity data were collected and analyzed for both experimental and control flies as previously described. The screen of putative miR-263b targets did not yield candidate target RNAs; all RNAi-expressing flies had normal rhythmicity (Table 3.4). Similarly, most flies expressing RNAi targeting predicted miR-274 gene targets had normal rhythms (Table 4.2). However, RNAi-mediated knockdown of two potential miR-274

targets, CG4328 and MESK2, resulted in significantly decreased rhythmicity. Glial expression of two different RNAi transgenes targeting CG4328, a predicted LIM-homeodomain transcription factor, resulted in significantly decreased rhythmicity, relative to the UAS-RNAi control (Student's *t*-test; $p<0.0001$; Fig. 3.12 A). Similarly, glial knockdown of MESK2 using either of two different RNAi transgenes also decreased rhythmicity (Student's *t*-test; $p<0.0001$; Fig. 3.12 B). MESK2, known to regulate Ras/ERK signaling, and has homology with mammalian NDRG2, a gene with astrocyte-specific expression.

I examined MESK2 RNA in flies overexpressing miR-274 to determine if transcript levels were decreased by miRNA expression. I used a pan-glial driver (repo-Gal4) to overexpress miR-274 in all glial cells. As this genotype is lethal before eclosion, I collected RNA from pupae (pharate adults) from the experimental cross and from UAS controls. Although there was a trend towards decreased MESK2 transcript levels for miR-274-expressing populations – suggesting altered RNA abundance – this effect was not statistically significant (n=4-7, Fig. 3.12 E).

miR-263b		
Gene	RNAi Line	Effect on DD Rhythmicity
Tsp5D	v45739	No Effect
	v45740	No Effect
	BL41681	No Effect
CG31345	BL61911	No Effect
	v104444	No Effect
Pex19	BL50702	No Effect
	v100746	No Effect
CG9951	v36172	No Effect
	v109399	No Effect
CDase	BL36764	No Effect
Ced-12	v10455	No Effect
	v107590	NA
	BL58153	No effect
	BL28556	No effect
x16	v31203	No Effect
	v100226	No Effect
	BL55642	No Effect
	BL51468	No Effect
bbc	BL50535	No Effect
	BL57704	No Effect
	v7988	No Effect
	v7989	No Effect
	v110371	No Effect
CG9649	BL55200	No Effect
	v11766	NA
	v100194	No Effect
CG8078	v11107	No Effect
	v104409	No Effect
Treh	BL51810	NA
	BL50585	NA
	v30730	NA
	v30731	No Effect
CG14229	BL60447	No Effect
	v109487	No Effect
miR-274		
Gene	RNAi Line	Effect on DD Rhythmicity
CG7927	v22627	No Effect
	v108125	No Effect
opa	BL34706	No Effect
	v51292	No Effect
	v101531	NA
MESK2	BL29380	Decreased Rhythmicity
	v19536	Decreased Rhythmicity
	v105492	NA

CG5068	v27352	NA
	v105759	No Effect
smid	v35965	No Effect
	v35967	NA
	v108178	No Effect
	BL36808	NA
	BL35704	NA
CG6770	v35825	No effect
	BL27561	No effect
GstD1	BL36818	no effect
	v103246	NA
D1	v7780	No Effect
	v101946	No Effect
	BL28616	No Effect
AdSS	v29519	No Effect
	v29520	No Effect
	v106314	No Effect
	BL33993	No Effect
Gγ1	v28844	NA
	BL34372	NA
	BL25934	NA
I-2	v39053	No Effect
	v39054	No Effect
	v101547	NA
	BL44050	No Effect
CG3793	v40475	No Effect
	v105652	No Effect
CG1552	v48550	No Effect
GckIII	BL31744	No Effect
	BL41596	No Effect
	BL57042	No Effect
	BL35339	NA
	v22024	No Effect
	v49558	No Effect
spir	BL43161	No Effect
	BL61283	No Effect
	BL30516	Decreased Rhythmicity
	v107335	No Effect
Vps4	v35125	NA
	v35126	NA
	v105977	NA
	BL31751	No Effect
Aats-thr	BL42902	No Effect
	BL34886	NA
	v7752	No Effect
	v107265	NA
CG1907	v1341	No Effect

	v1342	No Effect
	v103359	No Effect
	BL38998	No Effect
Aplip1	v50007	No Effect
	v109501	No Effect
	BL26024	No Effect
CG5355	v40588	No Effect
CG4328	v30516	Decreased Rhythmicity
	v30517	Decreased Rhythmicity
	BL27987	Decreased Rhythmicity

Table 3.4: RNAi lines screened for putative miRNA targets
RNAi strains from either BDSC (BL) or VDRC (v) were screened for effects on rhythmicity during DD. Each line was crossed with repoGal4 to drive expression in all glial cells. As a control, all lines were also crossed to w1118. CG3793 is a predicted target for both miR-263b and miR-274. The behavioral data is listed under miR-274. No effect denotes no decreased rhythmicity in DD. NA denotes the experimental cross was lethal or semi-lethal.

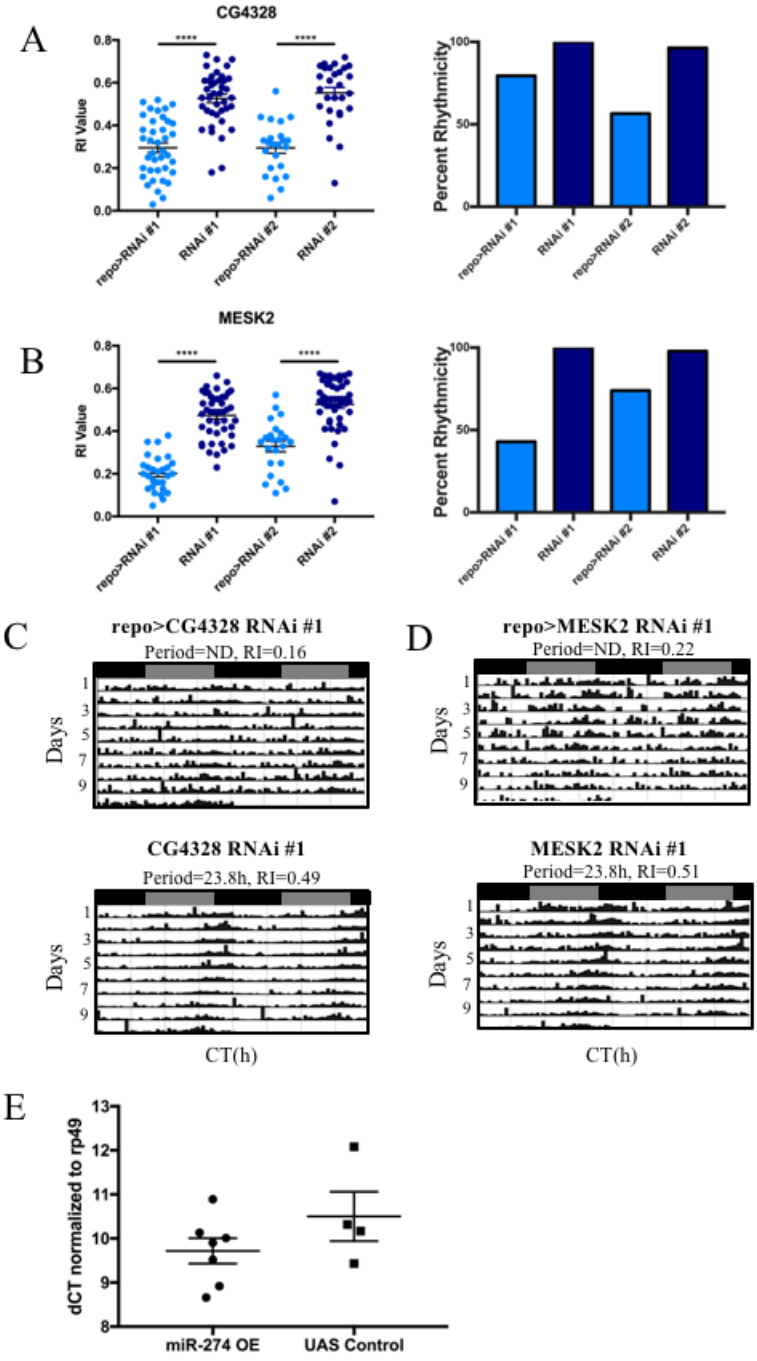

A
CG4328
RI Value
Percent Rhythmicity
repo>RNAi #1
RNAi #1
repo>RNAi #2
RNAi #2
B
MESK2
C
repo>CG4328 RNAi #1
Period=ND, RI=0.16
CG4328 RNAi #1
Period=23.8h, RI=0.49
Days
CT(h)
D
repo>MESK2 RNAi #1
Period=ND, RI=0.22
MESK2 RNAi #1
Period=23.8h, RI=0.51
E
dCT normalized to rp49
miR-274 OE
UAS Control

Figure 3.12: Glial knockdown of putative mir-274 targets causes arrhythmicity. Glial expression of either of two different CG4328 RNAi transgenes (A) or MESK2 RNAi transgenes (C) significantly decreased rhythmicity. Results are individual flies with mean ± SEM, two-tailed Students *t*-test, ****p<0.0001. Representative actograms for CG4328 (B) or MESK2 (D) knockdown flies and controls for one RNAi stain each. ND refers to not determinable. E) MESK2 RNA abundance in pupae with pan-glial overexpression of miR-274. Points represent biological replicates, each with 5-10 flies per replicate. Horizontal lines are mean ± SEM.

3.3 Identification of circadianly translated RNAs in astrocytes

For my astrocyte gene-profiling studies, I used a glial driver with expression predominantly in astrocytes (eaat1-Gal4). This driver also has expression in cortex glia and the T1 lamina neurons of the visual system (Hamanaka and Meinertzhagen, 2010; Stork et al., 2012). As such, I included the elav-Gal80 to inhibit Gal4 function in neurons. The elav-Gal80, eaat1-Gal4 flies were combined with a transgene containing a tagged ribosomal subunit (Huang et al., 2013) to generate flies that express EGFP-L10a predominantly in astrocytes. After an entrainment period of at least 4 days, flies were collected every four hours during DD1 and DD2 for a total of 12 time points. Using a high-affinity EGFP antibody, I performed immunoprecipitation of RNA bound to EGFP-tagged ribosomes, extracted from head lysates. The RNA was then assessed for quality using an Agilent Bioanalyzer. High-quality samples were then converted into RNA-seq libraries using the Illumina TruSeq RNA kit for sequencing. Libraries were sequenced using a multiplexing strategy, to produce single end, 100 base sequencing reads. In collaboration with Dr. Amy Yu, these reads were then mapped to the *Drosophila* reference genome and analyzed for sufficient mapping (≥60%). At this stage, elimination of poorly mapped samples was removed, and a single set of time-points was used for further analysis.

3.3.1 Comparison of genes derived from profiling studies using alrm-Gal4 or eaat1-Gal4

Previous astrocyte-profiling experiments were performed using the alrm-Gal4 driver, which is thought to be specific for astrocytes (Huang et al., 2015; Ng et al., 2016). In those studies, flies were collected at a single time point (ZT1). The driver used for the present studies (eaat1-Gal4) is predominantly astrocytic with some expression in cortex

glia. The two profiling experiments give me the opportunity to investigate which genes are high-confidence astrocyte genes. Conversely, genes only expressed in the alrm-Gal4 set or the eaat1-Gal4 set may reflect astrocyte heterogeneity or the gene expression of another glial cell-type. Another possibility is that the gene may be expressed at very low levels at the time of the alrm-Gal4 collection (CT1). Genes highly expressed at other time-points would thus be excluded. Both sets of profiling experiments were performed with elav-Gal80 in the genetic background to exclude expression in neurons. Of the 6712 genes generated in the alrm-Gal4 experiments and the 7669 genes in the eaat1-Gal4 experiments, 5200 genes overlap. This suggests the vast majority of genes identified in both experiments are high-confidence astrocyte genes. Among the high-confidence astrocyte genes are ten clock genes also identified in neuronal profiling studies (Thomas et al., 2012) (Table 3.5).

	Neurons elav-Gal4 (Thomas et al., 2012)	Astrocytes alrm-Gal4 and eaat1-Gal4 (Huang et al., 2015; Ng et al., 2016; current study)
per	Yes	Yes
tim	Yes	Yes
Clk	Yes	Yes
cyc	Yes	Yes
Pdp1	Yes	Yes
cwo	Yes	Yes
vri	Yes	Yes
cry	Yes	Yes
sgg	Yes	Yes
dbt	Yes	Yes

Table 3.5 TRAP identifies core clock genes in neurons and astrocytes.
Affinity purification of ribosome-bound RNA extracted from neurons (Thomas et al., 2012) and astrocytes (Huang et al., 2015; Ng et al., 2016) included the ten listed clock genes.

3.3.2 Astrocyte-Specific Expression Profiling Detects Circadianly Translated RNAs

The algorithm JTK-CYCLE was used to detect circadian rhythms in ribosome associated RNAs (Hughes et al., 2010). This algorithm also calculates period, phase and amplitude of cycling transcripts. 724 RNAs with significant circadian changes in abundance were identified from the initial analysis. Importantly, the clock genes *per*, *tim*, *vri* and *cyc* were among the genes identified as cycling (Fig. 3.13). Of those 724 cycling RNAs, 576 were identified as high-confidence astrocyte genes, and 88 were identified as "enriched" in astrocytes compared to total head RNA (Ng et al., 2016) (Fig. 3.14 A). The remaining 149 genes are presumed to be genes that cycle in non-astrocyte cells such as cortex glia or genes in astrocytes with low expression at CT1. Analysis of molecular

functions and biological processes for genes that cycle in astrocytes was performed using DAVID (Fig. 3.14 B, C). As expected from an analysis of cycling genes, circadian rhythms were among the overrepresented biological processes (Fig. 3.14 C).

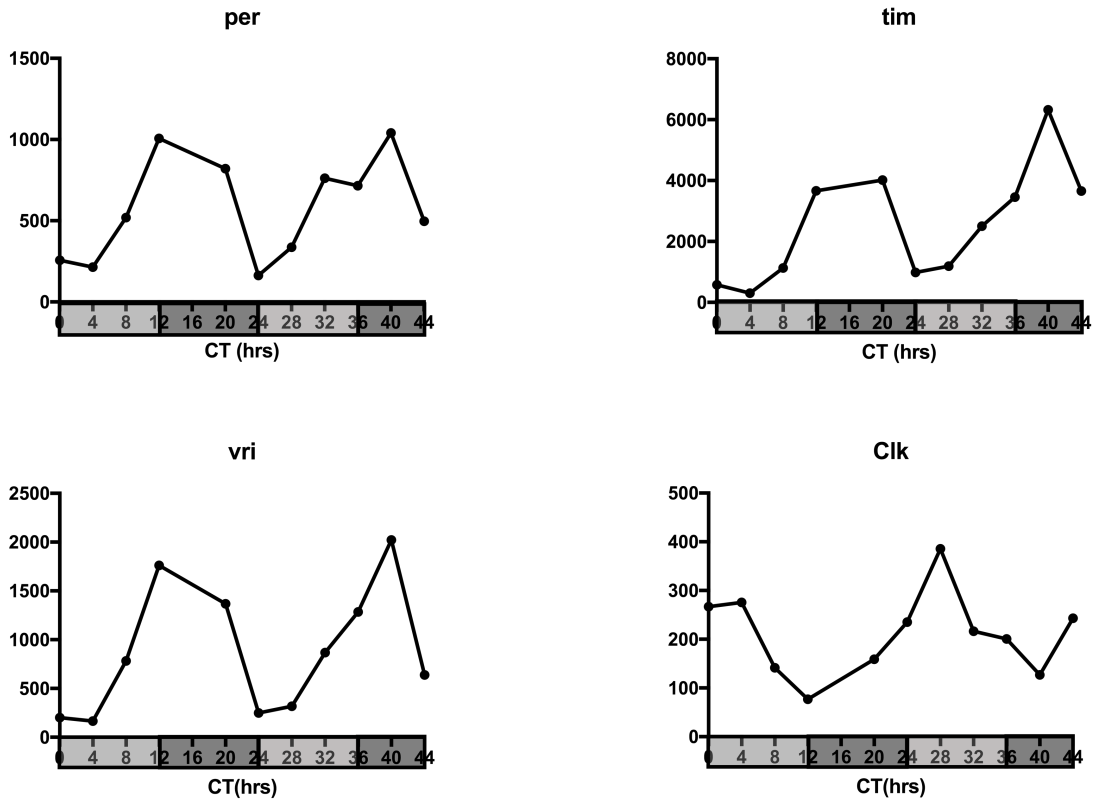


Figure 3.13: Cycling profile of known clock genes from eaat1-Gal4 gene profiling experiments.
Translational profile of known clock genes *per*, tim, *vri* and *Clk*. The y-axis represents normalized read counts. The entire cycle is in constant darkness with the light gray box representing "day" and dark gray box representing "night". $p<0.05$

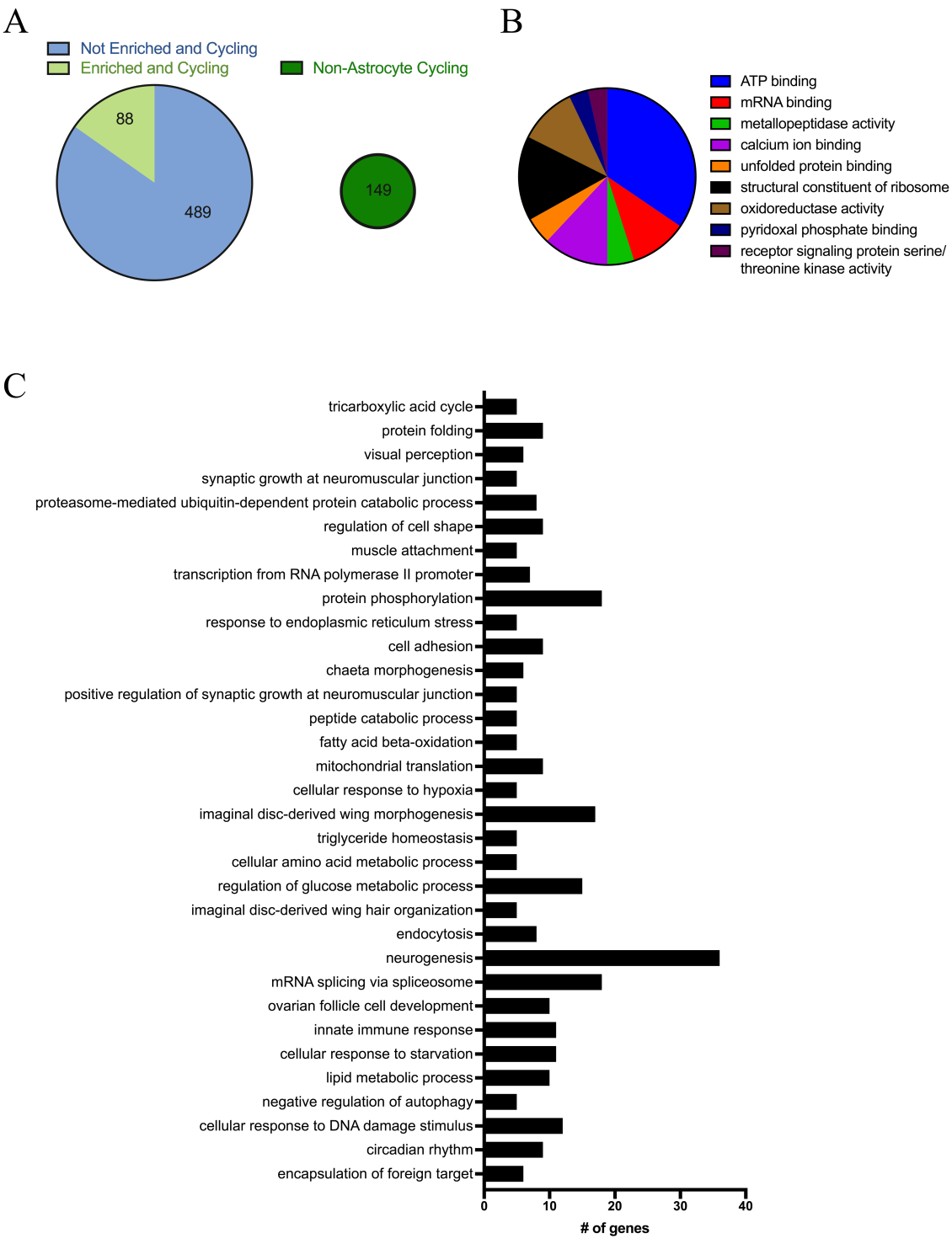

A
Not Enriched and Cycling
Enriched and Cycling
Non-Astrocyte Cycling
88
489
149
B
ATP binding
mRNA binding
metallopeptidase activity
calcium ion binding
unfolded protein binding
structural constituent of ribosome
oxidoreductase activity
pyridoxal phosphate binding
receptor signaling protein serine/ threonine kinase activity
C
tricarboxylic acid cycle
protein folding
visual perception
synaptic growth at neuromuscular junction
proteasome-mediated ubiquitin-dependent protein catabolic process
regulation of cell shape
muscle attachment
transcription from RNA polymerase II promoter
protein phosphorylation
response to endoplasmic reticulum stress
cell adhesion
chaeta morphogenesis
positive regulation of synaptic growth at neuromuscular junction
peptide catabolic process
fatty acid beta-oxidation
mitochondrial translation
cellular response to hypoxia
imaginal disc-derived wing morphogenesis
triglyceride homeostasis
cellular amino acid metabolic process
regulation of glucose metabolic process
imaginal disc-derived wing hair organization
endocytosis
neurogenesis
mRNA splicing via spliceosome
ovarian follicle cell development
innate immune response
cellular response to starvation
lipid metabolic process
negative regulation of autophagy
cellular response to DNA damage stimulus
circadian rhythm
encapsulation of foreign target
0
10
20
30
40
# of genes

Figure 3.14: Analysis of RNAs that cycle in fly astrocytes.
A) 724 genes were statistically identified as cycling using JTK_CYCLE. 576 genes are considered high-confidence astrocyte genes. 88 genes were previously identified as enriched in astrocytes. B) A pie chart showing overrepresented molecular functions of genes that are rhythmically expressed in astrocytes. C) Overrepresented biological processes of genes that are rhythmically expressed in astrocytes. Annotations were determined using DAVID v.6.8. Only categories with at least five genes are shown.

