Regulation of Circadian Behavior by Astroglial MicroRNAs in *Drosophila*

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ABSTRACT We describe a genome-wide microRNA (miRNA)-based screen to identify brain glial cell functions required for circadian behavior. To identify glial miRNAs that regulate circadian rhythmicity, we employed a collection of “miR-sponges” to inhibit miRNA function in a glia-specific manner. Our initial screen identified 20 glial miRNAs that regulate circadian behavior. We 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 effect on glial or clock neuronal cell viability. To identify potential RNA targets of miR-263b and miR-274, we screened 35 predicted miRNA targets, employing RNA interference-based approaches. 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.

KEYWORDS glial biology; astrocyte; circadian behavior; microRNA

THERE is pervasive regulation of animal physiology by circadian clocks located in the brain and peripheral tissues. Disruption of such clocks is associated with sleep and mood disorders, impaired cognitive function, dysregulated metabolism, and other pathophysiological states (Wulff *et al.* 2010; Zhang and Kay 2010). Most circadian clocks, including the one in the brain of *Drosophila*, consist of transcriptional/translational feedback loops that drive rhythmic changes in gene transcription and/or protein abundance (Hardin and Panda 2013). Although much circadian research has focused on the contribution of neurons to the maintenance of rhythmic behavior, it is now clear that glial cells of *Drosophila* and mammals, particularly astrocytes, are important components of the circadian circuitry [reviewed in Jackson *et al.* (2015)].

Drosophila and mammalian astrocytes are morphologically and functionally similar, and they have conserved gene

expression profiles (Jackson *et al.* 2015; Ng *et al.* 2016). Of interest, mammalian astrocytes contain PERIOD (PER)-based molecular oscillators (Prolo *et al.* 2005) that can be entrained by vasoactive intestinal polypeptide, a neuropeptide crucial for coordination of circadian pacemaker cells within the suprachiasmatic nucleus (SCN) (Marpegan *et al.* 2009). Moreover, there is circadian release of ATP from astrocytes of the SCN, a region that serves as the mammalian circadian pacemaker (Womac *et al.* 2009; Marpegan *et al.* 2011). Importantly, recent studies have also demonstrated that both fly and mouse astrocytes contribute to the regulation of circadian rhythms in locomotor activity *in vivo* (Ng *et al.* 2011; Barca-Mayo *et al.* 2017; Brancaccio *et al.* 2017; Tso *et al.* 2017).

In *Drosophila*, Ebony—a circadian, glia-specific N-β-alanyl biogenic amine synthetase that regulates neurotransmitter recycling by glia (Hovemann *et al.* 1998; Edwards and Meieritzhagen 2010)—is required for rhythmic *Drosophila* locomotor activity (Suh and Jackson 2007). More recently, it was demonstrated that astrocyte-specific manipulations of *Drosophila* vesicle trafficking/secretion result in behavioral arrhythmicity (Ng *et al.* 2011, 2016; Ng and Jackson 2015). Conditional adult disruption of glial secretion also decreases neuronal Pigment Dispersing Factor (PDF), an important circadian transmitter, within projections of the small ventral

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lateral (s-LN_v) clock neurons (Ng *et al.* 2011). This result revealed a role for glia-neuron signaling in the fly circadian system.

Although glia–neuron communication is clearly important for rhythmicity, little is known about the relevant mechanisms. In this study, we have taken advantage of *Drosophila* strains expressing genetic inhibitors of microRNAs (miRNAs) (Loya *et al.* 2009; Fulga *et al.* 2015) to identify glial miRNAs that are required for normal rhythmic behavior. Similar genetic screens recently identified miRNAs that regulate muscle formation (Fulga *et al.* 2015) and learning/memory (Busto *et al.* 2015), but those studies did not examine the contribution of glia to phenotypes. As each miRNA regulates many target RNAs, this strategy represents an efficient way to identify glial factors important for rhythmicity. miRNAs have been implicated in regulation of the molecular circadian clock or output pathways downstream of the clock (Cheng *et al.* 2007; Yang *et al.* 2008; Kadener *et al.* 2009; Shende *et al.* 2011; Luo and Sehgal 2012; Vodala *et al.* 2012; Chen *et al.* 2013; Zhang and Emery 2013; Chen and Rosbash 2016; Zhang *et al.* 2016); however, there are no studies, to our knowledge, of glial miRNAs that regulate rhythmic behavior.