Using annotations derived from FlyBase (FB2017_06), I manually sorted the list of cycling genes for circadian related biological processes (Table 3.6). 15 genes were identified in this manner including the clock genes *per*, *tim*, *vri* and *Pdp1*. Also identified was *ebony*, a previously studied glial cycling gene with relevance for circadian behavior (Fig. 3.15 A). Four of these genes function in the extracellular space, making them signaling candidates. Additionally, all four of these genes have similar phases of translational cycling (Fig. 3.15 D). I also sorted for genes that may be relevant for cell communication and identified a number of vesicle-related components along with transporters and their regulators (Table 3.6).

Another previously studied cycling astrocyte-enriched gene is *Trehalase (Treh),* which is responsible for converting trehalose into glucose (Fig. 3.15 B). We previously found that RNAi knockdown of *Treh* in glial cells significantly reduces activity level (Ng et al., 2016). Thrombospondin (Tsp) is known to be secreted from mammalian astrocytes and is involved in synaptogenesis (Christopherson et al., 2005) and response to injury(Andresen et al., 2014). Previous studies from the lab indicate that RNAi-mediated knockdown of *Tsp* in glia also reduced activity level (Ng et al., 2016). In this study, I found that *Tsp* also cycles in astrocytes (Fig. 3.15 C). The Jackson Lab previously proposed that HDAC6, an astrocyte-enriched cytoplasmic deacetylase, might play an astrocyte-specific role in regulating vesicle machinery (Huang et al., 2015). HDAC6 is necessary and sufficient for the deacetylation of Bruchpilot, a presynaptic component (Miskiewicz et al., 2014). Bruchpilot tethers synaptic vesicles and regulates release. In the current profiling study, I found that HDAC6 is also a translationally cycling gene (Table 3.6).

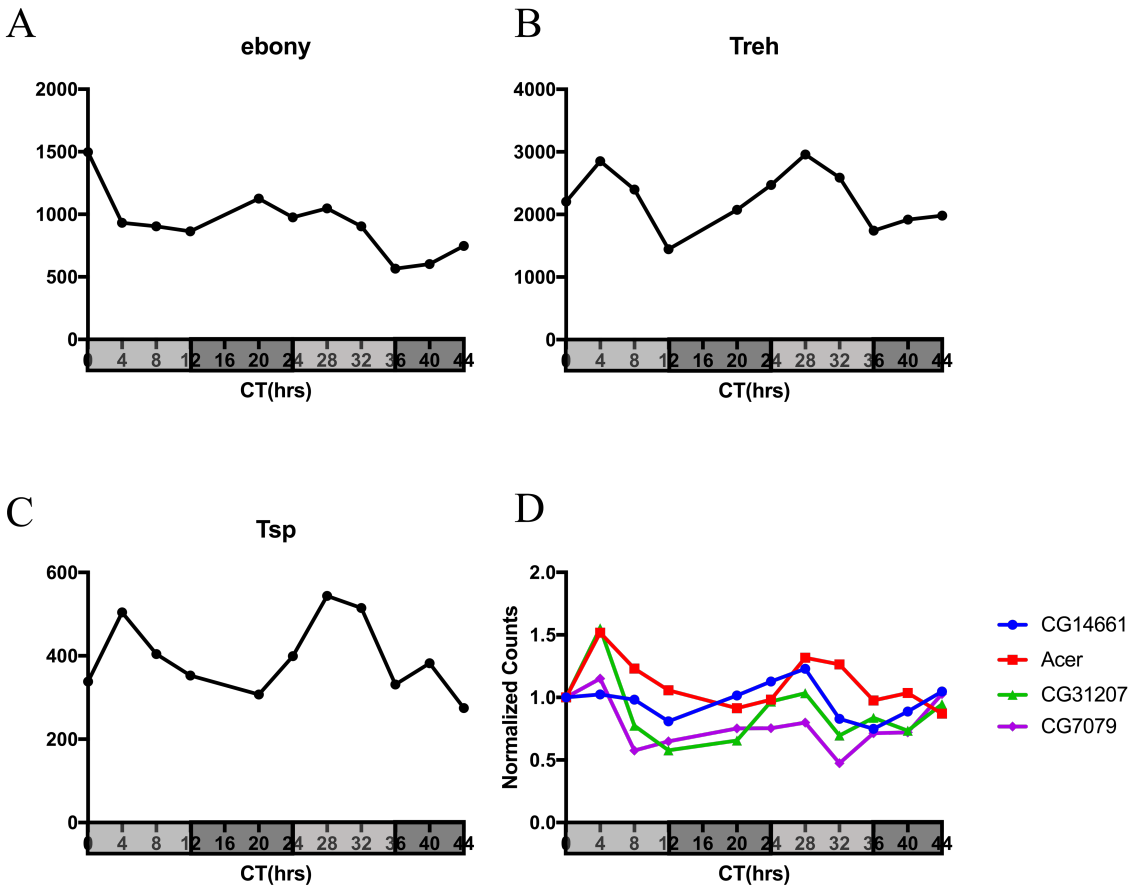


Figure 3.15: Identification of cycling glial genes.
Translation profile of known glial genes *ebony* (A), *Treh* (B) *and Tsp* (C). The y-axis represents normalized read counts. D) Translational profile of extracellular genes with circadian-related biological processes. Read counts were normalized to the counts at CT0 for all genes. The entire cycle is in constant darkness with the light gray box representing "day" and dark gray box representing "night".

GO Terms	Genes
Secretion, Vesicle, SNARE, Exocytosis, Transmitter	AP-1gamma, car, Syx18, Glt, Csp, MRP, GPHR, CG31729, beta'COP, jagn, Dab, CG5484, nemy, Snap29, cpx, Snx6, GAPsec, Rab14, CG7324, Gos28, HDAC6, GABPI, SH3PX1, Vps16B, dor, sesB, blot
Receptor	Rh2, msn, car, Srp54, sdk, kek4, aos, tkv, pyr, sax, Cul1, stumps, Fak, Mmp2, CoRest, Dab, AkhR, Tsp42El, ben, TBPH, pdgy, CG31195, 5-HT1B, fog, Grip, Med, dor, CG33639, hop, gl
Circadian	e, CG14661, inc, Plc21c, Acer, CG31207, CG5273, per, vri, tim, Pdp1, 5-HT1B, CG31189, CG7079, gl

Table 3.6: Vesicle-related, receptor-related and circadian-related genes identified as cycling in astrocytes
Genes were annotated with Flybase and sorted for potential glia-neuron signal components by looking for related biological process GO terms. Genes highlighted in red are located in the extracellular matrix or space.

3.3.2 Phases of translation

A previous study profiling clock cells revealed two major phases of rhythmic translation-during mid-day or mid-night (Huang et al., 2013). These time periods correspond to periods of low activity, potentially due to the lower metabolic expenditure of these time periods. Similarly, I found two major phases of rhythmic translation in astrocytes (Fig. 3.16 A-B). While profiling of clock cells revealed peak translation at CT8 and CT20, my astrocyte profiling reveals earlier peak translation at CT4 and CT16-18 (Fig. 3.16 A). The two phases of translation for astrocytes is similar in distribution to the two phases observed in the tim-Gal4 studies (Fig. 3.16 C compared to Fig. 3.16 D). These two studies stand in contrast to previous reported genome-wide profiling studies that showed translation throughout the day (Fig. 3.16 E). A more recent study focused on specific groups of clock cells found that the group most important for free-running rhythmicity, the LN_vs, also have two major phases of translation (Fig. 3.17 A)(Abruzzi et al., 2017). In contrast, the rhythmic profiles of the LN_ds and DN_1s have a unimodal distribution of translation (Fig. 3.17 B-C) (Abruzzi et al., 2017). I also looked to see what the distribution of translation was for the 149 cycling genes that were not considered high-confidence astrocyte genes. As previously discussed, this group of genes may represent cortex glia or alrm-Gal4 positive astrocytes with low expression at ZT1. I observed two major phases of translation, although the second phase contains twice as many genes as the first phase (Fig. 3.17 D).

The previous study utilized the tim-Gal4 driver, which is also expressed in glial cells (Huang et al., 2013). Surprisingly, there was little overlap between genes determined to be cycling in all clock cells and astrocytes. Out of 576 identified genes with rhythmic

translation in astrocytes, only 38 were also determined to be cycling in all clock cells. The lack of overlap is likely due to the cell-type specificity of the profiling studies. Certain genes that cycle in astrocytes may not do the same in other *tim*-positive cells, and therefore some genes that cycle in astrocyte would not have been identified in the tim-Gal4 experiments. Nevertheless, it is interesting that both profiling studies showed two phases of translation given such little overlap between the two lists.

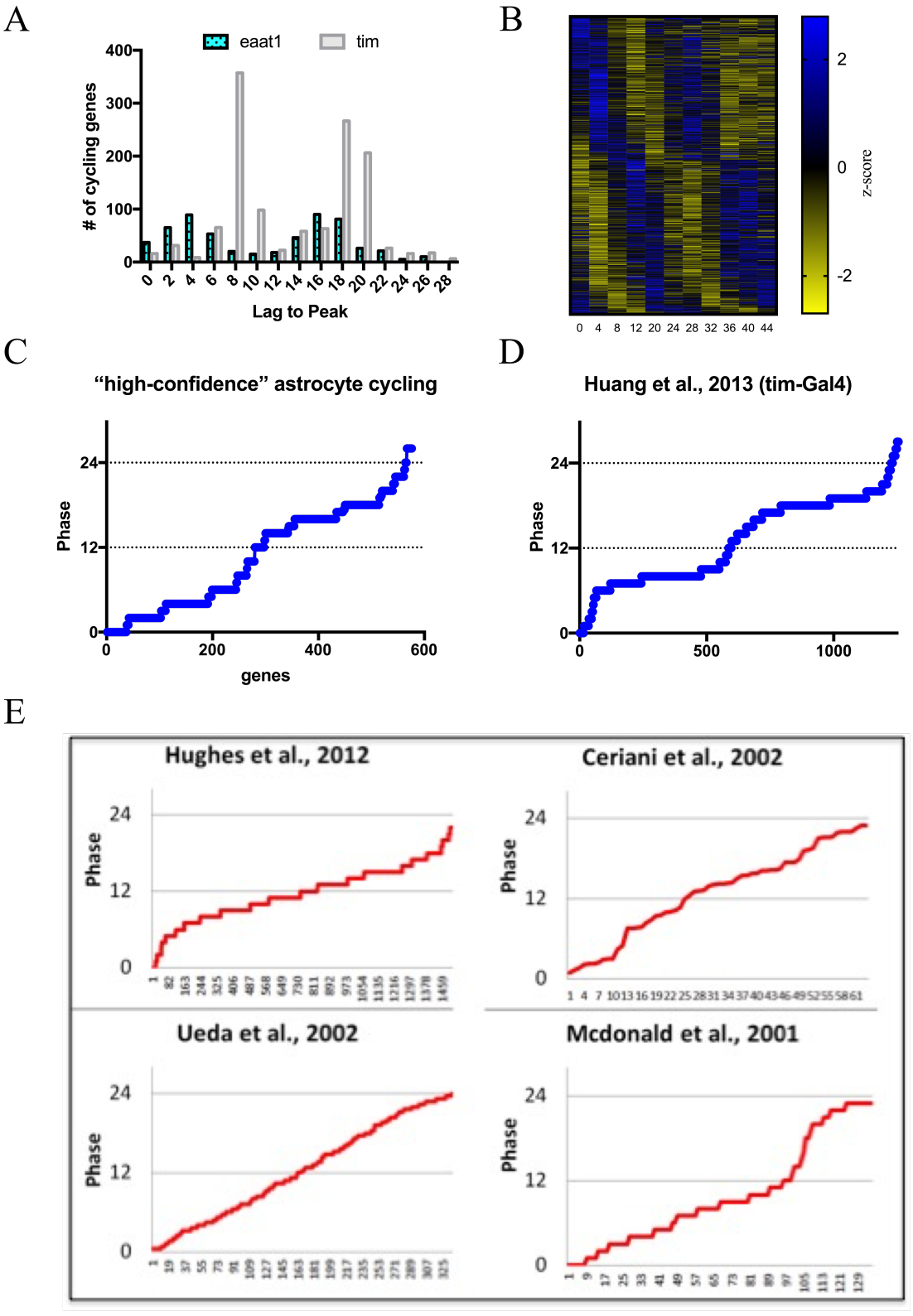

A
eaat1
tim
# of cycling genes
Lag to Peak
B
z-score
C
"high-confidence" astrocyte cycling
Phase
genes
D
Huang et al., 2013 (tim-Gal4)
Phase
E
Hughes et al., 2012
Ceriani et al., 2002
Ueda et al., 2002
Mcdonald et al., 2001
Phase

Figure 3.16: Protein synthesis in astrocytes occurs in two major phases
A) Histogram shows time of peak translation for all 576 genes cycling in astrocytes and 1255 genes cycling in the tim-Gal4 distribution. X-axis depicts the lag time in hours to peak translation. B) Heat map of read counts of genes normalized to z-score across two days. Genes were arranged based on peak translation. Blue represents higher abundance and yellow represents lower abundance. C) Graph depicts time of peak translation for 576 genes found to cycle. D) Graph depicts time of peak translation for 1255 cycling genes identified in Huang et al. 2013 E) Figure depicts peak translation of previously published genome-wide studies. The figure is adapted from Fig. S4 of Huang Y, Ainsley JA, Reijmers LG, Jackson FR (2013) Translational Profiling of Clock Cells Reveals Circadianly Synchronized Protein Synthesis. PLoS Biol 11(11): e1001703. doi:10.1371/journal.pbio.1001703. PLOS Biology is an open-access journal.

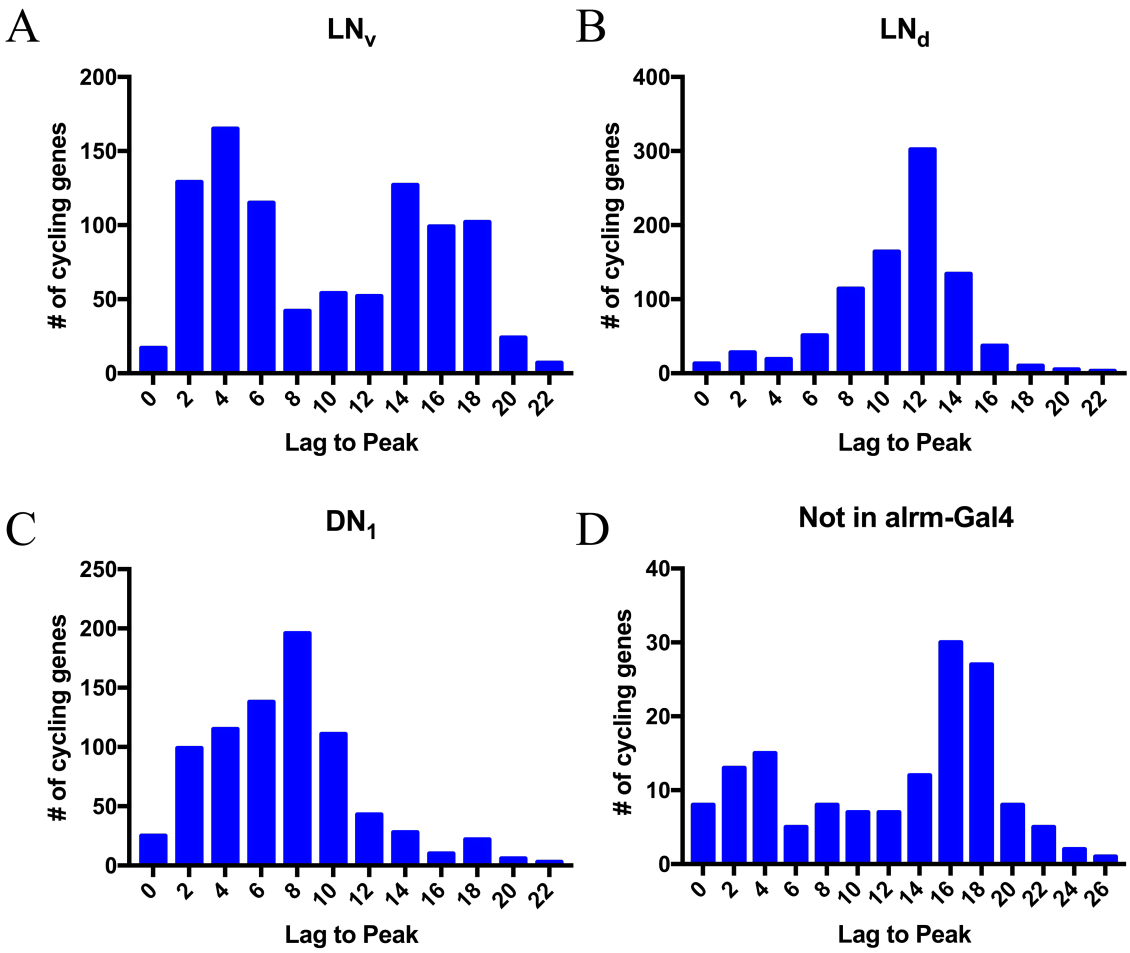


Figure 3.17: Phases of protein synthesis in subgroups of cells
A) The LN_v group of cells has two major phases of translation. The LN_d (B) and DN_1 (C) groups have a single peak of translation. Histograms (A-C) constructed from RNA-seq profiles of subgroups of clock cells (Abruzzi et al., 2017). D) The alrm-Gal4 negative, eaat1-Gal4 positive cycling genes (potential cortex glia) are translated in two major phases, with the second group containing twice as many genes as the first.

Chapter 4: Discussion

4.1 Overview

Chronobiologists have long endeavored to untangle the magic of our internal clocks. Similar to mechanical clocks, the clocks in our bodies operate through a series of molecular gears. We have evolved layer upon layer of control to ensure these clocks run on time. In spite of all the redundancy, circadian clocks can still malfunction, and the consequences of this dysfunction can be severe. Circadian research has come a long way since the initial observations of leaf movements. While much circadian research has focused on the neuronal control of rhythmic behavior, the role for glia in these circuits was identified in the early days of glial biology (Prolo et al., 2005; Zerr et al., 1990). We now know that astrocytes, a subclass of glia, are important for circadian behavior in both *Drosophila* and in mice (Barca-Mayo et al., 2017; Brancaccio et al., 2017; Ng et al., 2011; Tso et al., 2017). Still, there are many questions remaining about how these cells influence behavior. My thesis describes two approaches in an attempt to answer some of these questions.

In Chapter 3.1, I described the results of a genome-wide miRNA-based screen to identify miRNAs with functions relevant for circadian behavior in glial cells. Using genetically encoded miRNA inhibitors (miR-SPs) expressed in all glial cells, I performed a screen that identified multiple miRNAs with effects on circadian period, entrainment and/or activity level. I also identified 20 miRNAs whose inhibition in glial cells resulted in decreased rhythmicity in free-running conditions. I postulated that astrocytes would be the most likely group of cells responsible for these effects, and repeated my behavioral experiments using astrocyte-specific Gal4 drivers. Many, but not all of these candidate

miRNAs appear to have rhythmicity related functions in astrocytes. It is likely that other glial subclasses also contribute to regulation of circadian behavior. These data are the first to describe glial miRNAs relevant for normal circadian behavior.

In Chapter 3.2, I describe the in-depth characterization of two miRNAs – miR-263b and miR-274. Glial manipulation of both miRNAs decreased rhythmicity without causing any obvious defects on clock cell or glial cell development. This effect is specific to glial manipulation, as neuronal inhibition of either miRNA did not significantly decrease rhythmicity. Importantly, adult-specific inhibition of these miRNAs in astrocytes also significantly decreased rhythmicity, suggesting a physiological role in the maintenance of normal circadian behavior. I identified two putative RNA targets for miR-274, revealing potential mechanisms of action for the miRNA.

In Chapter 3.3, I describe the identification of ribosome-bound RNAs with cyclic changes in abundance in fly astrocytes utilizing the TRAP technique. Using a genetically expressed tagged-ribosomal subunit, I affinity purified ribosome-bound RNAs in astrocytes across the first two days of DD. I performed RNA-seq on isolated RNA and identified 576 high-confidence genes that cycle in astrocytes. These include many expected genes such as known clock and astrocyte genes along with many genes uncharacterized in the context of circadian rhythms. This database is the first to describe the identity of cycling genes in astrocytes.

4.2 miRNAs in circadian behavior

While miRNAs and glia have been separately implicated in the regulation of circadian behavior, there are no studies describing roles for glial miRNAs in rhythmicity. I report a

screen for miRNAs involved in the glial regulation of circadian behavior. To my knowledge, there has not been a published genome-wide screen to assess the function of individual miRNAs in regulating circadian behavior. The use of genetically encoded miRNA inhibitors, miR-SPs (Fulga et al., 2015), allowed me to test 145 miRNAs for loss-of-function circadian phenotypes in glial cells.

4.2.1 Identification of miRNAs important for glial regulation of circadian behavior

My primary screen identified 20 candidate miRNAs whose inhibition in glia decreased rhythmicity (Fig. 3.2). Additionally, four miRNAs were associated with altered circadian period, one with abnormal entrainment, and 23 with changes in activity level (Fig. 3.1). While there is little information about most of these miRNAs, a few of them were previously identified in other circadian studies. For example, miR-210, identified in my screen, was significantly increased in abundance in cyc^{01} mutants compared to control flies (Yang et al., 2008). The same study also identified miR-263b as an oscillating miRNA under clock control. miR-963 was previously identified as part of a cycling cluster that regulates innate immunity, metabolism and feeding behavior (Vodala et al., 2012). A number of my candidate miRNAs were previously identified as being expressed in *tim*-positive cells: miR-274, miR-285, miR-304, miR-305 and miR-317 (Kadener et al., 2009). These cells would include a subset of glial cells. While expression in clock cells is not a definitive sign of circadian function, it is indicative of potential relevance. Most previous studies of miRNAs in *Drosophila* do not distinguish between nervous system cell types. To the best of my knowledge, there is currently no information on the distribution of miRNAs in glial cells.

4.2.2 Characterization of miR-263b and miR-274

I performed detailed studies of two miRNAs: miR-263b and miR-274. miR-263b is a conserved miRNA with mammalian orthologs in the miR-183 family (miR-183, miR-96, miR-182) (Dambal et al., 2015). Of interest, the miR-183 family, similar to miR-263b, was found to exhibit diurnal variation in expression in mouse retina (Xu et al., 2007). Human miR-182 is known to target the *Clock* circadian gene, and a polymorphism in miR-182 was found to be associated with insomnia in major depression patients (Saus et al., 2010). In *Drosophila*, *Clk* was reported to be a potential target of miR-263b, based on *in silico* analysis, suggesting a possible conservation of function (Yang et al., 2008). However, I saw no effect on the PER-based molecular oscillator with inhibition of this miRNA (Fig. 3.8). This does not preclude the possibility that there may be an effect on another clock gene. As I did not observe any period phenotypes, it is more likely that the effect of miR-263b is downstream of the core clock.

As previously described, miR-263b is under circadian control (Yang et al., 2008), and glial inhibition or overexpression of the miRNA resulted in decreased rhythmicity (Fig. 3.3 A-C). Thus, it is possible that increased or decreased miR-263b disrupts the normal cycling of the miRNA, resulting in effects on activity rhythms. In unpublished microarray studies on pharate adults, the Jackson lab found that miR-274 also cycles in abundance (Y. Huang and F.R. Jackson, unpublished results). Similar to miR-263b, decreased or increased function for the miR-274 perturbs rhythmicity (Fig. 3.5 A, Fig. 3.9). MicroRNA-274 does not have direct mammalian orthologs but is conserved in 11 species of *Drosophila*, in the silkworm (*Bombyx mori*) and in the diamondback moth (*Plutella xylostella*) (miRBase).

The pro-apoptotic gene *hid* was previously identified as a target of miR-263b (Hilgers et al., 2010). As the miR-263b-SP disrupts miR-263b function, it was possible that the circadian phenotype resulted from cell death and abnormal development. Indeed, miRNAs are frequently studied in the context of disease and development, and I was concerned that inhibition of miRNAs throughout development might result in altered clock cell or glial development. However, I show that neither miR-263b-SP nor miR-274-SP expression resulted in abnormal development of clock cells as shown by staining for PER and PDF (Fig. 3.7 A-B, Fig. 3.8 A-B). Similarly, REPO and GAT distribution and abundance are normal in flies expressing these miR-sponges (Fig. 3.7 C-D, Fig. 3.8 C-D). These studies do not address whether there are functional differences due to miR-SP expression. To ensure that I am studying normal physiological phenomena, I used a temperature-sensitive Gal4 inhibitor ($Gal80^{ts}$) to turn "on" and "off" miR-SP expression at different times. Inhibition of either miR-263b or miR-274 during adulthood significantly decreased rhythmicity (Fig. 3.9, Fig. 3.10 C). Furthermore, flies with inhibition of these miRNAs during development but not during adulthood had normal behavioral rhythmicity (Fig. 3.9, Fig. 3.10 C). To ensure the adult-specific manipulation did not result in drastic glial cell death, I quantified REPO-positive cells at both "on" (30°C) and "off" (23°C) temperatures and found no difference in number (Fig. 3.10 A-B).

There are limited unbiased experimental methods for identifying miRNA target RNAs. The majority of the current experimental methods rely on immunoprecipitation of AGO-bound RNAs that are cross-linked together. These methods typically require large amounts of starting material, as their efficacy is currently low. Thus, I chose to combine

the predicted targets of two different programs (TargetScan and DIANA microT-CDS) in my analysis. Both programs incorporate how well the miRNA seed matches with a putative target. TargetScan also considers conservation while DIANA microT-CDS also incorporates a free-energy calculation (Paraskevopoulou et al., 2013; Reczko et al., 2012; Ruby et al., 2007). I limited candidate target RNAs to those identified by previous profiling studies as having enriched expression in astrocytes (in comparison to total brain RNA), as the observed miR-SP phenotypes were due to astrocyte-specific manipulation (Fig. 3.3) (Huang et al., 2015). While it is likely that I excluded *bona fide* targets by limiting my screen in this manner, I nonetheless identified at least two miRNAs with glia-specific functions affecting rhythmicity. Thus, I think that relevant circadian targets of the miRNAs are likely to have enriched astrocyte expression or a specific requirement within these glial cells. Due to the availability of RNAi strains directed against nearly every gene in the *Drosophila* genome, I decided to use RNAi to identify miRNA targets. Expression of a miR-SP would most likely increase the amount of the RNA target, while expression of RNAi would produce the opposite effect. As increased or decreased miRNA decreased rhythmicity, it is possible that increased or decreased target gene could also decrease rhythmicity. It is also possible that the decreased rhythmicity that is the result of miR-SP expression involves a different gene than the decreased rhythmicity due to miRNA overexpression. Using RNAi directed against each putative target, I screened a total of 35 genes for effects on rhythmicity (Table 3.4).

This genetic screen identified two putative targets for miR-274: CG4328 and MESK2 (Fig. 3.12). Pan-glial inhibition of CG4328 with two different RNAi strains significantly decreased rhythmicity (Fig.3.12 A, C). CG4328 encodes a transcription factor with

mammalian orthologs: Lmx1a and Lmx1b (FlyBase). CG4328 protein is ~40% identical and 55% similar to human LMX1A or LMX1B protein over a stretch of >300 amino acids. In addition, CG4328, LMX1a and LMX1b are predicted to have similar structure consisting of two LIM-domains followed by a homeodomain. In adult flies, CG4328 has a brain-specific pattern of expression, indicative of an adult function (FlyAtlas) (Chintapalli et al., 2007). Thus, it is of interest that mammalian LMX1A and B have been described as positive regulators of insulin synthesis (German et al., 1992). Consistent with such a role for CG4328, the encoded protein is also >50% similar to mouse and human insulin gene enhancer protein (ISL-2). Based on homology to LMX1 and ISL-2 proteins, I propose that CG4328 may be important for regulating genes encoding *Drosophila* insulin-like peptides (*dilps*) in glial cells. The Jackson lab previously showed that adult *Drosophila* astrocytes express such genes, including *dilp6* (Ng et al., 2016), whose secretion from glia has been tied to regulation of FOXO transcription factors and to *dilp2* expression in insulin producing neurosecretory cells (IPCs) of the fly brain (Okamoto and Nishimura, 2015). Whereas the regulation of glial *dilp* genes is not well understood, it is known that perturbation of insulin signaling in IPCs is important for circadian locomotor activity rhythms. Fly Fragile X protein (FMRP1), for example, regulates IPC *dilp* expression and is required in these cells for normal rhythmicity (Monyak et al., 2016). It was also recently reported that DILP2 is expressed in clock neurons of the adult fly brain (the LN_d and LN_v groups) and that deficits for this and other DILPs, or the *Drosophila* insulin receptor, DInR, resulted in reduced sleep (Cong et al., 2015). I suggest that CG4328 may affect rhythmicity by altering glial *dilp* expression and glia-neuron signaling.

Independent RNAi transgenes targeting MESK2, another potential miR-274 target, also resulted in decreased rhythmicity (Fig. 3.12 B, D). MESK2 was first identified from a screen for regulators of Ras (Huang and Rubin, 2000). It has four conserved isoforms in mammals known as N-myc downstream-regulated gene (NDRG) proteins. Of particular interest, NDRG2 has astrocyte-specific expression (Okuda et al., 2007). Beyond a possible regulation of Ras/ERK signaling, there is little known about the role of MESK2 in *Drosophila* or mammals. Intriguingly, sleep deprived mice have elevated levels of hyper-phosphorylated NDRG2 protein (Suzuki et al., 2013). I attempted to address whether miR-274 targets MESK2 by quantifying MESK2 RNA abundance in flies that overexpressed miR-274. While there was a trend in decreased MESK2 RNA, the result was not significant (Fig. 3.12 E). As I did not isolate glial cells for this experiment, it is possible that MESK2 in other cell types masks the effect of miR-274 overexpression. It is also possible that MESK2 abundance at the pharate adult stage is different than in the adult fly.

As miRNAs are capable of having hundreds of targets, it seems likely that any manipulation of a miRNA would influence a number of targets simultaneously. Thus, the decreased rhythmicity observed in the miR-SP experiments may be the combinatorial effect of many genes. Conversely, most genes are targeted by multiple miRNAs. Current technical limitations make it difficult to sufficiently inhibit more than one miRNA at a time in *vivo*.

4.2.3 Future directions for this project

The function of miRNAs in regulating circadian behavior resembles a rheostat more than a binary on/off switch. These small RNAs provide another layer of control over our

biological clocks, emphasizing the importance of circadian rhythmicity. As there is currently very little information on the distribution of miRNAs in glial cells, such studies would be hugely informative. A major caveat to these studies is the presumption that miR-SPs manipulate endogenous miRNAs and therefore, miR-SPs that altered circadian behavior represent miRNAs present in glial cells. To be certain, I can use expression profiling of glial cells and use RNA-seq or a microarray assay to determine which miRNAs are expressed in glia. In this case, I would mark glial cells using a genetically expressed reporter and isolate the cells using FACS. With this approach, I could collect glial cells at different time points throughout the day and determine which miRNAs cycle in abundance in astrocytes. Inhibition of miRNA function would likely interfere with normal miRNA cycling and subsequent translation of the miRNA target, providing an avenue for circadian dysfunction (also discussed in Chapter 4.3.5). Of course, it is not a necessity for miRNAs with circadian functions to cycle in abundance. For example, miR-122 targets the circadian deadenylase nocturnin but does not cycle in abundance (Kojima et al., 2010).

To verify CG4328 and MESK2 as targets of miR-274, 3' UTR reporter constructs can be made for both RNAs. The reporters would contain the 3' UTR of the miRNA target, along with a fluorescent reporter. As a control for specificity, reporters with modified (mutated) target recognition sequences in the 3'UTR can be used. If the miRNA targets the RNA in question, it would bind to the reporter and cause decreased reporter expression and fluorescence. These experiments are most easily performed in *vitro* by transfecting S2 cells with both the 3'UTR reporter and a miR-274 overexpression construct. Alternatively, in *vivo* experiments could be performed by genetic expression of

the 3'UTR reporter. Expression of miR-274-SP would increase fluorescence if miR-274 were present. Similar to the in *vitro* experiment described above, a reporter with a modified target recognition sequence could be used as a control for specificity.

Assuming the proposed RNA targets of miR-274 are *bona fide* targets, two different mechanisms are suggested. As CG4328 is implicated in insulin signaling, systematically screening components of insulin signaling pathways in glia could prove informative given that little is known about their function in glia and insulin signaling is implicated in circadian rhythms. Among the list of cycling high-confidence genes in astrocytes are two genes – *Cul1* and *pdgy* - involved in insulin signaling. I could also use RNAi directed against the *dilps* that are expressed in astrocytes to determine if any of these peptides are signals relevant for circadian behavior. There are eight *dilps* in the *Drosophila* genome; *dilp2* and *dilp4* are both considered high-confidence astrocyte genes, as determined by expression in both alrm-Gal4 and eaat1-Gal4 profiling experiments. *Dilp5* and *dilp6* are only represented in the alrm-Gal4 translatome. The receptor for insulin-like peptides, *dInR,* could also be examined; it is the only known insulin receptor in *Drosophila*. While abundantly expressed in the nervous system, there is not much information on its distribution among specific groups of cells. Locomotor behavior assays on strains with *dInR* RNAi expressed in different groups of clock neurons may give insight to whether insulin signaling is important for circadian behavior in those groups of cells.

Less is known about MESK2 but given its identification from a study on Ras/ERK signaling, analysis of this major signaling pathway would be informative. For example, this pathway can be activated through EGFR, which is a high-confidence astrocyte gene based on my profiling.

To provide a non-biased method of miRNA target identification, I can perform expression profiling using flies that overexpress miR-263b or miR-274. Overexpression of miRNAs may result in degradation of RNA targets, and so a comparison of RNA abundance between miRNA overexpression flies and background control flies will provide a list of probable targets. However, overexpression of miRNAs may create unnatural circumstances where miRNA expression is forced in cells that do not normally express it. Increased miRNA abundance may also interfere with miRNA processing components such as Ago. Inhibition of miRNAs provides another approach to verifying targets. Since this manipulation targets endogenous miRNAs, there may be fewer false positives. However, miRNA inhibitors do not necessarily result in the decay the miRNA targeted, and so it may be difficult to assess the potency of the inhibitor.

4.3 Identification of cycling genes in astrocytes using TRAP

This study is the first to identify the rhythmic expression of ribosome-bound RNAs in astrocytes. I used the TRAP method to isolate astrocyte-specific, ribosome-bound RNAs for RNA-seq. The sequencing of ribosome-bound RNAs is used as a proxy for the translational profile of these cells. While we assume this corresponds with amount of protein synthesis at the time of collection, it is not a definitive correlation. By restricting my profiling to astrocytes, I aimed to identify glial components important for glia-neuron communication. The Jackson lab previously used TRAP to profile larval and adult astrocytes at a single time point (Huang et al., 2015; Ng et al., 2016). Those studies provided information on the molecular makeup of fly astrocytes and found that ~70% of genes enriched in adult astrocytes have mammalian orthologs (Ng et al., 2016). Given the

similarities between fly and mouse astrocytes, *Drosophila* is an attractive model for forward genetic screens to study astrocyte function.

4.3.1 Building the astrocyte circadian translatome

I used TRAP to isolate ribosome-bound RNAs from fly astrocytes across the first two days of DD after entrainment. The isolated RNAs were then converted into cDNA libraries for sequencing. In collaboration with Dr. Amy Yu, the initial analysis identified 724 genes that express RNAs that cycle in abundance. I asserted that any gene represented in both the current study and in the previous astrocyte studies would be considered high-confidence astrocyte genes. Therefore, I identified the overlap between a previously generated list of astrocyte-expressed genes (using alrm-Gal4) and genes identified in the current study (using eaat1-Gal4). I identified 576 high-confidence cycling genes in fly astrocytes. Among these genes, importantly, are core clock genes and genes known to be expressed in astrocytes (Fig. 3.13 and 3.15). The presence of such genes in the list provides confidence in the fidelity of the collected samples and subsequent TRAP experiments. Of the 576 cycling genes, 88 were previously identified as having enriched expression in astrocytes compared to whole head RNA (Fig. 3.14 A).