By conducting a genome-wide screen of fly miRNAs, we discovered 20 glial miRNAs that regulate circadian behavior. Two of them, miR-263b and miR-274, were examined in detail and found to be required in astrocytes for normal rhythmic behavior. Importantly, we found no evidence of altered neural cell number or morphology with inhibition of these miRNAs, suggesting an adult physiological function. Indeed, we demonstrated that conditional inhibition of these miRNAs in adult astrocytes acutely alters rhythmic behavior. Finally, we predicted RNA targets of these two miRNAs, using *in silico* procedures, and performed genetic screens to determine which targets might be relevant for circadian behavior. Those screens identified two potential RNA targets for miR-274, one of which suggests a novel glia–neuron signaling mechanism that might regulate circadian behavior. Our study represents, to our knowledge, the first analysis of glial miRNAs in the context of behavior.

Materials and Methods

Fly strains and maintenance

Drosophila cultures were reared on standard cornmeal/sugar/wheat germ medium in an environmentally controlled incubator on a 12 hr:12 hr light/dark (LD) schedule. For the primary microRNA-sponge (miR-SP) screen, experimental flies were generated by crossing virgin female repo-Gal4 (w; Sp/SM1; repo-Gal4) with male 2× UAS-miR-SP (w¹¹¹⁸; UAS-miR-SP; UAS-miR-SP) (Fulga *et al.* 2015). As controls, both the driver and UAS strains were crossed to w¹¹¹⁸. For all constitutive miR-SP expression experiments, crosses were reared at 25°. To temporally restrict expression of the miR-SP to astrocytes, tubGal80^{ts}, alm-Gal4 was used to conditionally activate the UAS-miR-SP. Temperatures of 23 and 30°,

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 w¹¹¹⁸ to obtain heterozygous flies without any chromosome balancers. All strains were obtained from the Bloomington Stock or Vienna *Drosophila* Resource Centers.

Collection of locomotor activity and data analysis

Flies (<1 week old) were placed in *Drosophila* activity monitors (DAMS; Trikinetics) 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°. For conditional expression experiments, flies were entrained at 23° and free running behavior was assessed at different temperatures depending on the experiment (see *Results*). Behavioral data were collected as beam breaks in 30 min bins and analyzed using the MATLAB-based signal processing fly toolbox (Levine *et al.* 2002). Rhythmicity was assessed using rhythmicity index (RI) values and visual examination of actograms to verify arrhythmicity. By this analysis, arrhythmic flies of the sponge-expressing genotypes had RIs of 0.1–0.2, whereas rhythmic flies had average RIs of >0.3. Flies from experimental or control groups that did not entrain during LD were excluded from the analysis. To assess statistical significance of circadian data, we determined normality using the D'Agostino and Pearson omnibus normality test. If the data did not pass a normality test, we 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 (GraphPad). 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. Each candidate from the primary screen was repeated with an independent biological replicate to confirm results from the primary screen.

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 (1× PBS, 0.5% Triton X-100) on ice for 30 min. Brains were hand-dissected and then fixed in 4% PFA for 20 min. Fixed brains were then washed with PBST three times, for 10 min each, followed by blocking in 5% normal goat serum (in PBST) for 3 hr. Brains were incubated with the primary antibody overnight at 4°, washed with PBST three times quickly and three times for 25 min each, and then incubated with the secondary antibody overnight at 4°. Brains were washed again three

times quickly and three times for 25 min each, before mounting with VectaShield (Vector Laboratories, Burlingame, CA) to preserve fluorescence. For the GABA transporter (GAT) staining, we followed the general procedure with the following exceptions: pre-dissection flies were fixed for 17 min, post-dissection flies were fixed for 7 min, and blocking was restricted to 1 hr. Additionally, primary antibody incubation occurred over 2 days in 4°. We used 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 from R. Stanewsky), and rabbit anti-GAT (1:500, a gift from M. Freeman). Relevant secondary antibodies were used at 1:500 or 1:1000, including Alexa Fluor 488 goat anti-rabbit or Alexa Fluor 488 goat anti-mouse (both from Invitrogen, Carlsbad, CA).

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. We 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.

Quantification of *MESK2* RNA abundance

RNA was extracted from samples containing 5–10 pupae (pharate adults) and converted to complementary DNA using SuperScript II reverse transcriptase (Invitrogen) and random hexamers. The primers employed for *MESK2* were CTAC CACGATTGGGCCTCAA (Forward) and CAGCAGACCTCG CATCACTG (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.

Data and reagent availability

The authors declare that all data necessary for confirming the conclusions presented in the article are fully represented within the article.

Results

Drosophila glial miRNAs are required for normal circadian behavior

To identify glial miRNAs that regulate circadian behavior, we performed a genome-wide, cell-specific screen using a collection of Gal4-regulated miRNA sponges. We employed

transgenic miRNA inhibitor strains (miRNA sponges or miR-SP) targeting 145 different miRNAs that have previously been used in studies of memory or muscle formation (Busto *et al.* 2015; Fulga *et al.* 2015). Each strain carries multiple Gal4-regulated UAS-miR-SP transgenes expressing a specific sponge (see *Materials and Methods*); sponges contain 20 copies of a sequence complementary to a specific miRNA with deliberate mismatches at nucleotides 9–12 (Fulga *et al.* 2015). Additionally, each miR-SP contains an mCherry tag so that miR-SP expression can be visualized (Supplemental Material, Figure S1 in [File S2](#)). As an miR-SP binds to and inhibits a single miRNA, the expectation is that translation will be upregulated for the relevant target RNAs.

Sponge-expressing flies were generated by crossing UAS-miR-SP strains to those carrying the glial-specific repo-Gal4 driver. Background controls arose from a cross of the UAS-miR-SP or repo-Gal4 strains to *w¹¹¹⁸* flies. 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. Importantly, control flies from a cross of repo-Gal4 to *w¹¹¹⁸* had normal rhythmicity (Table 1).

The DAM system was employed to monitor locomotor activity in DD (Figure 1, see *Materials and Methods*). Activity was analyzed and rhythmicity assessed using two measures: the 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). Although it might seem that these two measures are synonymous, that is not the case as flies can have weak rhythmicity (*i.e.*, a low RI) but still be statistically rhythmic. Values for RI and percent rhythmicity were normalized by comparison of each experimental cross to its control. Table S1 in [File S1](#) shows normalized rhythmicity and activity indices for flies expressing each of 146 different miR-SPs (targeting 145 miRs).

To identify candidate miRNAs, we used a cutoff of 30% decreased RI and percent rhythmicity relative to controls (Figure 1B, candidates in red). This cutoff corresponds to statistical significance at $P \leq 0.0002$ for the hits compared with background controls (Table 1). Distributions of normalized RI and percent rhythmicity values (see *Materials and Methods*) are shown for all tested miR-SPs in Figure 1, C and D. Altogether, we identified 20 miRNAs causing significantly reduced rhythmicity (Table 1), for 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 Figure 1E; this panel also includes reference data for two miR-SPs that did not affect rhythmicity (miR-33-SP, miR-315-SP) and data from analysis of a scrambled SP sequence that served as a control for sponge-based manipulation. We note that glial expression of the scrambled SP did result in a modest but significant decrease in RI compared with UAS control flies. However, all flies expressing the scrambled SP were statistically rhythmic. In addition, the average RI for scrambled SP-expressing populations