4.3.2 Comparison of genes expressed in glial using alrm-Gal4 versus eaat1-Gal4

The current studies use the eaat1-Gal4 driver in concert with elav-Gal80 to inhibit Gal4 expression in neurons. This driver is predominantly expressed in astrocytes but is also expressed in some cortex glia. The previous astrocyte profiling studies utilized alrm-Gal4, which is thought to be specific for astrocytes. Given the amount of material needed for my circadian studies, I chose to use eaat1-Gal4 for these experiments because it is a stronger driver and therefore provides more robust EGFP-L10a expression. In comparing

the two experiments, there are 5200 genes in common; i.e., genes expressed in both arlm- and eaat1-containing cells. This represents 77% of genes in the alrm-Gal4 experiments and 68% of the genes in the eaat1-Gal4 experiments. Genes represented in both lists can be considered high-confidence astrocyte genes. As eaat1-Gal4 is also expressed in a population of cortex glia, it may be that the bulk of remaining genes (32%) not observed in the alrm-Gal4 profiling database represent those expressed in cortex glia. Alternatively, certain common genes may have been missed because they have low expression at ZT1, the only time assayed in the alrm-Gal4 experiments. The alrm-Gal4 expression profile also contains genes not expressed in the eaat1-Gal4 experiments. While alrm-Gal4 is thought to be astrocyte-specific, it is possible there is uncharacterized expression in other cell-types. It is also possible that the two drivers are expressed in overlapping but slightly different populations of astrocytes given the evidence for fly glial cell heterogeneity (Kremer et al., 2017). Finally, it is also possible that eaat1-Gal4 and alrm-Gal4 result in expression of EGFP-L10a at different levels in different cells.

4.3.3 Analysis of genes in astrocytes that cycle in abundance

Analysis of the high-confidence astrocyte cycling genes was done in two ways. To obtain a global perspective of all overrepresented cellular components (Fig. 3.14 B) and biological processes (Fig. 3.14 C), I used DAVID v.6.8 to process the list. The largest categories of cellular components were ATP binding (49) and structural component of ribosome (22). Additionally, there were multiple categories describing enzymatic functions, including those relevant for metabolism. To comprehensively identify genes of interest, I used FlyBase annotations to manually sort for biological processes relevant for glia-neuron communication (Table 3.6). Annotation of genes using GO terms associated

with biological processes identified 27 genes relevant for vesicle or secretion-related functions, indicating an important role for this process in rhythmicity.

I also identified 30 cycling receptors or modulators of receptors, including the serotonin receptor, 5-hydroxytryptamine receptor 1B (5-HT1B). Of the five *Drosophila* isoforms, 5-HT1B is the most similar to the mammalian serotonin receptor 5-HT1A (Saudou et al., 1992). There is evidence that serotonin signaling is important in the light-driven degradation of TIM in *Drosophila* (Yuan et al., 2005). The use of selective serotonin reuptake inhibitors (SSRIs) that target mammalian 5-HT1A as treatment for anxiety and major depression is now commonplace (Garcia-Garcia et al., 2014). 5-HT1A is expressed in high abundance in mammalian astrocytes (Whitaker-Azmitia et al., 1993). Much of the research on SSRIs is focused on neuronal response to serotonin but astrocytes also respond to SSRIs through calcium signaling (Schipke et al., 2011). Given the comorbidity of mood disorders and circadian disruption, astrocyte serotonin signaling may be important and provide another avenue for therapeutic treatments.

Analysis of circadian-related GO terms revealed 15 relevant genes, four of which have functions in extracellular space. These may be candidates for molecules that signal between glia and neurons. One identified astrocyte cycling gene is Angiotensin-converting enzyme-related (ACER). This gene was previously identified as cycling in whole head studies and found to be regulated by *Clk* (McDonald and Rosbash, 2001). This peptidase was determined to be important for nighttime sleep as mutants have decreased sleep and greater sleep fragmentation (Carhan et al., 2011). While that study found ACER in the fat body and not the adult brain, the investigators determined that

ACER is indeed a secreted protein as it is secreted into the hemolymph. It is possible that astrocytes also secrete ACER.

Another cycling gene is the enzyme *Treh,* which is responsible for metabolizing circulating trehalose into metabolites used by neurons in *Drosophila*. Trehalose is also present in the mammalian nervous system where it is made by astrocytes and metabolized by neurons (Martano et al., 2017). In mammals, glucose is the main source of energy for the brain. The glucose transporter GLUT1 is expressed in endothelial cells of brain vessels and in astrocytes that contact them (Leybaert, 2005). Astrocytes metabolize the glucose via glycolysis into pyruvate. It is then converted to lactate and shuttled to neurons to convert back to pyruvate to fuel the TCA cycle and respiratory chain. This series of interactions is named the astrocyte neuron lactate shuttle (ANLS) hypothesis (Schirmeier et al., 2016). Instead of glucose, trehalose is the major sugar in the hemolymph for *Drosophila*. The Jackson lab previously identified *Treh* as having enriched expression in astrocytes (Ng et al., 2016). Studies focused on surface glia, which comprise the *Drosophila* blood-brain barrier, found that these glial cells take up trehalose, express the transporter TRET1-1, and secrete alanine and lactate for neurons to metabolize (Volkenhoff et al., 2015). Furthermore, inhibition of glial glycolysis results in severe neurodegeneration. In contrast, neuronal glycolysis appears to be mostly dispensable (Volkenhoff et al., 2015). Along those lines, we found that glial knockdown of *Treh* significantly decreased activity level (Ng et al., 2016). I have shown that inhibition of two miRNAs (let-7 and miR-79) predicted to target *Treh* also decreased activity (Fig. 3.1 C). While inhibition of a miRNA that targets *Treh* should result in increased TREH synthesis, it is possible that both increased and decreased metabolism of

trehalose are similarly detrimental to the fly. In the present astrocyte profiling study, I found robust cycling of *Treh* translation in astrocytes (Fig. 3.15 B). While little is known about the function of *Treh* in astrocytes and how it may differentiate from its function in surface glia, functions similar to the mammalian ANLS hypothesis seem reasonable. Fly astrocytes may metabolize trehalose into lactate and other metabolites, and then shuttle these products to neurons to use for energy.

4.3.4 Two phases of astrocyte protein synthesis

Previous profiling studies on clock cells utilizing tim-Gal4 found that there are two major phases of translation (Huang et al., 2013). Peak translation occurred during mid-day or mid-night, periods of behavioral quiescence. In comparison, most genome-wide studies observed translation throughout the day (Ceriani et al., 2002; Claridge-Chang et al., 2001; Hughes et al., 2012; McDonald and Rosbash, 2001; Ueda et al., 2002). In profiling astrocytes, I also observed two major phases of protein synthesis. Peak translation in astrocytes appears to occur a few hours earlier than in clock cells (Fig. 3.16). Because there is only a single set of data for the astrocyte circadian profiling experiments, it is possible this difference is an experimental artifact but recent mammalian studies have shown temporal differences between astrocyte and neuronal activity (Brancaccio et al., 2017). A recent study profiled different subgroups of clock cells and observed that the LN_vs have two peaks of translation while LN_ds and DN1s have one peak (Fig. 3.17) (Abruzzi et al., 2017). The experiments of Abruzzi *et al.* were performed in LD conditions whereas the present study was performed in DD. As such, the list generated by Abruzzi *et al.* likely includes genes that cycle in abundance due to influence by light.

Surprisingly, the overlap of genes identified as cycling in the clock-cell profiling studies and the current study is low. Only 38 genes were found to be cycling in both studies. The previous tim-Gal4 profiling experiments included all cells with PER-based oscillators including astrocytes. It is possible that the addition of other cell types by both profiling studies accounts for this difference. For example, a gene that is cycling in astrocytes may cycle in other cell types in a different phase, or it may be expressed in other cell types at constant levels throughout the day.

4.3.5 Future directions for this project

While the single set of data is informative, a second set of data is necessary to confirm my results. For specific genes of interest, use of qPCR can also provide confirmation of cycling. As genes of interest may be expressed in low amounts or may only cycle in astrocytes, it is still imperative to probe these genes in a cell-type specific manner. As there are multiple genome-wide RNAi collections, a targeted screen of cycling genes could be performed to identify those important in astrocytes.

During the course of my experiments, new glial Gal4 drivers were characterized (Kremer et al., 2017). The authors annotated the distribution of about 500 glial-specific Gal4 drivers and determined the best Gal4 drivers for each glial subclass. R86E01-Gal4 and 55B03-Gal4 were identified as the best astrocyte drivers. Given the disparity between the alrm-Gal4 translational profile and the eaat1-Gal4 translational profile, performing TRAP with one of these new drivers could improve the accuracy of the astrocyte translational profile. In addition, I can begin to explore the genetic identity of other glial subclasses. In particular, the ensheathing glia is also neuropil associated and surrounds large sections of neuropil. The cortex glia surround neuronal cell bodies, making them

well situated to interacting with the soma. An ongoing interest of the Jackson Lab is to identify specific subgroups of astrocytes that have circadian functions. Intersectional techniques allow for manipulations of smaller subsets of cells within a Gal4 population. If I am able to identify these subgroups, I can use TRAP to characterize their molecular identity. Determining the molecular identity of these different groups of glial cells will add to our understanding of how these cells influence neuronal behavior.

While knockdown of PER using RNAi in fly glial cells does not alter circadian rhythms (Ng et al., 2011), manipulation of the core clock in mammalian astrocytes does result in changes in circadian period (Barca-Mayo et al., 2017; Brancaccio et al., 2017; Tso et al., 2017). It is possible that mammalian astrocyte clocks have diverged from fly astrocyte clocks. It is also possible that the clocks in fly astrocytes have different components. While the RNAi directed against PER appeared to eliminate all PER from glial cells, it is possible very low but sufficient amounts were still present. It would be of interest to use genetic editing techniques such as the CRISPR/Cas9 technique to delete individual clock genes specifically from astrocytes. The mutant strains could be probed for effects on circadian rhythms using the previously described locomotor behavior assay. Circadian profiling of astrocytes in these mutants might provide insight into which genes are specifically under the control of the endogenous astrocyte clock.

Using the list of genes with cyclic expression in astrocytes, I can investigate whether miRNAs contribute to the cycling of genes in astrocytes. As previously discussed, a number of miRNAs have been identified as cycling in abundance (summarized in Chapter 1.3.2). It is possible that the rhythmic expression of a miRNA may contribute to the rhythmic expression of its target genes. Manipulation of miRNAs through inhibition

(miR-SP) or overexpression (miRNA transgene) could be used to interfere with the normal cycling of a specific miRNA. Disruption of this cycling may result in the dampened or elimination of rhythmic expression of target genes. By combining miRNA manipulation with astrocyte circadian profiling, I could determine what influence the miRNA may have on its target. This is not possible with the potential miR-274 targets identified in Chapter 3.2.6, as neither CG4328 nor MESK2 appear to be rhythmically expressed. Nevertheless, this does not preclude cycling miRNA influence on other cycling RNAs.

4.4 Concluding remarks

My thesis has described two complementary methods of investigating *Drosophila* glia. The results are focused primarily on the role of astrocytes in circadian behavior but also touch on the potential contribution of other glial classes. I have established a role for glial miRNAs in altering circadian rhythms. In particular, miRNAs can have physiological contributions to the maintenance of normal circadian behavior in adulthood. I have also identified ribosome-bound RNAs that cycle in abundance in fly astrocytes. The findings presented in this thesis support a better understanding of the role of astrocytes in circadian rhythms and the pathologies resulting from altered rhythmic functions.

Chapter 5: Appendix

The following appendices contain experiments that were not described in the results chapter due to their preliminary nature (Chapter 5.1-2, 4). Also included are the full results of the miRNA glial screen (Chapter 5.3).

5.1 miR-263b-SP increases *hid* 3'UTR expression

A previous study identified the pro-apoptotic gene *hid* as a target of miR-263b (Hilgers et al., 2010). As a test of the specificity of miR-263b-SP, I wanted to see if the sponge would have an effect on hid. I expressed both miR-263b-SP and a *hid* 3'UTR sensor tagged with GFP ubiquitously using actin-Gal4. Brains were dissected from experimental and control flies (no sponge). Brains were imaged using a confocal microscope at 40X and z-projections were created. I then used ImageJ to measure pixel intensity. Brains with miR-263b-SP expression were significantly brighter than brains without the sponge. I did not include this experiment in the main text because I do not have a control sensor or control sponge to ensure that the effect is specific.

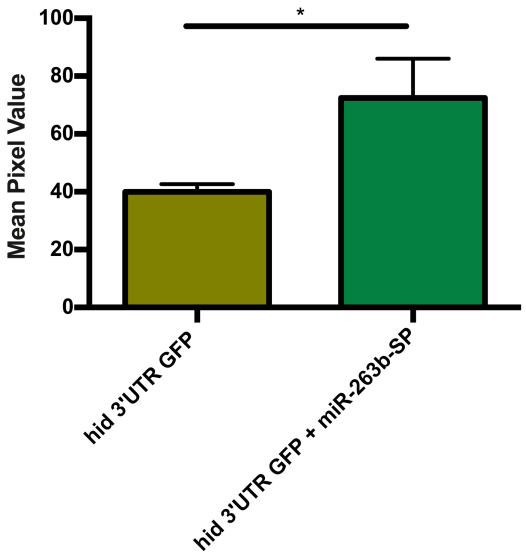


Figure 5.1: miR-263b-SP expression increases hid 3'UTR sensor expression
Ubiquitous miR-263b-SP expression with actin-Gal4 significantly increased *hid* 3'UTR GFP fluorescence compared to the no-sensor control. Results are mean ± SEM, n=7, two-tailed Student's *t*-test, *p<0.05.

5.2 Glial miR-274-SP expression rescues decreased rhythmicity caused by MESK2 RNAi

I identified MESK2 as a potential miR-274 target in Chapter 3.2.6 using MESK2 RNAi strains. To probe whether MESK2 is a *bona fide* target of miR-274, I performed genetic rescue experiments. If miR-274 targets MESK2 then inhibition of miR-274 with miR-274-SP should result in increased MESK2. As shown in Fig. 5.2, flies that express both MESK2 RNAi and miR-274-SP in glial cells have normal rhythmicity compared to flies that MESK2 RNAi alone.

The miR-SP strains contain two copies of sponge, one on the second chromosome and one on the third chromosome. The "rescue" strains also contain a MESK2 RNAi on the second chromosome. While not definitive, it is possible that three UAS transgene might dilute Gal4 amounts. The MESK2 RNAi experiments all contained a single UAS transgene and therefore may have more penetrance in this genotype. To assess the potential contribution of Gal4 dilution, I planned to use the scramble-SP. Unfortunately, the flies constructed for these experiments were unhealthy and the line did not survive so the experiment could not be replicated with appropriate controls.

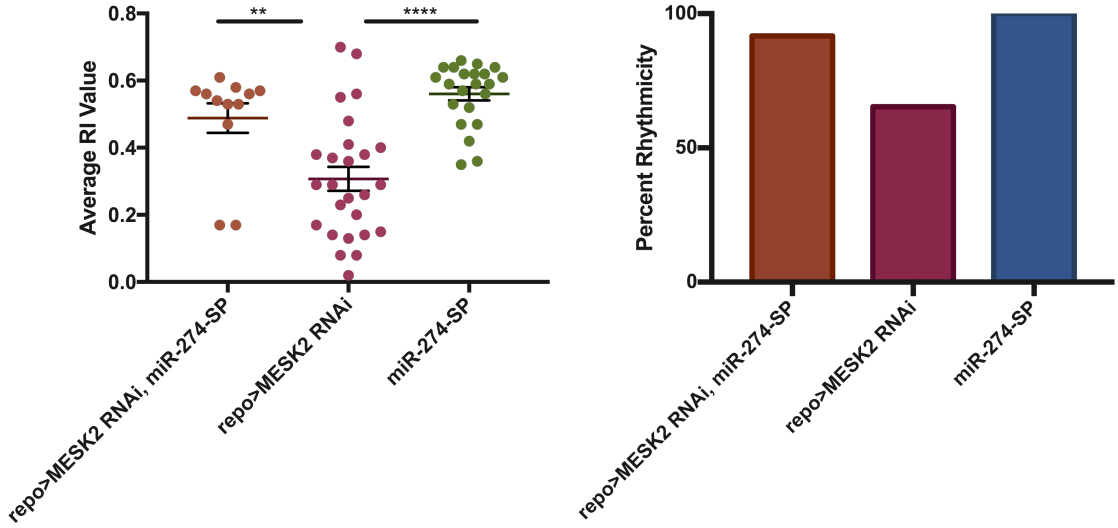


Figure 5.2: miR-274-SP rescues arrhythmicity caused by MESK2 RNAi
Pan-glial expression of MESK2 RNAi and miR-274-SP significantly more robust rhythmicity compared to MESK2 RNAi alone. Results are individual flies ± SEM, one-way ANOVA with Tukey's multiple comparison's test, **p<0.01, ***p<0.001.

5.3 Behavioral results from glial expression of 146 different miR-SPs.

miR-SP	Normalized Percent Entrainment	Normalized Percent Rhythmicity	Period Change in Minutes	Normalized Activity Level
R x 2xUAS-bantam SP	0.80	0.88	-23.96	0.86
R x 2X UAS-miR-let-7-SP	1.03	0.87	4.79	0.68
R x 2x UAS-miR-1 SP	0.83	0.83	-3.13	0.94
R x 2x UAS-miR-2a SP	0.80	0.80	-10.75	0.97
R x 2x UAS-miR-2b SP	0.92	0.75	-11.12	0.94
R x 2x UAS-miR-2c SP	0.88	0.79	-5.14	0.83
R x 2x UAS-miR-3 SP	0.94	0.93	-33.60	0.90
R x 2x UAS-miR-4 SP	0.94	0.94	-19.13	0.69
R x 2x UAS-miR-5 SP	1.03	0.86	-39.72	1.01
R x 2x UAS-miR-6 SP	1.00	0.90	-22.74	0.73
TR x 2x UAS-miR-7 SP	0.94	1.00	0.40	1.45
R x 2x UAS-miR-8 SP	1.00	0.92	-14.88	0.92
TR x 2x UAS-miR-9b SP	1.00	1.15	-32.00	1.19
R x 2X UAS-miR-9c-SP	0.93	0.73	2.05	0.87
R x 2x UAS-miR-10 SP	1.00	0.73	-3.03	1.15
R x 2x UAS-miR-11 SP	0.89	1.00	-12.69	1.25
R x 2x UAS-miR-12 SP	0.81	0.76	-17.89	0.90
R x 2x UAS-miR-13a SP	0.88	1.00	-26.14	1.10
R x 2x UAS-miR-13b SP	0.62	0.75	-10.71	1.24
R x 2X UAS-miR-14 SP	0.99	1.00	-11.57	0.75
R x 2X UAS-miR-31a-SP	0.86	0.78	-32.76	0.85
R x 2X UAS-miR-31b-SP	0.75	1.04	1.40	1.20

miR-SP	Normalized Percent Entrainment	Normalized Percent Rhythmicity	Period Change in Minutes	Normalized Activity Level
R x 2X UAS-miR-33-SP	0.94	1.01	-8.57	1.15
R x 2X UAS-miR-34-SP	0.99	0.91	3.84	0.93
R x 2X UAS-miR-79-SP	0.74	0.63	7.40	0.65
R x 2X UAS-miR-79-SP	0.91	0.59	-32.62	0.71
R x 2X UAS-miR-87-SP	1.08	0.78	13.10	0.59
R x 2X UAS-miR-92a-SP	1.17	0.92	17.77	0.75
R x 2X UAS-miR-92b-SP	0.98	0.89	-26.21	0.93
R x 2X UAS-miR-100-SP	0.82	1.00	-27.17	0.95
R x 2xUAS-miR-124 SP	0.75	0.89	-31.42	1.24
R x 2X UAS-miR-125-SP	1.00	0.93	-25.12	0.63
R x 2xUAS-miR-133 SP	0.87	0.92	-1.44	1.23
R x 2X UAS-miR-137-SP	1.07	0.98	-30.53	0.98
R x 2X UAS-miR-184-SP	0.63	0.86	12.60	0.89
R x 2X UAS-miR-190-SP	0.89	0.84	-12.33	0.88
R x 2X UAS-miR-193-SP	0.87	0.74	-8.91	0.98
R x 2X UAS-miR-210 SP	0.93	0.49	-10.19	0.99
TR x 2xUAS-miR-219 SP	1.14	0.84	-0.85	1.00
R x 2X UAS-miR-252-SP	0.75	0.89	-3.13	0.87
R x 2xUAS-miR-263a SP	0.91	0.83	-18.98	0.72
R x 2X UAS-miR-263b-SP	0.87	0.62	10.33	0.74
R x 2X UAS-miR-274-SP	0.83	0.68	-20.10	1.00
R x 2X UAS-miR-275-SP	1.00	0.87	-18.72	1.05
R x 2X UAS-miR-276*-SP	0.81	0.92	-30.14	1.26