Table 1 Glial expression of each of 20 different miR-SPs decreases rhythmicity

Genotype	Experimental		Percent rhythmic	Control		Percent rhythmic	P value	Biological replicates
	RI $\pm$ SEM	n		RI $\pm$ SEM	n			
miR-79-SP	0.25 $\pm$ 0.03	18	50.00	0.47 $\pm$ 0.04	20	80.00	<0.0001	2
miR-79-SP (2)	0.25 $\pm$ 0.02	16	50.00	0.44 $\pm$ 0.03	26	84.62	0.0002	2
miR-210-SP	0.28 $\pm$ 0.02	25	44.00	0.51 $\pm$ 0.02	32	90.63	<0.0001	2
miR-263b-SP	0.24 $\pm$ 0.03	20	60.00	0.55 $\pm$ 0.02	31	96.77	<0.0001	2
miR-274-SP	0.31 $\pm$ 0.03	20	65.00	0.48 $\pm$ 0.02	25	96.00	<0.0001	2
miR-281-1-SP	0.30 $\pm$ 0.03	23	60.87	0.53 $\pm$ 0.03	30	96.67	<0.0001	2
miR-285-SP	0.32 $\pm$ 0.02	21	52.38	0.49 $\pm$ 0.03	27	92.59	<0.0001	2
miR-304-SP	0.28 $\pm$ 0.02	31	51.61	0.50 $\pm$ 0.02	30	96.67	<0.0001	2
miR-305-SP	0.27 $\pm$ 0.02	44	54.41	0.51 $\pm$ 0.02	44	90.00	<0.0001	4
miR-309-SP	0.28 $\pm$ 0.02	35	57.14	0.51 $\pm$ 0.02	44	97.73	<0.0001	2
miR-310-SP	0.26 $\pm$ 0.03	34	57.14	0.50 $\pm$ 0.02	39	94.74	<0.0001	4
miR-317-SP	0.29 $\pm$ 0.03	22	57.14	0.51 $\pm$ 0.02	37	100.00	<0.0001	4
miR-927-SP	0.27 $\pm$ 0.03	28	53.57	0.51 $\pm$ 0.02	36	97.22	<0.0001	2
miR-963-SP	0.28 $\pm$ 0.03	26	53.85	0.49 $\pm$ 0.02	30	96.67	<0.0001	2
miR-967-SP	0.25 $\pm$ 0.03	25	56.00	0.45 $\pm$ 0.02	45	93.33	<0.0001	3
miR-971-SP	0.28 $\pm$ 0.04	23	60.87	0.51 $\pm$ 0.02	31	100.00	<0.0001	2
miR-981-SP	0.32 $\pm$ 0.02	41	67.39	0.50 $\pm$ 0.02	41	95.65	<0.0001	3
miR-990-SP	0.28 $\pm$ 0.04	13	60.00	0.47 $\pm$ 0.02	29	92.50	<0.0001	3
miR-992-SP	0.34 $\pm$ 0.02	67	67.16	0.52 $\pm$ 0.01	75	96.00	<0.0001	4
miR-994-SP	0.24 $\pm$ 0.02	28	32.14	0.45 $\pm$ 0.02	30	100.00	<0.0001	2
miR-iab-4-3p-SP	0.31 $\pm$ 0.03	26	65.38	0.45 $\pm$ 0.02	30	96.67	0.0002	2
miR-33-SP	0.39 $\pm$ 0.04	14	92.86	0.40 $\pm$ 0.03	13	92.31	NS	1
miR-315-SP	0.49 $\pm$ 0.03	14	100.00	0.52 $\pm$ 0.02	16	100.00	NS	1
Scramble-SP	0.49 $\pm$ 0.02	45	97.78	0.54 $\pm$ 0.02	32	100.00	0.0009	2
repoGal4 ^a				0.52 $\pm$ 0.03	14	100.00	NA	

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.

^arepoGal4 was included as a control in every behavioral experiment. A representative result is shown.

was equal to or greater than that observed for several other UAS-miR-SP control populations. Thus, the behavioral phenotypes associated with the 20 miR-SP “hits” do not result from nonspecific miR-SP expression in glial cells. Two miR-SPs (263b and 274) with robust effects on rhythmicity were chosen for further analysis.