miR-SP	Normalized Percent Entrainment	Normalized Percent Rhythmicity	Period Change in Minutes	Normalized Activity Level
R x 2X UAS-miR-276a-SP	0.92	0.62	-14.86	1.29
R x 2X UAS-miR-276b-SP	0.85	0.71	6.57	1.20
R x 2X UAS-miR-277-SP	0.95	0.71	-0.58	1.08
R x 2X UAS-miR-278-SP	0.76	0.80	2.66	0.55
R x 2X UAS-miR-279-SP	0.68	0.96	-19.36	0.73
R x 2X UAS-miR-281-SP	1.07	0.93	8.34	0.89
R x 2X UAS-miR-2811-SP	1.00	0.63	-26.41	0.93
R x 2X UAS-miR-2812-SP	0.86	0.93	-14.00	0.61
R x 2X UAS-miR-282-SP	0.75	1.00	-1.60	1.01
R x 2X UAS-miR-283-SP	0.93	0.80	-27.27	0.91
R x 2X UAS-miR-284-SP	1.00	1.00	-9.75	0.97
R x 2X UAS-miR-285-SP	0.92	0.57	-26.95	0.93
R x 2X UAS-miR-286-SP	0.94	0.82	-6.00	1.24
R x 2X UAS-miR-287-SP	1.00	0.94	-13.18	1.26
R x 2X UAS-miR-288-SP	1.14	1.08	-2.03	1.10
R x 2X UAS-miR-289-SP	1.00	1.00	3.70	0.81
R x 2X UAS-miR-303-SP	1.00	0.93	-6.95	0.77
R x 2X UAS-miR-304-SP	1.00	0.53	-4.26	0.84
R x 2X UAS-miR-305-SP	0.98	0.49	-14.38	0.78
R x 2X UAS-miR-306-SP	0.87	0.92	3.35	1.13
R x 2X UAS-miR-307-SP	1.01	0.87	-6.97	0.83
R x 2X UAS-miR-308-SP	0.85	0.95	-25.21	0.82
R x 2X UAS-miR-309-SP	0.93	0.59	-16.43	0.89

miR-SP	Normalized Percent Entrainment	Normalized Percent Rhythmicity	Period Change in Minutes	Normalized Activity Level
R x 2X UAS-miR-310-SP	1.02	0.60	-23.96	0.98
R x 2X UAS-miR-311-SP	1.08	1.00	-17.50	0.84
R x 2X UAS-miR-312-SP	0.84	0.93	-7.91	0.90
R x 2X UAS-miR-313-SP	1.00	1.00	-17.14	0.66
R x 2X UAS-miR-314-SP	0.80	0.83	0.25	0.99
R x 2X UAS-miR-315-SP	0.88	1.00	1.39	0.80
R x 2X UAS-miR-316-SP	1.07	0.86	10.29	0.70
R x 2X UAS-miR-317-SP	0.83	0.57	-12.57	0.96
R x 2X UAS-miR-318-SP	0.94	0.86	-6.43	0.77
R x 2X UAS-miR-375-SP	1.07	0.94	10.30	0.70
R x 2X UAS-miR-927-SP	0.95	0.55	-10.98	0.85
R x 2X UAS-miR-929-SP	1.00	0.89	-18.51	0.75
R x 2X UAS-miR-932-SP	0.94	0.94	4.18	0.77
R x 2X UAS-miR-954-SP	0.86	0.86	-3.86	0.68
R x 2X UAS-miR-955-SP	1.00	1.00	-10.00	0.92
R x 2X UAS-miR-956-SP	0.99	0.86	-7.92	0.61
R x 2x UAS-miR-957-SP	0.91	0.91	-4.76	0.99
R x 2x UAS-miR-958-SP	0.98	0.98	7.18	0.78
R x 2x UAS-miR-959-SP	0.82	0.82	-17.56	0.88
R x 2x UAS-miR-960-SP	0.81	0.85	-13.00	0.94
R x 2x UAS-miR-961-SP	0.89	0.80	-18.44	0.90
R x 2x UAS-miR-962-SP	0.87	0.87	-13.20	0.76
R x 2x UAS-miR-963-SP	0.87	0.56	-32.46	0.94

miR-SP	Normalized Percent Entrainment	Normalized Percent Rhythmicity	Period Change in Minutes	Normalized Activity Level
R x 2X UAS-miR-964-SP	0.84	0.78	-4.67	0.75
R x 2X UAS-miR-965-SP	1.00	1.00	-20.49	1.02
R x 2X UAS-miR-966-SP	0.83	0.83	-16.75	1.03
R x 2X UAS-miR-967-SP	0.77	0.60	-15.36	0.93
R x 2X UAS-miR-968-SP	0.93	0.77	-20.60	0.84
R x 2X UAS-miR-969-SP	0.81	0.81	-18.75	0.66
R x 2X UAS-miR-970-SP	0.93	0.93	-15.00	0.79
R x 2X UAS-miR-971-SP	0.95	0.61	-21.79	0.91
R x 2X UAS-miR-972-SP	0.92	0.92	-1.90	0.98
R x 2X UAS-miR-973-SP	0.99	0.90	-7.07	0.63
R x 2X UAS-miR-974-SP	0.95	0.73	-12.80	0.54
R x 2X UAS-miR-975-SP	1.00	0.84	-19.86	0.85
R x 2X UAS-miR-976-SP	0.95	1.08	-11.54	0.79
R x 2X UAS-miR-977-SP	1.00	0.93	-22.29	0.73
R x 2X UAS-miR-978-SP	0.94	0.87	-15.37	0.74
R x 2X UAS-miR-979-SP	1.00	0.82	-26.62	1.01
R x 2X UAS-miR-980-SP	1.12	1.00	-32.35	0.92
R x 2X UAS-miR-981-SP	1.02	0.70	-32.61	0.84
R x 2X UAS-miR-982-SP	1.07	1.00	-20.90	0.75
R x 2X UAS-miR-983-SP	1.07	1.00	-19.71	0.63
R x 2X UAS-miR-984 SP	0.83	0.85	-38.40	0.84
R x 2X UAS-miR-985 SP	0.93	0.88	5.30	0.82
R x 2X UAS-miR-986 SP	0.94	0.73	-16.40	1.05

miR-SP	Normalized Percent Entrainment	Normalized Percent Rhythmicity	Period Change in Minutes	Normalized Activity Level
R x 2X UAS-miR-987 SP	0.92	0.95	-6.50	0.54
R x 2X UAS-miR-988 SP	0.81	0.66	2.22	1.26
R x 2X UAS-miR-989 SP	1.00	0.62	-11.54	1.27
R x 2X UAS-miR-990 SP	0.74	0.65	-7.65	0.87
R x 2X UAS-miR-991 SP	0.85	0.98	-28.86	0.86
R x 2X UAS-miR-992 SP	0.93	0.70	-21.93	0.81
R x 2X UAS-miR-993 SP	0.80	0.92	-19.63	0.75
R x 2X UAS-miR-994 SP	0.93	0.32	-17.10	1.09
R x 2X UAS-miR-996 SP	0.87	0.92	-23.94	0.73
R x 2X UAS-miR-997 SP	0.97	1.09	0.40	1.09
R x 2X UAS-miR-998 SP	0.82	0.89	-47.96	0.57
R x 2X UAS-miR-999 SP	0.87	0.83	-20.15	0.53
R x 2X UAS-miR-1000 SP	0.94	1.00	-20.30	0.79
R x 2X UAS-miR-1001 SP	0.87	0.77	-14.11	0.82
R x 2X UAS-miR-1002 SP	0.90	0.75	-14.15	0.88
R x 2X UAS-miR-1003 SP	0.99	0.85	2.47	0.98
R x 2X UAS-miR-1004 SP	0.81	0.84	-18.75	1.11
R x 2X UAS-miR-1005 SP	1.07	0.94	-13.27	1.16
R x 2X UAS-miR-1006 SP	0.84	0.80	-16.70	1.29
R x 2X UAS-miR-1007 SP	0.94	0.79	-22.14	1.18
R x 2X UAS-miR-1008 SP	1.00	0.79	6.86	0.69
R x 2X UAS-miR-1009 SP	1.00	0.73	-33.07	0.67
R x 2X UAS-miR-1010 SP	1.00	0.79	-28.39	0.73

miR-SP	Normalized Percent Entrainment	Normalized Percent Rhythmicity	Period Change in Minutes	Normalized Activity Level
R x 2X UAS-miR-1011 SP	0.87	0.90	3.14	1.16
R x 2X UAS-miR-1012 SP	0.74	0.72	-37.44	0.85
R x 2X UAS-miR-1013 SP	0.99	0.77	-22.49	0.61
R x 2X UAS-miR-1014 SP	0.74	0.71	-6.98	0.92
R x 2X UAS-miR-1015 SP	0.81	0.90	-2.54	1.18
R x 2X UAS-miR-1016 SP	0.87	1.00	-13.62	0.58
R x 2X UAS-miR-1017 SP	0.92	0.88	-20.80	0.88
R x 2X UAS-miR-iab-4-3p SP	1.00	0.68	-10.48	0.96
R x 2X UAS-miR-iab-4-5p SP	0.93	0.93	-22.29	0.88

Table 5.1: Behavioral results from glial expression of 146 different miR-SPs
Data points represent experimental results normalized to those of UAS-miR-SP controls. Crosses were performed with the repo-Gal4 pan glial driver (R). miR-7, -9b, -219 were tested using conditional repo-Gal4 expression (TR).

5.4 Exploration of glia important for rhythmicity via TRPA1

Since discovering that astrocytes participate in the regulation of circadian behavior, the Jackson lab has been interested in identifying specific groups of cells that may be relevant. Given the heterogeneity of astrocytes, it is possible that a subset of these cells may be particularly important in this context. I performed preliminary experiments exploring the use of the temperature-sensitive ion channel TRPA1. I generated experimental flies by combining a TRPA1 transgene with an astrocyte driver (elav-Gal80, eaat1-Gal4 and alrm-Gal4). As a control, the UAS-TrpA1 was crossed to w^{1118}. The crosses were kept at 23°C during development and during the entrainment portion of the behavioral run. The temperature was then increased to 28°C for the remainder of the experiment (5 days of DD). Activation of TRPA1 significantly decreased rhythmicity compared to the UAS-control (Fig. 5.3 A).

To use TRPA1 to manipulate subsets of cells, I tested a method called ET-FLP-induced intersectional GAL80/GAL4 repression (FINGR) (Bohm et al., 2010). This is an intersectional strategy used to study subsets of cells within Gal4 patterns. Cell-type specific Gal80 can be used to restrict GAL4 expression. The recombinase Flippase (FLP) removes sequences located between two FLP recognition target sequences (FRT, >). FINGR uses a flip-in Gal80 construct (tub^{P}>Gal80>stop) in conjunction with tissue-specific enhancer-trapping FLP (ET-FLP) to express Gal80. By combining Gal4, flip-in Gal80, ET-FLP and UAS, I can repress UAS expression from cell-types with both Gal4 and Gal80 expressed, resulting in expression only in cells Gal4-positive and Gal80-negative. I used strains with flip-in Gal80, and TRPA1 expressed in all glial cells (tub^{P}>Gal80>stop; UAS-TrpA1; repo-Gal4, TTR). On its own, Gal80 is expressed

ubiquitously and therefore Gal4 is also inhibited ubiquitously. The result should be no TRPA1 expression. TTR females were crossed to different male ET-FLP strains. I tested six different ET-FLP lines for TRPA1 induced effects on rhythmicity. Activation of TRPA1 in experiments with two ET-FL lines, 168 and 197B, resulted in decreased rhythmicity compared to controls (ET-FLP alone or Gal4 inhibited) (Fig. 5.3 B). TTRxET-FLP 197B strains have TRPA1 expression in the optic lobes and also some expression in the neuropil (unpublished data). ET-FLP 168 is under the control of enhancer elements near *kra*, a translational regulator with expression in astrocytes (represented in both alrm-Gal4 and eaat1-Gal4 profiling experiments). By systematically manipulating subsets of glial cells, I can map out which cells are relevant for circadian behavior. Once interesting subsets of glia cells are identified, this method could be modified so that different manipulations can be utilized.

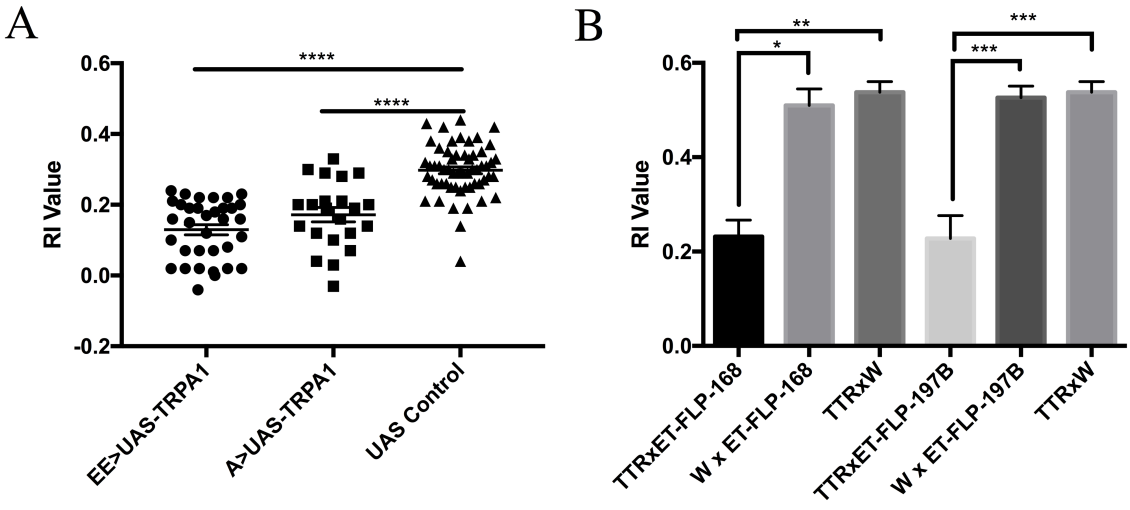


Figure 5.3: Use of TRPA1 to manipulate behavior in glial cells
A) TRPA1 activation using elav-Gal80, eaat1-Gal4 (EE) or alrm-Gal4 (A). Results are individual flies ± SEM, n=22-57, two-tailed Student's *t*-test, ****p<0.0001. B) TRPA1 activation in a subset of repo-GAL4 positive glial cells using FINGR. TTR represents repo-Gal4, UAS-TrpA1 and flip-in Gal80. Results are mean ± SEM, n=6-14, Kruskal-Wallis with Dunn's multiple comparison test *p<0.05, **p<0.01, ***p<0.001.

Chapter 6: Bibliography

Abruzzi, K.C., Zadina, A., Luo, W., Wiyanto, E., Rahman, R., Guo, F., Shafer, O., Rosbash, M., 2017. RNA-seq analysis of Drosophila clock and non-clock neurons reveals neuron-specific cycling and novel candidate neuropeptides. PLOS Genet. 13, e1006613. https://doi.org/10.1371/journal.pgen.1006613

Akten, B., Jauch, E., Genova, G.K., Kim, E.Y., Edery, I., Raabe, T., Jackson, F.R., 2003. A role for CK2 in the Drosophila circadian oscillator. Nat. Neurosci. 6, 251–257. https://doi.org/10.1038/nn1007

Allada, R., White, N.E., So, W.V., Hall, J.C., Rosbash, M., 1998. A mutant Drosophila homolog of mammalian Clock disrupts circadian rhythms and transcription of period and timeless. Cell 93, 791–804.

Alvarez-Saavedra, M., Antoun, G., Yanagiya, A., Oliva-Hernandez, R., Cornejo-Palma, D., Perez-Iratxeta, C., Sonenberg, N., Cheng, H.-Y.M., 2011. miRNA-132 orchestrates chromatin remodeling and translational control of the circadian clock. Hum. Mol. Genet. 20, 731–751. https://doi.org/10.1093/hmg/ddq519

Andresen, L., Hampton, D., Taylor-Weiner, A., Morel, L., Yang, Y., Maguire, J., Dulla, C.G., 2014. Gabapentin attenuates hyperexcitability in the freeze-lesion model of developmental cortical malformation. Neurobiol. Dis. 71, 305–316. https://doi.org/10.1016/j.nbd.2014.08.022

Araque, A., Li, N., Doyle, R.T., Haydon, P.G., 2000. SNARE protein-dependent glutamate release from astrocytes. J. Neurosci. Off. J. Soc. Neurosci. 20, 666–673.

Araque, A., Parpura, V., Sanzgiri, R.P., Haydon, P.G., 1999. Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci. 22, 208–215.

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

Aton, S.J., Colwell, C.S., Harmar, A.J., Waschek, J., Herzog, E.D., 2005. Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons. Nat. Neurosci. https://doi.org/10.1038/nn1419

Awasaki, T., Lai, S.-L., Ito, K., Lee, T., 2008. Organization and postembryonic development of glial cells in the adult central brain of Drosophila. J. Neurosci. Off. J. Soc. Neurosci. 28, 13742–13753. https://doi.org/10.1523/JNEUROSCI.4844-08.2008

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

Bargiello, T.A., Jackson, F.R., Young, M.W., 1984. Restoration of circadian behavioural rhythms by gene transfer in Drosophila. Nature 312, 752–754. https://doi.org/10.1038/312752a0

Bejarano, F., Bortolamiol-Becet, D., Dai, Q., Sun, K., Saj, A., Chou, Y.-T., Raleigh, D.R., Kim, K., Ni, J.-Q., Duan, H., Yang, J.-S., Fulga, T.A., Van Vactor, D., Perrimon, N., Lai, E.C., 2012. A genome-wide transgenic resource for conditional

expression of Drosophila microRNAs. Development 139, 2821–2831. https://doi.org/10.1242/dev.079939
Bernstein, E., Caudy, A.A., Hammond, S.M., Hannon, G.J., 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409, 363–366. https://doi.org/10.1038/35053110
Bezzi, P., Gundersen, V., Galbete, J.L., Seifert, G., Steinhauser, C., Pilati, E., Volterra, A., 2004. Astrocytes contain a vesicular compartment that is competent for regulated exocytosis of glutamate. Nat. Neurosci. 7, 613–620. https://doi.org/10.1038/nn1246
Bohm, R., Welch, W., Goodnight, L., Cox, L., Henry, L., Gunter, T., Bao, H., Zhang, B., 2010. A genetic mosaic approach for neural circuit mapping in Drosophila. Proc. Natl. Acad. Sci. U. S. A. 107, 16378–16383. https://doi.org/10.1073/pnas.1004669107
Bohnsack, M.T., Czaplinkski, K., Gorlich, D., 2004. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA 10, 185–191. https://doi.org/10.1261/rna.5167604
Brancaccio, M., Patton, A.P., Chesham, J.E., Maywood, E.S., Hastings, M.H., 2017. Astrocytes Control Circadian Timekeeping in the Suprachiasmatic Nucleus via Glutamatergic Signaling. Neuron 93, 1420–1435.e5. https://doi.org/10.1016/j.neuron.2017.02.030
Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Dev. Camb. Engl. 118, 401–415.
Bushong, E.A., Martone, M.E., Jones, Y.Z., Ellisman, M.H., 2002. Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. J. Neurosci. Off. J. Soc. Neurosci. 22, 183–192.
Busto, G.U., Guven-Ozkan, T., Fulga, T.A., Van Vactor, D., Davis, R.L., 2015. microRNAs That Promote or Inhibit Memory Formation in Drosophila melanogaster. Genetics 200, 569–580. https://doi.org/10.1534/genetics.114.169623
Busza, A., Emery-Le, M., Rosbash, M., Emery, P., 2004. Roles of the two Drosophila CRYPTOCHROME structural domains in circadian photoreception. Science 304, 1503–1506. https://doi.org/10.1126/science.1096973
Cai, X., Hagedorn, C.H., Cullen, B.R., 2004. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA 10, 1957–1966. https://doi.org/10.1261/rna.7135204
Cajochen, C., Frey, S., Anders, D., Späti, J., Bues, M., Pross, A., Mager, R., Wirz-Justice, A., Stefani, O., 2011. Evening exposure to a light-emitting diodes (LED)-backlit computer screen affects circadian physiology and cognitive performance. J. Appl. Physiol. 110, 1432–1438. https://doi.org/10.1152/japplphysiol.00165.2011
Carhan, A., Tang, K., Shirras, C.A., Shirras, A.D., Isaac, R.E., 2011. Loss of Angiotensin-converting enzyme-related (ACER) peptidase disrupts night-time sleep in adult Drosophila melanogaster. J. Exp. Biol. 214, 680–686. https://doi.org/10.1242/jeb.049353
Cavanaugh, D.J., Geratowski, J.D., Wooltorton, J.R.A., Spaethling, J.M., Hector, C.E., Zheng, X., Johnson, E.C., Eberwine, J.H., Sehgal, A., 2014. Identification of a

Circadian Output Circuit for Rest:Activity Rhythms in Drosophila. Cell 157, 689–701. https://doi.org/10.1016/j.cell.2014.02.024
Ceriani, M.F., Darlington, T.K., Staknis, D., Más, P., Petti, A.A., Weitz, C.J., Kay, S.A., 1999. Light-dependent sequestration of TIMELESS by CRYPTOCHROME. Science 285, 553–556.
Ceriani, M.F., Hogenesch, J.B., Yanovsky, M., Panda, S., Straume, M., Kay, S.A., 2002. Genome-wide expression analysis in Drosophila reveals genes controlling circadian behavior. J. Neurosci. Off. J. Soc. Neurosci. 22, 9305–9319.
Charles, A.C., Merrill, J.E., Dirksen, E.R., Sanderson, M.J., 1991. Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. Neuron 6, 983–992.
Chen, R., D'Alessandro, M., Lee, C., 2013. miRNAs Are Required for Generating a Time Delay Critical for the Circadian Oscillator. Curr. Biol. 23, 1959–1968. https://doi.org/10.1016/j.cub.2013.08.005
Chen, W., Liu, Z., Li, T., Zhang, R., Xue, Y., Zhong, Y., Bai, W., Zhou, D., Zhao, Z., 2014. Regulation of Drosophila circadian rhythms by miRNA let-7 is mediated by a regulatory cycle. Nat. Commun. 5, 5549. https://doi.org/10.1038/ncomms6549
Chen, X., Rosbash, M., 2016. mir-276a strengthens *Drosophila* circadian rhythms by regulating *timeless* expression. Proc. Natl. Acad. Sci. 113, E2965–E2972. https://doi.org/10.1073/pnas.1605837113
Chen, Y.-W., Song, S., Weng, R., Verma, P., Kugler, J.-M., Buescher, M., Rouam, S., Cohen, S., 2014. Systematic Study of Drosophila MicroRNA Functions Using a Collection of Targeted Knockout Mutations. Dev. Cell 31, 784–800. https://doi.org/10.1016/j.devcel.2014.11.029
Chendrimada, T.P., Gregory, R.I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K., Shiekhattar, R., 2005. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature 436, 740–744. https://doi.org/10.1038/nature03868
Cheng, H.-Y.M., Papp, J., Varlamova, O., Dziema, H., Russell, B., Curfman, J., Nakazawa, T., Shimizu, K., Okamura, H., Impey, S., Obrietan, K., 2007. microRNA modulation of circadian-clock period and entrainment. Neuron 54, 813–829. https://doi.org/10.1016/j.neuron.2007.05.017
Chintapalli, V.R., Wang, J., Dow, J.A.T., 2007. Using FlyAtlas to identify better Drosophila melanogaster models of human disease. Nat. Genet. 39, 715–720. https://doi.org/10.1038/ng2049
Christopherson, K.S., Ullian, E.M., Stokes, C.C.A., Mullowney, C.E., Hell, J.W., Agah, A., Lawler, J., Mosher, D.F., Bornstein, P., Barres, B.A., 2005. Thrombospondins Are Astrocyte-Secreted Proteins that Promote CNS Synaptogenesis. Cell 120, 421–433. https://doi.org/10.1016/j.cell.2004.12.020
Claridge-Chang, A., Wijnen, H., Naef, F., Boothroyd, C., Rajewsky, N., Young, M.W., 2001. Circadian regulation of gene expression systems in the Drosophila head. Neuron 32, 657–671.
Cong, X., Wang, H., Liu, Z., He, C., An, C., Zhao, Z., 2015. Regulation of Sleep by Insulin-like Peptide System in Drosophila melanogaster. Sleep 38, 1075–1083. https://doi.org/10.5665/sleep.4816

Cornell-Bell, A.H., Finkbeiner, S.M., Cooper, M.S., Smith, S.J., 1990. Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. Science 247, 470–473.

Cyran, S.A., Buchsbaum, A.M., Reddy, K.L., Lin, M.-C., Glossop, N.R.J., Hardin, P.E., Young, M.W., Storti, R.V., Blau, J., 2003. vrille, Pdp1, and dClock form a second feedback loop in the Drosophila circadian clock. Cell 112, 329–341.

Dambal, S., Shah, M., Mihelich, B., Nonn, L., 2015. The microRNA-183 cluster: the family that plays together stays together. Nucleic Acids Res. 43, 7173–7188. https://doi.org/10.1093/nar/gkv703

Dani, J.W., Chernjavsky, A., Smith, S.J., 1992. Neuronal activity triggers calcium waves in hippocampal astrocyte networks. Neuron 8, 429–440. https://doi.org/10.1016/0896-6273(92)90271-E

Darlington, T.K., Wager-Smith, K., Ceriani, M.F., Staknis, D., Gekakis, N., Steeves, T.D., Weitz, C.J., Takahashi, J.S., Kay, S.A., 1998. Closing the circadian loop: CLOCK-induced transcription of its own inhibitors per and tim. Science 280, 1599–1603.

Denli, A.M., Tops, B.B.J., Plasterk, R.H.A., Ketting, R.F., Hannon, G.J., 2004. Processing of primary microRNAs by the Microprocessor complex. Nature 432, 231–235. https://doi.org/10.1038/nature03049

Doherty, J., Logan, M., Taşdemir, Ö., Freeman, M., 2009. Ensheathing glia function as phagocytes in the adult Drosophila brain. J. Neurosci. Off. J. Soc. Neurosci. 29, 4768–4781. https://doi.org/10.1523/JNEUROSCI.5951-08.2009

Doyle, J.P., Dougherty, J.D., Heiman, M., Schmidt, E.F., Stevens, T.R., Ma, G., Bupp, S., Shrestha, P., Shah, R.D., Doughty, M.L., Gong, S., Greengard, P., Heintz, N., 2008. Application of a Translational Profiling Approach for the Comparative Analysis of CNS Cell Types. Cell 135, 749 762. https://doi.org/10.1016/j.cell.2008.10.029

Egan, E.S., Franklin, T.M., Hilderbrand-Chae, M.J., McNeil, G.P., Roberts, M.A., Schroeder, A.J., Zhang, X., Jackson, F.R., 1999. An extraretinally expressed insect cryptochrome with similarity to the blue light photoreceptors of mammals and plants. J. Neurosci. Off. J. Soc. Neurosci. 19, 3665–3673.

Emery, P., So, W.V., Kaneko, M., Hall, J.C., 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. https://doi.org/10.1016/S0092-8674(00)81637-2

Emery, P., Stanewsky, R., Hall, J.C., Rosbash, M., 2000. A unique circadian-rhythm photoreceptor. Nature 404, 456–457. https://doi.org/10.1038/35006558

Etain, B., Milhiet, V., Bellivier, F., Leboyer, M., 2011. Genetics of circadian rhythms and mood spectrum disorders. Eur. Neuropsychopharmacol. J. Eur. Coll. Neuropsychopharmacol. 21 Suppl 4, S676-682. https://doi.org/10.1016/j.euroneuro.2011.07.007

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391, 806–811. https://doi.org/10.1038/35888

Förstemann, K., Tomari, Y., Du, T., Vagin, V.V., Denli, A.M., Bratu, D.P., Klattenhoff, C., Theurkauf, W.E., Zamore, P.D., 2005. Normal microRNA Maturation and

Germ-Line Stem Cell Maintenance Requires Loquacious, a Double-Stranded RNA-Binding Domain Protein. PLoS Biol. 3, e236. https://doi.org/10.1371/journal.pbio.0030236
Freeman, M.R., Delrow, J., Kim, J., Johnson, E., Doe, C.Q., 2003. Unwrapping glial biology: Gcm target genes regulating glial development, diversification, and function. Neuron 38, 567–580.
Friedman, R.C., Farh, K.K.-H., Burge, C.B., Bartel, D.P., 2009. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 19, 92–105. https://doi.org/10.1101/gr.082701.108
Fulga, T.A., McNeill, E.M., Binari, R., Yelick, J., Blanche, A., Booker, M., Steinkraus, B.R., Schnall-Levin, M., Zhao, Y., DeLuca, T., Bejarano, F., Han, Z., Lai, E.C., Wall, D.P., Perrimon, N., Van Vactor, D., 2015. A transgenic resource for conditional competitive inhibition of conserved Drosophila microRNAs. Nat. Commun. 6, 7279. https://doi.org/10.1038/ncomms8279
Gao, Q., Zhou, L., Yang, S.-Y., Cao, J.-M., 2016. A novel role of microRNA 17-5p in the modulation of circadian rhythm. Sci. Rep. 6. https://doi.org/10.1038/srep30070
Garaulet, M., Gómez-Abellán, P., Alburquerque-Béjar, J.J., Lee, Y.-C., Ordovás, J.M., Scheer, F.A.J.L., 2013. Timing of food intake predicts weight loss effectiveness. Int. J. Obes. 37, 604–611. https://doi.org/10.1038/ijo.2012.229
Garcia-Garcia, A.L., Newman-Tancredi, A., Leonardo, E.D., 2014. P5-HT1A receptors in mood and anxiety: recent insights into autoreceptor versus heteroreceptor function. Psychopharmacology (Berl.) 231, 623–636. https://doi.org/10.1007/s00213-013-3389-x
German, M.S., Wang, J., Chadwick, R.B., Rutter, W.J., 1992. Synergistic activation of the insulin gene by a LIM-homeo domain protein and a basic helix-loop-helix protein: building a functional insulin minienhancer complex. Genes Dev. 6, 2165–2176. https://doi.org/10.1101/gad.6.11.2165
Glossop, N.R., Lyons, L.C., Hardin, P.E., 1999. Interlocked feedback loops within the Drosophila circadian oscillator. Science 286, 766–768.
Glossop, N.R.J., Houl, J.H., Zheng, H., Ng, F.S., Dudek, S.M., Hardin, P.E., 2003. VRILLE feeds back to control circadian transcription of Clock in the Drosophila circadian oscillator. Neuron 37, 249–261.
Gooley, J.J., Chamberlain, K., Smith, K.A., Khalsa, S.B.S., Rajaratnam, S.M.W., Van Reen, E., Zeitzer, J.M., Czeisler, C.A., Lockley, S.W., 2011. Exposure to Room Light before Bedtime Suppresses Melatonin Onset and Shortens Melatonin Duration in Humans. J. Clin. Endocrinol. Metab. 96, E463–E472. https://doi.org/10.1210/jc.2010-2098
Gramates, L.S., Marygold, S.J., Santos, G. dos, Urbano, J.-M., Antonazzo, G., Matthews, B.B., Rey, A.J., Tabone, C.J., Crosby, M.A., Emmert, D.B., Falls, K., Goodman, J.L., Hu, Y., Ponting, L., Schroeder, A.J., Strelets, V.B., Thurmond, J., Zhou, P., the?FlyBase?Consortium, 2017. FlyBase at 25: looking to the future. Nucleic Acids Res. 45, D663–D671. https://doi.org/10.1093/nar/gkw1016
Gregory, R.I., Yan, K., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., Shiekhattar, R., 2004. The Microprocessor complex mediates the genesis of microRNAs. Nature 432, 235–240. https://doi.org/10.1038/nature03120

Grima, B., Chélot, E., Xia, R., Rouyer, F., 2004. Morning and evening peaks of activity rely on different clock neurons of the Drosophila brain. Nature 431, 869–873. https://doi.org/10.1038/nature02935

Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G., Mello, C.C., 2001. Genes and Mechanisms Related to RNA Interference Regulate Expression of the Small Temporal RNAs that Control C. elegans Developmental Timing. Cell 106, 23–34. https://doi.org/10.1016/S0092-8674(01)00431-7

Guo, F., Cerullo, I., Chen, X., Rosbash, M., 2014. PDF neuron firing phase-shifts key circadian activity neurons in Drosophila. eLife 3. https://doi.org/10.7554/eLife.02780

Guthrie, P.B., Knappenberger, J., Segal, M., Bennett, M.V., Charles, A.C., Kater, S.B., 1999. ATP released from astrocytes mediates glial calcium waves. J. Neurosci. Off. J. Soc. Neurosci. 19, 520–528.

Guven-Ozkan, T., Busto, G.U., Schutte, S.S., Cervantes-Sandoval, I., O'Dowd, D.K., Davis, R.L., 2016. MiR-980 Is a Memory Suppressor MicroRNA that Regulates the Autism-Susceptibility Gene A2bp1. Cell Rep. 14, 1698–1709. https://doi.org/10.1016/j.celrep.2016.01.040

Haase, A.D., Jaskiewicz, L., Zhang, H., Lainé, S., Sack, R., Gatignol, A., Filipowicz, W., 2005. TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. EMBO Rep. 6, 961–967. https://doi.org/10.1038/sj.embor.7400509

Halassa, M.M., Fellin, T., Haydon, P.G., 2007. The tripartite synapse: roles for gliotransmission in health and disease. Trends Mol. Med. 13, 54 63. https://doi.org/10.1016/j.molmed.2006.12.005

Hamanaka, Y., Meinertzhagen, I.A., 2010. Immunocytochemical localization of synaptic proteins to photoreceptor synapses of *Drosophila melanogaster*. J. Comp. Neurol. 518, 1133–1155. https://doi.org/10.1002/cne.22268

Han, J., 2004. The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev. 18, 3016–3027. https://doi.org/10.1101/gad.1262504

Hao, H., Allen, D.L., Hardin, P.E., 1997. A circadian enhancer mediates PER-dependent mRNA cycling in Drosophila melanogaster. Mol. Cell. Biol. 17, 3687–3693.

Haraguchi, T., Ozaki, Y., Iba, H., 2009. Vectors expressing efficient RNA decoys achieve the long-term suppression of specific microRNA activity in mammalian cells. Nucleic Acids Res. 37, e43–e43. https://doi.org/10.1093/nar/gkp040

Hardin, P.E., Hall, J.C., Rosbash, M., 1990. Feedback of the Drosophila period gene product on circadian cycling of its messenger RNA levels. Nature 343, 536–540. https://doi.org/10.1038/343536a0

Hassinger, T.D., Atkinson, P.B., Strecker, G.J., Whalen, L.R., Dudek, F.E., Kossel, A.H., Kater, S.B., 1995. Evidence for glutamate-mediated activation of hippocampal neurons by glial calcium waves. J. Neurobiol. 28, 159–170. https://doi.org/10.1002/neu.480280204

Hassinger, T.D., Guthrie, P.B., Atkinson, P.B., Bennett, M.V., Kater, S.B., 1996. An extracellular signaling component in propagation of astrocytic calcium waves. Proc. Natl. Acad. Sci. U. S. A. 93, 13268–13273.

Heiman, M., Schaefer, A., Gong, S., Peterson, J.D., Day, M., Ramsey, K.E., Suarez-Farinas, M., Schwarz, C., Stephan, D.A., Surmeier, D.J., Greengard, P., Heintz, N., 2008. A translational profiling approach for the molecular characterization of CNS cell types. Cell 135, 738–748. https://doi.org/10.1016/j.cell.2008.10.028

Helfrich-Forster, C., 1995. The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of Drosophila melanogaster. Proc. Natl. Acad. Sci. 92, 612–616. https://doi.org/10.1073/pnas.92.2.612

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

Hendricks, J.C., Finn, S.M., Panckeri, K.A., Chavkin, J., Williams, J.A., Sehgal, A., Pack, A.I., 2000. Rest in Drosophila Is a Sleep-like State. Neuron 25, 129–138. https://doi.org/10.1016/S0896-6273(00)80877-6

Henneberger, C., Papouin, T., Oliet, S.H.R., Rusakov, D.A., 2010. Long-term potentiation depends on release of d-serine from astrocytes. Nature 463, 232–236. https://doi.org/10.1038/nature08673

Hilgers, V., Bushati, N., Cohen, S.M., 2010. Drosophila microRNAs 263a/b Confer Robustness during Development by Protecting Nascent Sense Organs from Apoptosis. PLoS Biol. 8, e1000396. https://doi.org/10.1371/journal.pbio.1000396

Huang, A.M., Rubin, G.M., 2000. A misexpression screen identifies genes that can modulate RAS1 pathway signaling in Drosophila melanogaster. Genetics 156, 1219–1230.