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). We found that inhibition of miR-263b specifically in glial cells significantly decreased rhythmicity compared with both the UAS-miR-SP control and the repo-Gal4 control (Kruskal–Wallis test; *P* < 0.0001; Figure 2, A and B). In unpublished studies, we previously found that a second candidate, miR-274, cycles in pharate adult flies (data not shown), which may be indicative of a role in circadian behavior. Similar to results with miR-263b, glial inhibition of miR-274 resulted in significantly decreased rhythmicity compared with UAS and Gal4 controls (Kruskal–Wallis test; *P* < 0.0001; Figure 2A).

As miR-SP inhibition of miRNA function is predicted to result in increased expression of target RNAs, we 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). miRNA 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), we 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; Figure 2C). 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), precluding behavioral analysis (but see Figure S5 in File S2 for adult-specific overexpression).

We also asked if the observed behavioral phenotype was a glia-specific effect. To answer this question, we 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 with the corresponding UAS-miR-SP control (Figure S2 in File S2). These results indicate a glial-specific function for these miRNAs in the context of circadian behavior.

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

To assess the specificity of the miR-263b- and miR-274-SPs, we utilized recently described knockout strains for both

Figure 1 miR-SP screen and results. (A) Schematic of an 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 20 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 *w¹¹¹⁸* were crossed to the UAS-miR-SP strain (experimental and control groups, respectively). Flies were entrained for 4 days to LD 12 hr:12 hr, then transferred to DD for 10 days. For each experimental and control group, $n \geq 8$. (B) Normalized percent rhythmicity vs. 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 1 for full list). Results are mean RI $\pm$ SEM ($n = 13$ –44; two-tailed Student's *t*-test, $P < 0.05$ for all comparisons of experimental to control populations).

miRNAs (Chen *et al.* 2014). For either miRNA knockout, mean RI was significantly decreased compared with controls (Student's *t*-test; $P < 0.0001$; Figure 2D), and the distributions of RI values were visually different in knockouts vs. controls. Percent rhythmicity was decreased only slightly for the mutants (for miR-263b, 82% for knockout vs. 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.

Figure 2 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 with UAS-miR-SP and repoGal4 controls. RI value (left) results shown are individual flies with mean $\pm$ 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. (C) Glial overexpression of miR-263b decreased rhythmicity relative to the UAS-miR-263b control. Results are individual flies with mean $\pm$ SEM, $n = 28$ –46 (two biological replicates), two-tailed Student’s t -test, **** $P < 0.0001$. (D) Both miR-263b and miR-274 knockout flies had decreased robustness of rhythmicity when compared with heterozygous controls. Controls were generated by crossing mutant strains to w^{1118} flies. Results are individual flies with mean $\pm$ SEM, $n = 50$ –69, two-tailed Student’s t -test, **** $P < 0.0001$.

We also performed experiments using mutant strains outcrossed for four generations and obtained similar results, albeit a weaker effect for the miR-263b knockout (Figure S2C in File S2). Thus, phenotypes for an miRNA knockout are similar to those observed with miR-SP expression, indicative of specificity.

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

Published results indicate that miR-263b or miR-274 knockout has no effect on survival to adulthood (Chen *et al.* 2014), indicative of normal development. Furthermore, flies with pan-glial expression of the miR-274-SP or miR-263b-SP had

seemingly normal health and viability, and they survived for the course of an activity experiment (at least several weeks) in a manner similar to controls. As we previously showed that severe perturbations of glial cells result in lethality within a few days (Ng *et al.* 2011), we do not think glial cell physiology or overall health is severely affected in sponge-expressing animals. However, we wondered whether the behavioral phenotypes resulted from alterations of clock cells, as miRNAs often function in the context of development. To examine development of the entire circadian circuit, we stained for the core clock protein PERIOD (PER) at four time points (CT0, CT6, CT12, and CT18). As shown in Figure 3A, repo-Gal4 > UAS-miR-274-SP (experimental) and UAS-miR-274-SP (control) brains exhibited similar PER immunoreactivity for the ventral lateral neurons (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 (Figure 3A; $n = 6$ –8 hemispheres). At CT12 and CT18, we observed low or absent immunosignal in both experimental and control genotypes (Figure 3A), and did not see detectable signal in all PER-containing cells. Therefore, we were 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 (Figure S3A in File S2).

We next examined PDF, as it is the principal circadian transmitter in *Drosophila*, and its rhythmic secretion from the LN_vs is important for maintenance of rhythmicity (e.g., Shafer and Yao 2014; Klose *et al.* 2016). However, comparison of PDF staining between experimental and control brains did not reveal any obvious differences in peptide immunoreactivity or s-LN_v projection morphology (Figure 3B, $n = 3$). Given these results, it is unlikely that the observed decreased rhythmicity is a result of altered clock cell development or molecular clock function.

We also asked whether glial expression of these miR-SPs altered glial cell development. To assess this, we examined glia of experimental and control brains using a REPO antibody, which stains all glia of the adult brain. We detected no gross differences between brain types. In addition, we 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; $P = 0.636$, Figure 3C; $P = 0.109$, Figure S3C in File S2). In addition, we examined synaptic neuropil regions, which contain astrocytic processes, of miR-263b-SP- and miR-274-SP-expressing brains. To accomplish that, we performed antibody staining against GAT, which is specific for astrocytes (Stork *et al.* 2014). For each brain, we 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; $P = 0.754$, Figure 3D; $P = 0.908$, Figure S3D in File S2). From these experiments we 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.

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

In previous studies, we found that the astrocyte class of glial cells is important for regulation of the circadian circuit (Ng *et al.* 2011). Given that finding, we wished to determine if any of our candidate miRNAs were required in astrocytes for circadian behavior. Using astrocyte-specific drivers, we identified a number of miR-SPs that resulted in significantly decreased rhythmicity ($\geq 30\%$) as assessed by RI value (data not shown). 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 determine whether miR-263b or miR-274 was required in mature astrocytes, we 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 temperatures but allow activity at high temperatures. In our studies, we utilized the alm-Gal4 astrocyte driver (Doherty *et al.* 2009). In a first set of experiments, tubGal80^{ts}, alm-Gal4 > 2 × UAS-miR-263b-SP (experimental), and UAS-miR-263b-SP (control) flies were reared at 23° to inhibit Gal4 expression. The behavior of experimental and control populations were then assessed at 23° to determine if Gal4 activity was sufficiently inhibited. We found no significant difference between experimental and control groups (left bars, Student's *t*-test; $P = 0.215$; Figure 4A and Table S2 in File S1). We next assessed rhythmicity in both groups when flies were reared and entrained to LD at 23°, but then monitored in DD at 30°. In those conditions, there was significantly decreased rhythmicity in the miR-263b-SP-expressing flies compared with UAS-miR-263b-SP controls (middle bars, Student's *t*-test; $P < 0.0001$; Figure 4A and Table S2 in File S1). Finally, we performed the reverse experiment, rearing flies at 30° and then monitoring activity at 23°, 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°, 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; $P = 0.132$; Figure 4A and Table S2 in File S1). We performed an identical set of studies using the miR-274-SP strain, with similar results (Figure 4, B and C and Table S2 in File S1). We 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.

Figure 3 Glial manipulation of miR-274 does not alter glial cell number or clock neuronal morphology. (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 two 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 $\pm$ 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. 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 $\pm$ 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 $\pm$ SEM; $n = 5$ brains for each group (two-tailed Student's t -test, $P = 0.755$). All images were acquired with a 40 $\times$ objective with 2 μ m optical z-steps. Bar, 50 μ m.

To ensure that these acute manipulations did not result in glial cell death, we counted the number of REPO-positive cells in the optic lobe of flies kept at 30 $^{\circ}$ (experimental) and 23 $^{\circ}$ (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; $P = 0.154$; Figure S4A in File S2) or miR-274-SP (Student's t -test; $P = 0.546$; Figure S4B in File S2). These results strongly support the idea

that miR-263b and miR-274 have physiological functions relevant for rhythmicity in adult astrocytes.