Huang, D.W., Sherman, B.T., Lempicki, R.A., 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57. https://doi.org/10.1038/nprot.2008.211

Huang, Y., Ainsley, J.A., Reijmers, L.G., Jackson, F.R., 2013. Translational Profiling of Clock Cells Reveals Circadianly Synchronized Protein Synthesis. PLoS Biol. 11, e1001703. https://doi.org/10.1371/journal.pbio.1001703

Huang, Y., Ng, F.S., Jackson, F.R., 2015. Comparison of Larval and Adult Drosophila Astrocytes Reveals Stage-Specific Gene Expression Profiles. G3 5, 551. https://doi.org/10.1534/g3.114.016162

Hughes, M.E., Grant, G.R., Paquin, C., Qian, J., Nitabach, M.N., 2012. Deep sequencing the circadian and diurnal transcriptome of Drosophila brain. Genome Res. 22, 1266–1281. https://doi.org/10.1101/gr.128876.111

Hughes, M.E., Hogenesch, J.B., Kornacker, K., 2010. JTK_CYCLE: an efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets. J. Biol. Rhythms 25, 372–380. https://doi.org/10.1177/0748730410379711

Hunter-Ensor, M., Ousley, A., Sehgal, A., 1996. Regulation of the Drosophila protein timeless suggests a mechanism for resetting the circadian clock by light. Cell 84, 677–685.
Huntwork, S., Littleton, J.T., 2007. A complexin fusion clamp regulates spontaneous neurotransmitter release and synaptic growth. Nat. Neurosci. 10, 1235–1237. https://doi.org/10.1038/nn1980
Hutvagner, G., McLachlan, J., Pasquinelli, A.E., Balint, E., Tuschl, T., Zamore, P.D., 2001. A Cellular Function for the RNA-Interference Enzyme Dicer in the Maturation of the let-7 Small Temporal RNA. Science 293, 834–838. https://doi.org/10.1126/science.1062961
Ito, C., Tomioka, K., 2016. Heterogeneity of the Peripheral Circadian Systems in Drosophila melanogaster: A Review. Front. Physiol. 7. https://doi.org/10.3389/fphys.2016.00008
Kadener, S., Menet, J., Sugino, K., Horwich, M., Weissbein, U., Nawathean, P., Vagin, V., Zamore, P., Nelson, S., Rosbash, M., 2009. A role for microRNAs in the Drosophila circadian clock. Genes Dev. 23, 2179–2191. https://doi.org/10.1101/gad.1819509
Kadener, S., Stoleru, D., McDonald, M., Nawathean, P., Rosbash, M., 2007. Clockwork Orange is a transcriptional repressor and a new Drosophila circadian pacemaker component. Genes Dev. 21, 1675–1686. https://doi.org/10.1101/gad.1552607
Kaneko, H., Head, L.M., Ling, J., Tang, X., Liu, Y., Hardin, P.E., Emery, P., Hamada, F.N., 2012. Circadian Rhythm of Temperature Preference and Its Neural Control in Drosophila. Curr. Biol. 22, 1851–1857. https://doi.org/10.1016/j.cub.2012.08.006
Kaneko, M., Hall, J.C., 2000. Neuroanatomy of cells expressing clock genes in Drosophila: transgenic manipulation of the period and timeless genes to mark the perikarya of circadian pacemaker neurons and their projections. J. Comp. Neurol. 422, 66–94.
Kaneko, M., Helfrich-Förster, C., Hall, J.C., 1997. Spatial and temporal expression of the period and timeless genes in the developing nervous system of Drosophila: newly identified pacemaker candidates and novel features of clock gene product cycling. J. Neurosci. Off. J. Soc. Neurosci. 17, 6745–6760.
Kang, J., Jiang, L., Goldman, S.A., Nedergaard, M., 1998. Astrocyte-mediated potentiation of inhibitory synaptic transmission. Nat. Neurosci. 1, 683–692. https://doi.org/10.1038/3684
Karlsson, B., Knutsson, A., Lindahl, B., 2001. Is there an association between shift work and having a metabolic syndrome? Results from a population based study of 27 485 people. Occup. Environ. Med. 58, 747–752. https://doi.org/10.1136/oem.58.11.747
Kawamata, T., Tomari, Y., 2010. Making RISC. Trends Biochem. Sci. 35, 368–376. https://doi.org/10.1016/j.tibs.2010.03.009
Ketting, R.F., Fischer, S.E.J., Bernstein, E., Sijen, T., Hannon, G.J., Plasterk, R.H.A., 2001. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev. 15, 2654–2659. https://doi.org/10.1101/gad.927801

Klose, M., Duvall, L.B., Li, W., Liang, X., Ren, C., Steinbach, J.H., Taghert, P.H., 2016. Functional PDF Signaling in the Drosophila Circadian Neural Circuit Is Gated by Ral A-Dependent Modulation. Neuron 90, 781–794. https://doi.org/10.1016/j.neuron.2016.04.002

Kloss, B., Price, J.L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C.S., Young, M.W., 1998. The Drosophila clock gene double-time encodes a protein closely related to human casein kinase Iepsilon. Cell 94, 97–107.

Kloss, B., Rothenfluh, A., Young, M.W., Saez, L., 2001. Phosphorylation of period is influenced by cycling physical associations of double-time, period, and timeless in the Drosophila clock. Neuron 30, 699–706.

Knight, S.W., Bass, B.L., 2001. A Role for the RNase III Enzyme DCR-1 in RNA Interference and Germ Line Development in Caenorhabditis elegans. Science 293, 2269–2271. https://doi.org/10.1126/science.1062039

Koh, K., Zheng, X., Sehgal, A., 2006. JETLAG Resets the Drosophila Circadian Clock by Promoting Light-Induced Degradation of TIMELESS. Science 312, 1809–1812. https://doi.org/10.1126/science.1124951

Kohsaka, A., Laposky, A.D., Ramsey, K.M., Estrada, C., Joshu, C., Kobayashi, Y., Turek, F.W., Bass, J., 2007. High-Fat Diet Disrupts Behavioral and Molecular Circadian Rhythms in Mice. Cell Metab. 6, 414–421. https://doi.org/10.1016/j.cmet.2007.09.006

Kojima, S., Gatfield, D., Esau, C.C., Green, C.B., 2010. MicroRNA-122 modulates the rhythmic expression profile of the circadian deadenylase Nocturnin in mouse liver. PloS One 5, e11264. https://doi.org/10.1371/journal.pone.0011264

Konopka, R.J., Benzer, S., 1971. Clock mutants of Drosophila melanogaster. Proc. Natl. Acad. Sci. U. S. A. 68, 2112–2116.

Kozomara, A., Griffiths-Jones, S., 2014. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res. 42, D68–D73. https://doi.org/10.1093/nar/gkt1181

Kremer, M.C., Jung, C., Batelli, S., Rubin, G.M., Gaul, U., 2017. The glia of the adult *Drosophila* nervous system: Glia Anatomy in Adult Drosophila Nervous System. Glia 65, 606–638. https://doi.org/10.1002/glia.23115

Kula-Eversole, E., Nagoshi, E., Shang, Y., Rodriguez, J., Allada, R., Rosbash, M., 2010. Surprising gene expression patterns within and between PDF-containing circadian neurons in Drosophila. Proc. Natl. Acad. Sci. U. S. A. 107, 13497–13502. https://doi.org/10.1073/pnas.1002081107

Kume, K., Kume, S., Park, S.K., Hirsh, J., Jackson, F.R., 2005. Dopamine is a regulator of arousal in the fruit fly. J. Neurosci. Off. J. Soc. Neurosci. 25, 7377–7384. https://doi.org/10.1523/JNEUROSCI.2048-05.2005

Kunst, M., Hughes, M.E., Raccuglia, D., Felix, M., Li, M., Barnett, G., Duah, J., Nitabach, M.N., 2014. Calcitonin Gene-Related Peptide Neurons Mediate Sleep-Specific Circadian Output in Drosophila. Curr. Biol. 24, 2652–2664. https://doi.org/10.1016/j.cub.2014.09.077

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., Tuschl, T., 2001. Identification of Novel Genes Coding for Small Expressed RNAs. Science 294, 853–858. https://doi.org/10.1126/science.1064921

Lai, S.-L., Lee, T., 2006. Genetic mosaic with dual binary transcriptional systems in Drosophila. Nat. Neurosci. 9, 703–709. https://doi.org/10.1038/nn1681
Lamia, K.A., Storch, K.-F., Weitz, C.J., 2008. Physiological significance of a peripheral tissue circadian clock. Proc. Natl. Acad. Sci. U. S. A. 105, 15172–15177. https://doi.org/10.1073/pnas.0806717105
Landthaler, M., Yalcin, A., Tuschl, T., 2004. The Human DiGeorge Syndrome Critical Region Gene 8 and Its D. melanogaster Homolog Are Required for miRNA Biogenesis. Curr. Biol. 14, 2162–2167. https://doi.org/10.1016/j.cub.2004.11.001
Lau, N.C., Lim, L.P., Weinstein, E.G., Bartel, D.P., 2001. An Abundant Class of Tiny RNAs with Probable Regulatory Roles in Caenorhabditis elegans. Science 294, 858–862. https://doi.org/10.1126/science.1065062
Lee, C., Bae, K., Edery, I., 1998. The Drosophila CLOCK protein undergoes daily rhythms in abundance, phosphorylation, and interactions with the PER-TIM complex. Neuron 21, 857–867.
Lee, C., Parikh, V., Itsukaichi, T., Bae, K., Edery, I., 1996. Resetting the Drosophila clock by photic regulation of PER and a PER-TIM complex. Science 271, 1740–1744.
Lee, R.C., Ambros, V., 2001. An Extensive Class of Small RNAs in Caenorhabditis elegans. Science 294, 862–864. https://doi.org/10.1126/science.1065329
Lee, R.C., Feinbaum, R.L., Ambros, V., 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75, 843–854. https://doi.org/10.1016/0092-8674(93)90529-Y
Lee, T., Luo, L., 1999. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron 22, 451–461.
Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Rådmark, O., Kim, S., Kim, V.N., 2003. The nuclear RNase III Drosha initiates microRNA processing. Nature 425, 415–419. https://doi.org/10.1038/nature01957
Lee, Y., Hur, I., Park, S.-Y., Kim, Y.-K., Suh, M.R., Kim, V.N., 2006. The role of PACT in the RNA silencing pathway. EMBO J. 25, 522–532. https://doi.org/10.1038/sj.emboj.7600942
Lee, Y., Jeon, K.J., Lee, J.T., Kim, S., Kim, N., 2002. MicroRNA maturation: stepwise processing and subcellular localization. EMBO J. 21, 4663–4670. https://doi.org/10.1093/emboj/cdf476
Lee, Y., Kim, M., Han, J., Yeom, K.-H., Lee, S., Baek, S.H., Kim, V.N., 2004. MicroRNA genes are transcribed by RNA polymerase II. EMBO J. 23, 4051–4060. https://doi.org/10.1038/sj.emboj.7600385
Levine, J.D., Funes, P., Dowse, H.B., Hall, J.C., 2002. Signal analysis of behavioral and molecular cycles. BMC Neurosci. 3, 1.
Lewy, A.J., Wehr, T.A., Goodwin, F.K., Newsome, D.A., Markey, S.P., 1980. Light suppresses melatonin secretion in humans. Science 210, 1267–1269.
Leybaert, L., 2005. Neurobarrier Coupling in the Brain: A Partner of Neurovascular and Neurometabolic Coupling? J. Cereb. Blood Flow Metab. 25, 2–16. https://doi.org/10.1038/sj.jcbfm.9600001
Lim, C., Chung, B.Y., Pitman, J.L., McGill, J.J., Pradhan, S., Lee, J., Keegan, K.P., Choe, J., Allada, R., 2007. Clockwork orange encodes a transcriptional repressor

important for circadian-clock amplitude in Drosophila. Curr. Biol. CB 17, 1082–1089. https://doi.org/10.1016/j.cub.2007.05.039
Lin, J.-M., Kilman, V.L., Keegan, K., Paddock, B., Emery-Le, M., Rosbash, M., Allada, R., 2002. A role for casein kinase 2α in the Drosophila circadian clock. Nature 420, 816–820. https://doi.org/10.1038/nature01235
Lin, Y., Han, M., Shimada, B., Wang, L., Gibler, T.M., Amarakone, A., Awad, T.A., Stormo, G.D., Van Gelder, R.N., Taghert, P.H., 2002. Influence of the period-dependent circadian clock on diurnal, circadian, and aperiodic gene expression in Drosophila melanogaster. Proc. Natl. Acad. Sci. U. S. A. 99, 9562–9567. https://doi.org/10.1073/pnas.132269699
Lin, Y., Stormo, G.D., Taghert, P.H., 2004. The neuropeptide pigment-dispersing factor coordinates pacemaker interactions in the Drosophila circadian system. J. Neurosci. Off. J. Soc. Neurosci. 24, 7951–7957. https://doi.org/10.1523/JNEUROSCI.2370-04.2004
Liu, X., Zwiebel, L.J., Hinton, D., Benzer, S., Hall, J.C., Rosbash, M., 1992. The period gene encodes a predominantly nuclear protein in adult Drosophila. J. Neurosci. Off. J. Soc. Neurosci. 12, 2735–2744.
Loya, C.M., Lu, C.S., Van Vactor, D., Fulga, T.A., 2009. Transgenic microRNA inhibition with spatiotemporal specificity in intact organisms. Nat Meth 6, 897–903.
Lund, E., Güttinger, S., Calado, A., Dahlberg, J.E., Kutay, U., 2004. Nuclear Export of MicroRNA Precursors. Science 303, 95–98. https://doi.org/10.1126/science.1090599
Luo, W., Sehgal, A., 2012. Regulation of circadian behavioral output via a MicroRNA-JAK/STAT circuit. Cell 148, 765–779. https://doi.org/10.1016/j.cell.2011.12.024
Marpegan, L., Krall, T.J., Herzog, E.D., 2009. Vasoactive Intestinal Polypeptide Entrains Circadian Rhythms in Astrocytes. J. Biol. Rhythms 24, 135–143. https://doi.org/10.1177/0748730409332042
Martano, G., Gerosa, L., Prada, I., Garrone, G., Krogh, V., Verderio, C., Passafaro, M., 2017. Biosynthesis of Astrocytic Trehalose Regulates Neuronal Arborization in Hippocampal Neurons. ACS Chem. Neurosci. 8, 1865–1872. https://doi.org/10.1021/acschemneuro.7b00177
Martinek, S., Inonog, S., Manoukian, A.S., Young, M.W., 2001. A role for the segment polarity gene shaggy/GSK-3 in the Drosophila circadian clock. Cell 105, 769–779.
Matsumoto, A., Ukai-Tadenuma, M., Yamada, R.G., Houl, J., Uno, K.D., Kasukawa, T., Dauwalder, B., Itoh, T.Q., Takahashi, K., Ueda, R., Hardin, P.E., Tanimura, T., Ueda, H.R., 2007. A functional genomics strategy reveals clockwork orange as a transcriptional regulator in the Drosophila circadian clock. Genes Dev. 21, 1687–1700. https://doi.org/10.1101/gad.1552207
McDonald, M.J., Rosbash, M., 2001. Microarray analysis and organization of circadian gene expression in Drosophila. Cell 107, 567–578.
McGuire, S.E., Mao, Z., Davis, R.L., 2004. Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila. Sci. STKE Signal Transduct. Knowl. Environ. 2004, pl6. https://doi.org/10.1126/stke.2202004pl6

Meissner, R.-A., Kilman, V.L., Lin, J.-M., Allada, R., 2008. TIMELESS Is an Important Mediator of CK2 Effects on Circadian Clock Function In Vivo. J. Neurosci. 28, 9732–9740. https://doi.org/10.1523/JNEUROSCI.0840-08.2008

Miskiewicz, K., Jose, L.E., Yeshaw, W.M., Valadas, J.S., Swerts, J., Munck, S., Feiguin, F., Dermaut, B., Verstreken, P., 2014. HDAC6 Is a Bruchpilot Deacetylase that Facilitates Neurotransmitter Release. Cell Rep. 8, 94–102. https://doi.org/10.1016/j.celrep.2014.05.051

Miyazaki, M., Schroder, E., Edelmann, S.E., Hughes, M.E., Kornacker, K., Balke, C.W., Esser, K.A., 2011. Age-Associated Disruption of Molecular Clock Expression in Skeletal Muscle of the Spontaneously Hypertensive Rat. PLoS ONE 6, e27168. https://doi.org/10.1371/journal.pone.0027168

Mohawk, J.A., Green, C.B., Takahashi, J.S., 2012. Central and peripheral circadian clocks in mammals. Annu. Rev. Neurosci. 35, 445–462. https://doi.org/10.1146/annurev-neuro-060909-153128

Monyak, R.E., Emerson, D., Schoenfeld, B.P., Zheng, X., Chambers, D.B., Rosenfelt, C., Langer, S., Hinchey, P., Choi, C.H., McDonald, T.V., Bolduc, F.V., Sehgal, A., McBride, S.M.J., Jongens, T.A., 2016. Insulin signaling misregulation underlies circadian and cognitive deficits in a Drosophila fragile X model. Mol. Psychiatry. https://doi.org/10.1038/mp.2016.51

Murad, A., Emery-Le, M., Emery, P., 2007. A subset of dorsal neurons modulates circadian behavior and light responses in Drosophila. Neuron 53, 689–701. https://doi.org/10.1016/j.neuron.2007.01.034

Myers, M.P., Wager-Smith, K., Rothenfluh-Hilfiker, A., Young, M.W., 1996. Light-induced degradation of TIMELESS and entrainment of the Drosophila circadian clock. Science 271, 1736–1740.

Na, Y.-J., Sung, J.H., Lee, S.C., Lee, Y.-J., Choi, Y.J., Park, W.-Y., Shin, H.S., Kim, J.H., 2009. Comprehensive analysis of microRNA-mRNA co-expression in circadian rhythm. Exp. Mol. Med. 41, 638. https://doi.org/10.3858/emm.2009.41.9.070

Nagel, R., Clijsters, L., Agami, R., 2009. The miRNA-192/194 cluster regulates the Period gene family and the circadian clock: Regulation of the circadian clock by miR-192/194. FEBS J. 276, 5447–5455. https://doi.org/10.1111/j.1742-4658.2009.07229.x

Nagoshi, E., Sugino, K., Kula, E., Okazaki, E., Tachibana, T., Nelson, S., Rosbash, M., 2010. Dissecting differential gene expression within the circadian neuronal circuit of Drosophila. Nat. Neurosci. 13, 60–68. https://doi.org/10.1038/nn.2451

Nedergaard, M., 1994. Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. Science 263, 1768–1771.

Newman, E.A., Zahs, K.R., 1998. Modulation of neuronal activity by glial cells in the retina. J. Neurosci. Off. J. Soc. Neurosci. 18, 4022–4028.

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

Ng, F.S., Jackson, F.R., 2015. The ROP vesicle release factor is required in adult Drosophila glia for normal circadian behavior. Front. Cell. Neurosci. 9. https://doi.org/10.3389/fncel.2015.00256
Ng, F.S., Sengupta, S., Huang, Y., Yu, A.M., You, S., Roberts, M.A., Iyer, L.K., Yang, Y., Jackson, F.R., 2016. TRAP-seq Profiling and RNAi-Based Genetic Screens Identify Conserved Glial Genes Required for Adult Drosophila Behavior. Front. Mol. Neurosci. 9. https://doi.org/10.3389/fnmol.2016.00146
Nitabach, M., Wu, Y., Sheeba, V., Lemon, W., Strumbos, J., Zelensky, P., White, B., Holmes, T., 2006. Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J. Neurosci. Off. J. Soc. Neurosci. 26, 479–489. https://doi.org/10.1523/JNEUROSCI.3915-05.2006
Okamoto, N., Nishimura, T., 2015. Signaling from Glia and Cholinergic Neurons Controls Nutrient-Dependent Production of an Insulin-like Peptide for Drosophila Body Growth. Dev. Cell 35, 295–310. https://doi.org/10.1016/j.devcel.2015.10.003
Okuda, T., Kokame, K., Miyata, T., 2007. Differential Expression Patterns of NDRG Family Proteins in the Central Nervous System. J. Histochem. Cytochem. 56, 175–182. https://doi.org/10.1369/jhc.7A7323.2007
Paraskevopoulou, M.D., Georgakilas, G., Kostoulas, N., Vlachos, I.S., Vergoulis, T., Reczko, M., Filippidis, C., Dalamagas, T., Hatzigeorgiou, A.G., 2013. DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows. Nucleic Acids Res. 41, W169–W173. https://doi.org/10.1093/nar/gkt393
Parpura, V., Basarsky, T.A., Liu, F., Jeftinija, K., Jeftinija, S., Haydon, P.G., 1994. Glutamate-mediated astrocyte–neuron signalling. Nature 369, 744–747. https://doi.org/10.1038/369744a0
Parpura, V., Haydon, P.G., 2000. Physiological astrocytic calcium levels stimulate glutamate release to modulate adjacent neurons. Proc. Natl. Acad. Sci. U. S. A. 97, 8629–8634.
Pascual, O., Casper, K.B., Kubera, C., Zhang, J., Revilla-Sanchez, R., Sul, J.-Y., Takano, H., Moss, S.J., McCarthy, K., Haydon, P.G., 2005. Astrocytic Purinergic Signaling Coordinates Synaptic Networks. Science 310, 113 116. https://doi.org/10.1126/science.1116916
Peschel, N., Veleri, S., 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. 103, 17313–17318. https://doi.org/10.1073/pnas.0606675103
Picao-Osorio, J., Lago-Baldaia, I., Patraquim, P., Alonso, C.R., 2017. Pervasive Behavioral Effects of MicroRNA Regulation in Drosophila. Genetics 206, 1535–1548. https://doi.org/10.1534/genetics.116.195776
Piggins, H.D., Antle, M.C., Rusak, B., 1995. Neuropeptides phase shift the mammalian circadian pacemaker. J. Neurosci. Off. J. Soc. Neurosci. 15, 5612–5622.
Porter, J.T., McCarthy, K.D., 1997. Astrocytic neurotransmitter receptors in situ and in vivo. Prog. Neurobiol. 51, 439–455.