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

Since glial overexpression of miR-274 throughout development resulted in lethality, we utilized the aforementioned TARGET system to manipulate astrocyte miR-274 abundance in adult

Figure 4 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 *alm-Gal4, tub-Gal80^{ts} > miR-SP* (TA) flies. In one set of experiments (23 > 30°, third and fourth 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° during DD to effect miR-SP expression. In an independent set of experiments (30 > 23°, fifth and sixth 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, a third set of experiments was performed with crosses reared and kept at 23° to inhibit miR-SP expression during development and the behavior run (23°, first and second columns). Results are individual flies with mean $\pm$ SEM, two-tailed Student's *t*-test, **** $P < 0.0001$. (C) Representative actograms for each set of experiments summarized in (B). See Figure S4C in File S2 for representative actograms of experiments depicted in (A).

flies. Flies were reared and entrained at 23° before transfer to 30° for assessment of behavior in DD conditions. Adult, astrocyte-specific overexpression of miR-274 resulted in significantly decreased rhythmicity compared with the UAS control (Student's *t*-test; $P < 0.01$; Figure S5 in File S2). Thus, overexpression of either miR-263b (Figure 2C) or miR-274 results

in arrhythmicity, and this predicts that a knockdown of a target RNA will also result in arrhythmic behavior.

Identification of putative miRNA targets

To search for RNA targets of miR-263b and miR-274, we first utilized target prediction algorithms (TargetScan-FLY and

DIANA microT-CDS) (Ruby *et al.* 2007; Reczko *et al.* 2012; Paraskevopoulou *et al.* 2013). For both miRNAs, we compiled a list of potential targets from the combined predictions of both programs. We then compared that list to one derived from our earlier cell type-specific profiling studies that defined transcripts with enriched expression in astrocytes [1.5-fold enriched expression relative to total RNA from whole heads (Huang *et al.* 2015)]. In this manner, we identified astrocyte-expressed RNAs that were predicted to be targets of either miRNA. We note that enriched expression does not mean high-level astrocyte expression; indeed, expression of a putative target might be quite low in astrocytes, but higher than that observed in total RNA.

Based on our results with miRNA overexpression, we predicted that decreased RNA target abundance would perturb rhythmicity. Therefore, we performed RNA interference (RNAi)-based screens to identify predicted astrocyte target RNAs that function in circadian behavior. Altogether, we examined 12 and 20 putative targets for miR-263b and miR-274, respectively, and one putative target of both miRNAs (Table S3 in File S1). For these screens, we utilized existing collections of UAS-RNAi strains that represent nearly all genes of the fly genome. When possible, we screened each of our putative targets using multiple RNAi lines. Similar to the miR-SP screen, we crossed each UAS-RNAi strain to *w¹¹¹⁸* 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 S3 in File S1). Similarly, most flies expressing RNAi targeting predicted miR-274 gene targets had normal rhythms (Table S3 in File S1). 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; $P < 0.0001$; Figure 5, A and B). Similarly, glial knockdown of MESK2 using either of two different RNAi transgenes also decreased rhythmicity (Student's *t*-test; $P < 0.0001$; Figure 5, C and D). MESK2, known to be involved with Ras/ERK signaling, has homology with mammalian NDRG2, a gene with astrocyte-specific expression (see Discussion).

We examined MESK2 RNA in flies overexpressing miR-274 to determine if transcript levels were decreased by miRNA expression. We used a pan-glial driver (repo-Gal4) to overexpress miR-274 in all glial cells. As this genotype is lethal before eclosion, we collected RNA from pupae (pharate adults) from the experimental cross and from UAS controls. Although there was a trend toward decreased MESK2 transcript levels for miR-274-expressing populations, suggesting altered RNA abundance; this effect was not statistically significant ($n = 4$ –7, Figure S5B in File S2).

Discussion

We report a screen for miRNAs involved in the glial regulation of circadian behavior. To our knowledge, there have been no published genome-wide screens to assess the function of individual miRNAs in regulating circadian behavior. Of interest, however, Goodwin *et al.* recently completed a similar screen that identified multiple miRNAs required for fly sleep (P. R. Goodwin and L. C. Griffith, personal communication). In addition, a recent study identified miRNAs that regulate *Drosophila* larval behavior (Picao-Osorio *et al.* 2017). The use of genetically encoded miRNA inhibitors (Fulga *et al.* 2015) allowed us to test 145 miRNAs, of the 256 listed in MirBase, for loss-of-function circadian phenotypes in glial cells. Our primary screen identified 20 candidate miRNAs with circadian functions in glia (Figure 1). Although there is little information about most of these candidate miRNAs, a few of them were previously identified in other circadian studies. For example, miR-210, identified in our screen, was significantly increased in abundance in *cyc⁰¹* mutants compared with control flies (Yang *et al.* 2008). The same study also identified miR-263b as an oscillating miRNA under clock control. miR-963, another hit from our screen, was identified as part of a cycling cluster that regulates innate immunity, metabolism, and feeding behavior (Vodala *et al.* 2012). Most previous studies of fly miRNAs do not distinguish between neural cell types and therefore there is currently no information on the distribution of miRNAs in glial cells.

We 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 patients with major depression (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).

As mentioned, miR-263b is under circadian control (Yang *et al.* 2008), and glial inhibition or overexpression of the miR resulted in decreased rhythmicity (Figure 2). 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 studies, we found that miR-274 is also under circadian control. Similar to miR-263b, decreased or increased function for the miRNA perturbs rhythmicity (Figure 2A and Figure S4 in File S2). miR-274 does not have direct mammalian orthologs but is conserved in 11 species of *Drosophila* and in the silkworm (*Bombyx mori*) and diamond-back moth (*Plutella xylostella*) (miRBase).

The proapoptotic gene *hid* was previously experimentally identified as a target of miR-263b (Hilgers *et al.* 2010). As the miR-263b-SP disrupts miR-263b function, it was possible that our circadian phenotype resulted from cell death and altered

Figure 5 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 $\pm$ 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.

development. However, we show that neither miR-263b-SP nor miR-274-SP expression resulted in abnormal development of clock cells (Figure 3, A and B and Figure S3, A and B in [File S2](#)). Similarly, REPO and GAT distribution and abundance are normal in flies expressing these miR-sponges (Figure 2, C and D and Figure 3, C and D). In addition, we show that conditional miR-SP expression in adult astrocytes

leads to arrhythmicity without affecting cell death or neural morphology.

There are limited, unbiased experimental methods for identifying miRNA target RNAs. Thus, we chose to combine the predicted targets of two different programs (TargetScan and microT-CDS) in our analysis. We limited candidate target RNAs to those identified by previous profiling studies as

having enriched expression in astrocytes (in comparison with total brain RNA) (Huang *et al.* 2015), as the observed miR-SP phenotypes were due to astrocyte-specific manipulation. A screen of predicted miR-274 target genes identified two relevant for rhythmicity: CG4328 and MESK2. CG4328 is known to be expressed in adult astrocytes (Ng *et al.* 2016) and encodes a transcription factor with mammalian orthologs: LMX1a and LMX1b [FlyBase (Gramates *et al.* 2017)]. Mammalian LMX1A and LMX1B have been described as positive regulators of insulin synthesis (German *et al.* 1992), and fly glial cells, including astrocytes, express genes encoding insulin-like peptides (Chell and Brand 2010; Okamoto and Nishimura 2015; Ng *et al.* 2016). Of interest, recent studies suggest that insulin signaling may be important for fly circadian behavior and sleep (Cong *et al.* 2015; Monyak *et al.* 2015).

Independent RNAi transgenes targeting MESK2, another potential miR-274 target, also resulted in decreased rhythmicity. MESK2 was first identified from a screen for regulators of Ras (Huang and Rubin 2000). MESK2 has mammalian orthologs and one called N-myc downstream-regulated gene 2 (NDRG2) is known to have astrocyte-specific expression (Okuda *et al.* 2007). Beyond a possible regulation of Ras/ERK signaling, there is little known about the biochemical role of MESK2 in *Drosophila* or mammals. Intriguingly, however, sleep-deprived mice have elevated levels of hyperphosphorylated NDRG2 protein (Suzuki *et al.* 2013).

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