Porter, J.T., McCarthy, K.D., 1996. Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals. J. Neurosci. Off. J. Soc. Neurosci. 16, 5073–5081.
Potter, C.J., Tasic, B., Russler, E.V., Liang, L., Luo, L., 2010. The Q system: a repressible binary system for transgene expression, lineage tracing, and mosaic analysis. Cell 141, 536–548. https://doi.org/10.1016/j.cell.2010.02.025
Price, J.L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B., Young, M.W., 1998. double-time is a novel Drosophila clock gene that regulates PERIOD protein accumulation. Cell 94, 83–95.
Prolo, L.M., Takahashi, J.S., Herzog, E.D., 2005. Circadian rhythm generation and entrainment in astrocytes. J. Neurosci. 25, 404–408. https://doi.org/10.1523/JNEUROSCI.4133-04.2005
Prosser, R.A., Edgar, D.M., Craig Heller, H., Miller, J.D., 1994. A possible glial role in the mammalian circadian clock. Brain Res. 643, 296–301. https://doi.org/10.1016/0006-8993(94)90036-1
Reczko, M., Maragkakis, M., Alexiou, P., Grosse, I., Hatzigeorgiou, A.G., 2012. Functional microRNA targets in protein coding sequences. Bioinforma. Oxf. Engl. 28, 771–776. https://doi.org/10.1093/bioinformatics/bts043
Reed, H.E., Meyer-Spasche, A., Cutler, D.J., Coen, C.W., Piggins, H.D., 2001. Vasoactive intestinal polypeptide (VIP) phase-shifts the rat suprachiasmatic nucleus clock *in vitro*: VIP phase-shifts the rat SCN clock *in vitro*. Eur. J. Neurosci. 13, 839–843. https://doi.org/10.1046/j.0953-816x.2000.01437.x
Renn, S.C.P., Park, J.H., Rosbash, M., Hall, J.C., 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. https://doi.org/10.1016/S0092-8674(00)81676-1
Richier, B., Michard-Vanhée, C., Lamouroux, A., Papin, C., Rouyer, F., 2008. The clockwork orange Drosophila protein functions as both an activator and a repressor of clock gene expression. J. Biol. Rhythms 23, 103–116. https://doi.org/10.1177/0748730407313817
Robles, M.S., Humphrey, S.J., Mann, M., 2017. Phosphorylation Is a Central Mechanism for Circadian Control of Metabolism and Physiology. Cell Metab. 25, 118–127. https://doi.org/10.1016/j.cmet.2016.10.004
Roybal, K., Theobold, D., Graham, A., DiNieri, J.A., Russo, S.J., Krishnan, V., Chakravarty, S., Peevey, J., Oehrlein, N., Birnbaum, S., Vitaterna, M.H., Orsulak, P., Takahashi, J.S., Nestler, E.J., Carlezon, W.A., McClung, C.A., 2007. Mania-like behavior induced by disruption of CLOCK. Proc. Natl. Acad. Sci. U. S. A. 104, 6406–6411. https://doi.org/10.1073/pnas.0609625104
Ruben, M., Drapeau, M.D., Mizrak, D., Blau, J., 2012. A Mechanism for Circadian Control of Pacemaker Neuron Excitability. J. Biol. Rhythms 27, 353–364. https://doi.org/10.1177/0748730412455918
Ruby, J.G., Stark, A., Johnston, W.K., Kellis, M., Bartel, D.P., Lai, E.C., 2007. Evolution, biogenesis, expression, and target predictions of a substantially expanded set of Drosophila microRNAs. Genome Res. 17, 1850–1864. https://doi.org/10.1101/gr.6597907

Rutila, J.E., Suri, V., Le, M., So, W.V., Rosbash, M., 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.
Sack, R.L., Auckley, D., Auger, R.R., Carskadon, M.A., Wright, K.P., Vitiello, M.V., Zhdanova, I.V., American Academy of Sleep Medicine, 2007a. Circadian rhythm sleep disorders: part I, basic principles, shift work and jet lag disorders. An American Academy of Sleep Medicine review. Sleep 30, 1460–1483.
Sack, R.L., Auckley, D., Auger, R.R., Carskadon, M.A., Wright, K.P., Vitiello, M.V., Zhdanova, I.V., American Academy of Sleep Medicine, 2007b. Circadian rhythm sleep disorders: part II, advanced sleep phase disorder, delayed sleep phase disorder, free-running disorder, and irregular sleep-wake rhythm. An American Academy of Sleep Medicine review. Sleep 30, 1484–1501.
Saez, L., Young, M.W., 1996. Regulation of nuclear entry of the Drosophila clock proteins period and timeless. Neuron 17, 911–920.
Saito, K., Ishizuka, A., Siomi, H., Siomi, M.C., 2005. Processing of Pre-microRNAs by the Dicer-1–Loquacious Complex in Drosophila Cells. PLoS Biol. 3, e235. https://doi.org/10.1371/journal.pbio.0030235
Saudou, F., Boschert, U., Amlaiky, N., Plassat, J.L., Hen, R., 1992. A family of Drosophila serotonin receptors with distinct intracellular signalling properties and expression patterns. EMBO J. 11, 7–17.
Saus, E., Soria, V., Escaramis, G., Vivarelli, F., Crespo, J.M., Kagerbauer, B., Menchon, J.M., Urretavizcaya, M., Gratacos, M., Estivill, X., 2010. Genetic variants and abnormal processing of pre-miR-182, a circadian clock modulator, in major depression patients with late insomnia. Hum. Mol. Genet. 19, 4017–4025. https://doi.org/10.1093/hmg/ddq316
Schertel, C., Rutishauser, T., Förstemann, K., Basler, K., 2012. Functional characterization of Drosophila microRNAs by a novel in vivo library. Genetics 192, 1543–1552. https://doi.org/10.1534/genetics.112.145383
Schipke, C.G., Heuser, I., Peters, O., 2011. Antidepressants act on glial cells: SSRIs and serotonin elicit astrocyte calcium signaling in the mouse prefrontal cortex. J. Psychiatr. Res. 45, 242–248. https://doi.org/10.1016/j.jpsychires.2010.06.005
Schirmeier, S., Matzat, T., Klämbt, C., 2016. Axon ensheathment and metabolic supply by glial cells in Drosophila. Brain Res. 1641, 122–129. https://doi.org/10.1016/j.brainres.2015.09.003
Sehgal, A., Rothenfluh-Hilfiker, A., Hunter-Ensor, M., Chen, Y., Myers, M.P., Young, M.W., 1995. Rhythmic expression of timeless: a basis for promoting circadian cycles in period gene autoregulation. Science 270, 808–810.
Shafer, O.T., Helfrich-Förster, C., Renn, S.C.P., Taghert, P.H., 2006. Reevaluation of Drosophila melanogaster's neuronal circadian pacemakers reveals new neuronal classes. J. Comp. Neurol. 498, 180–193. https://doi.org/10.1002/cne.21021
Shafer, O.T., Yao, Z., 2014. Pigment-dispersing factor signaling and circadian rhythms in insect locomotor activity. Curr. Opin. Insect Sci. 1, 73–80. https://doi.org/10.1016/j.cois.2014.05.002

Shanahan, T.L., Czeisler, C.A., 1991. Light exposure induces equivalent phase shifts of the endogenous circadian rhythms of circulating plasma melatonin and core body temperature in men. J. Clin. Endocrinol. Metab. 73, 227–235. https://doi.org/10.1210/jcem-73-2-227

Shaw, P.J., Cirelli, C., Greenspan, R.J., Tononi, G., 2000. Correlates of Sleep and Waking in Drosophila melanogaster. Science 287, 1834–1837. https://doi.org/10.1126/science.287.5459.1834

Shende, V.R., Goldrick, M.M., Ramani, S., Earnest, D.J., 2011. Expression and Rhythmic Modulation of Circulating MicroRNAs Targeting the Clock Gene Bmal1 in Mice. PLoS ONE 6, e22586. https://doi.org/10.1371/journal.pone.0022586

Shi, F., Chen, X., Fu, A., Hansen, J., Stevens, R., Tjonneland, A., Vogel, U.B., Zheng, T., Zhu, Y., 2013. Aberrant DNA methylation of miR-219 promoter in long-term night shiftworkers: Shiftwork and miR-219. Environ. Mol. Mutagen. 54, 406–413. https://doi.org/10.1002/em.21790

Shi, L., Ko, M.L., Ko, G.Y.-P., 2009. Rhythmic Expression of MicroRNA-26a Regulates the L-type Voltage-gated Calcium Channel α1C Subunit in Chicken Cone Photoreceptors. J. Biol. Chem. 284, 25791–25803. https://doi.org/10.1074/jbc.M109.033993

Siwicki, K.K., Eastman, C., Petersen, G., Rosbash, M., 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.

Somjen, G.G., 1988. Nervenkitt: notes on the history of the concept of neuroglia. Glia 1, 2–9. https://doi.org/10.1002/glia.440010103

Spanagel, R., Pendyala, G., Abarca, C., Zghoul, T., Sanchis-Segura, C., Magnone, M.C., Lascorz, J., Depner, M., Holzberg, D., Soyka, M., Schreiber, S., Matsuda, F., Lathrop, M., Schumann, G., Albrecht, U., 2005. The clock gene Per2 influences the glutamatergic system and modulates alcohol consumption. Nat. Med. 11, 35–42. https://doi.org/10.1038/nm1163

Stephan, F.K., Zucker, I., 1972. Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. Proc. Natl. Acad. Sci. U. S. A. 69, 1583–1586.

Stoleru, D., Peng, Y., Agosto, J., Rosbash, M., 2004. Coupled oscillators control morning and evening locomotor behaviour of Drosophila. Nature 431, 862–868. https://doi.org/10.1038/nature02926

Stork, T., Bernardos, R., Freeman, M., 2012. Analysis of glial cell development and function in Drosophila. Cold Spring Harb. Protoc. 2012, 1–17. https://doi.org/10.1101/pdb.top067587

Stork, T., Sheehan, A., Tasdemir-Yilmaz, O.E., Freeman, M.R., 2014. Neuron-Glia Interactions through the Heartless FGF Receptor Signaling Pathway Mediate Morphogenesis of Drosophila Astrocytes. Neuron 83, 388–403. https://doi.org/10.1016/j.neuron.2014.06.026

Suh, J., Jackson, F.R., 2007. Drosophila Ebony Activity Is Required in Glia for the Circadian Regulation of Locomotor Activity. Neuron 55, 435–447. https://doi.org/10.1016/j.neuron.2007.06.038

Suh, Y.S., Bhat, S., Hong, S.-H., Shin, M., Bahk, S., Cho, K.S., Kim, S.-W., Lee, K.-S., Kim, Y.-J., Jones, W.D., Yu, K., 2015. Genome-wide microRNA screening reveals that the evolutionary conserved miR-9a regulates body growth by targeting sNPFR1/NPYR. Nat. Commun. 6, 7693. https://doi.org/10.1038/ncomms8693

Suzuki, A., Sinton, C.M., Greene, R.W., Yanagisawa, M., 2013. Behavioral and biochemical dissociation of arousal and homeostatic sleep need influenced by prior wakeful experience in mice. Proc. Natl. Acad. Sci. 110, 10288–10293. https://doi.org/10.1073/pnas.1308295110

Szabó, Á., Papin, C., Zorn, D., Ponien, P., Weber, F., Raabe, T., Rouyer, F., 2013. The CK2 Kinase Stabilizes CLOCK and Represses Its Activity in the Drosophila Circadian Oscillator. PLoS Biol. 11, e1001645. https://doi.org/10.1371/journal.pbio.1001645

Terazono, H., Mutoh, T., Yamaguchi, S., Kobayashi, M., Akiyama, M., Udo, R., Ohdo, S., Okamura, H., Shibata, S., 2003. Adrenergic regulation of clock gene expression in mouse liver. Proc. Natl. Acad. Sci. 100, 6795–6800. https://doi.org/10.1073/pnas.0936797100

Thimgan, M.S., Berg, J.S., Stuart, A.E., 2006. Comparative sequence analysis and tissue localization of members of the SLC6 family of transporters in adult Drosophila melanogaster. J. Exp. Biol. 209, 3383–3404. https://doi.org/10.1242/jeb.02328

Thomas, A., Lee, P.-J., Dalton, J.E., Nomie, K.J., Stoica, L., Costa-Mattioli, M., Chang, P., Nuzhdin, S., Arbeitman, M.N., Dierick, H.A., 2012. A Versatile Method for Cell-Specific Profiling of Translated mRNAs in Drosophila. PLoS ONE 7, e40276. https://doi.org/10.1371/journal.pone.0040276

Tso, C.F., Simon, T., Greenlaw, A.C., Puri, T., Mieda, M., Herzog, E.D., 2017. Astrocytes Regulate Daily Rhythms in the Suprachiasmatic Nucleus and Behavior. Curr. Biol. 27, 1055–1061. https://doi.org/10.1016/j.cub.2017.02.037

Turek, F.W., Joshu, C., Kohsaka, A., Lin, E., Ivanova, G., McDearmon, E., Laposky, A., Losee-Olson, S., Easton, A., Jensen, D.R., Eckel, R.H., Takahashi, J.S., Bass, J., 2005. Obesity and Metabolic Syndrome in Circadian Clock Mutant Mice. Science 308, 1043–1045. https://doi.org/10.1126/science.1108750

Ueda, H.R., Matsumoto, A., Kawamura, M., Iino, M., Tanimura, T., Hashimoto, S., 2002. Genome-wide transcriptional orchestration of circadian rhythms in Drosophila. J. Biol. Chem. 277, 14048–14052. https://doi.org/10.1074/jbc.C100765200

van Ooijen, G., Millar, A.J., 2012. Non-transcriptional oscillators in circadian timekeeping. Trends Biochem. Sci. 37, 484–492. https://doi.org/10.1016/j.tibs.2012.07.006

Verkhratsky, A., Butt, A., 2007. Glial Neurobiology. https://doi.org/10.1002/9780470517796

Vodala, S., Pescatore, S., Rodriguez, J., Buescher, M., Chen, Y.-W., Weng, R., Cohen, S.M., Rosbash, M., 2012. The Oscillating miRNA 959-964 Cluster Impacts Drosophila Feeding Time and Other Circadian Outputs. Cell Metab. 16, 601–612. https://doi.org/10.1016/j.cmet.2012.10.002

Volkenhoff, A., Weiler, A., Letzel, M., Stehling, M., Klämbt, C., Schirmeier, S., 2015. Glial Glycolysis Is Essential for Neuronal Survival in Drosophila. Cell Metab. 22, 437–447. https://doi.org/10.1016/j.cmet.2015.07.006

Vosshall, L.B., Price, J.L., Sehgal, A., Saez, L., Young, M.W., 1994. Block in nuclear localization of period protein by a second clock mutation, timeless. Science 263, 1606–1609.

Wang, J., Mauvoisin, D., Martin, E., Atger, F., Galindo, A.N., Dayon, L., Sizzano, F., Palini, A., Kussmann, M., Waridel, P., Quadroni, M., Dulić, V., Naef, F., Gachon, F., 2017. Nuclear Proteomics Uncovers Diurnal Regulatory Landscapes in Mouse Liver. Cell Metab. 25, 102–117. https://doi.org/10.1016/j.cmet.2016.10.003

Wang, X., Lou, N., Xu, Q., Tian, G.F., Peng, W.G., Han, X., Kang, J., Takano, T., Nedergaard, M., 2006. Astrocytic Ca2+ signaling evoked by sensory stimulation in vivo. Nat. Neurosci. 9, 816–823. https://doi.org/10.1038/nn1703

Wang, Z., Haydon, P.G., Yeung, E.S., 2000. Direct observation of calcium-independent intercellular ATP signaling in astrocytes. Anal. Chem. 72, 2001–2007.

Whitaker-Azmitia, P.M., Clarke, C., Azmitia, E.C., 1993. Localization of 5-HT1A receptors to astroglial cells in adult rats: Implications for neuronal-glial interactions and psychoactive drug mechanism of action. Synapse 14, 201–205. https://doi.org/10.1002/syn.890140303

Wightman, B., Ha, I., Ruvkun, G., 1993. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 75, 855–862. https://doi.org/10.1016/0092-8674(93)90530-4

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

Xu, K., DiAngelo, J.R., Hughes, M.E., Hogenesch, J.B., Sehgal, A., 2011. The Circadian Clock Interacts with Metabolic Physiology to Influence Reproductive Fitness. Cell Metab. 13, 639–654. https://doi.org/10.1016/j.cmet.2011.05.001

Xu, S., Witmer, P.D., Lumayag, S., Kovacs, B., Valle, D., 2007. MicroRNA (miRNA) Transcriptome of Mouse Retina and Identification of a Sensory Organ-specific miRNA Cluster. J. Biol. Chem. 282, 25053–25066. https://doi.org/10.1074/jbc.M700501200

Yang, M., Lee, J.-E., Padgett, R.W., Edery, I., 2008. Circadian regulation of a limited set of conserved microRNAs in Drosophila. BMC Genomics 9, 83. https://doi.org/10.1186/1471-2164-9-83

Yi, R., Qin, Y., Macara, I.G., Cullen, B.R., 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev. 17, 3011–3016. https://doi.org/10.1101/gad.1158803

Yuan, Q., Lin, F., Zheng, X., Sehgal, A., 2005. Serotonin Modulates Circadian Entrainment in Drosophila. Neuron 47, 115–127. https://doi.org/10.1016/j.neuron.2005.05.027

Zehring, W.A., Wheeler, D.A., Reddy, P., Konopka, R.J., Kyriacou, C.P., Rosbash, M., Hall, J.C., 1984. P-element transformation with period locus DNA restores

rhythmicity to mutant, arrhythmic Drosophila melanogaster. Cell 39, 369–376.
Zeng, H., Qian, Z., Myers, M.P., Rosbash, M., 1996. A light-entrainment mechanism for the Drosophila circadian clock. Nature 380, 129–135. https://doi.org/10.1038/380129a0
Zerr, D.M., Hall, J.C., Rosbash, M., Siwicki, K.K., 1990. Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of Drosophila. J. Neurosci. Off. J. Soc. Neurosci. 10, 2749–2762.
Zhang, Q., Pangrsic, T., Kreft, M., Krzan, M., Li, N., Sul, J.-Y., Halassa, M., Van Bockstaele, E., Zorec, R., Haydon, P.G., 2004. Fusion-related release of glutamate from astrocytes. J. Biol. Chem. 279, 12724–12733. https://doi.org/10.1074/jbc.M312845200
Zhang, Y., Emery, P., 2013. GW182 Controls Drosophila Circadian Behavior and PDF-Receptor Signaling. Neuron 78, 152–165. https://doi.org/10.1016/j.neuron.2013.01.035
Zhang, Y., Lamba, P., Guo, P., Emery, P., 2016. miR-124 Regulates the Phase of Drosophila Circadian Locomotor Behavior. J. Neurosci. 36, 2007–2013. https://doi.org/10.1523/JNEUROSCI.3286-15.2016