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# Circadian rhythm in mRNA expression of the glutathione synthesis gene *Gclc* is controlled by peripheral glial clocks in *Drosophila melanogaster*

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## Abstract

Circadian coordination of metabolism, physiology, and behaviour is found in all living kingdoms. Clock genes are transcriptional regulators, and their rhythmic activities generate daily rhythms in clock-controlled genes which result in cellular and organismal rhythms. Insects provide numerous examples of rhythms in behaviour and reproduction, but less is known about control of metabolic processes by circadian clocks in insects. Recent data suggest that several pathways involved in protecting cells from oxidative stress may be modulated by the circadian system, including genes involved in glutathione (GSH) biosynthesis. Specifically, rhythmic expression of the gene encoding the catalytic subunit (*Gclc*) of the rate-limiting GSH biosynthetic enzyme was detected in *Drosophila melanogaster* heads. The aim of this study was to determine which clocks in the fly multi-oscillatory circadian system are responsible for *Gclc* rhythms. Genetic disruption of tissue-specific clocks in *D. melanogaster* revealed that transcriptional rhythms in *Gclc* mRNA levels occur independently of the central pacemaker neurons, because these rhythms persisted in heads of behaviourally arrhythmic flies with a disabled central clock but intact peripheral clocks. Disrupting the clock specifically in glial cells abolished rhythmic expression of *Gclc*, suggesting that glia play an important role in *Gclc* transcriptional regulation, which may contribute to maintaining homeostasis in the fly nervous system.

### Keywords

Central pacemaker; circadian clock; circadian rhythms; glutathione biosynthesis; peripheral clocks

## Introduction

Circadian rhythms regulate many physiological, metabolic, and behavioural functions with an approximately 24 h periodicity. This way of molecular timekeeping has likely evolved in organisms to provide for optimal survival in a diurnally changing environment. In both insects and mammals, circadian regulation is achieved by a negative feedback loop

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consisting of transcriptional activators and repressors, which among insects, are best understood in *Drosophila melanogaster* (Hardin & Panda, 2013). The core clock genes are *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), and *cycle* (*cyc*). CLK and CYC induce transcription of *per* and *tim* mRNA. As PER and TIM proteins accumulate in the early night, they form heterodimers and repress CLK-CYC activity, thus leading to the suppression of their own transcription. This suppression eventually subsides when PER and TIM are degraded, starting another clock cycle over again. The clock feedback loops are cell autonomous and are known to operate in many different cell types.

The multi-oscillatory circadian system consists of a “central clock” and cell autonomous clocks in peripheral tissues (Tomioka *et al.*, 2012). In flies, the central pacemaker neurons comprise several dozen of lateral and dorsal neurons, which control different aspects of rest/activity rhythms (Rieger *et al.*, 2006). In addition, circadian oscillators are present in retinal photoreceptors, olfactory and gustatory sensory neurons, glia, and most other tissues. These clocks are called peripheral, as their function is not necessary for behavioural rest/activity rhythms (Glossop & Hardin, 2002), but rather for the control of tissue-specific rhythmic processes (Tomioka *et al.*, 2012). These rhythmic processes are initiated by transcriptional regulation of clock-controlled genes (CCGs). Several genome-wide studies have shown that a substantial number of genes exhibit circadian expression in heads of *Drosophila melanogaster* (Keegan *et al.*, 2007; Rodriguez *et al.*, 2013; Hughes *et al.*, 2012) and other insects (Leming *et al.*, 2014) but functional significance of oscillatory CCG expression is poorly understood.

Recent studies have suggested that circadian clocks have a role in regulating oxidative stress responses. Flies with a null mutation in the core clock gene *per* show increased susceptibility to hydrogen peroxide (Krishnan *et al.*, 2008), and their aging is associated with accelerated neurodegeneration in the brain and reduced lifespan following hyperoxia exposure (Krishnan *et al.*, 2009, 2012). This role of the circadian system appears to be conserved, as clock-deficient mice have elevated oxidative damage and accelerated aging symptoms (Kondratova & Kondratov, 2012).

Glutathione (GSH) is an essential molecule for defence against toxins and oxidative insult. GSH levels have previously been demonstrated to oscillate in heads of wild type *D. melanogaster* but not in $per^{01}$ or $cyc^{01}$ clock mutants (Beaver *et al.*, 2012). The first, rate-limiting reaction in GSH biosynthesis is catalyzed by the holoenzyme glutathione cysteine ligase. Glutathione cysteine ligase is composed of two subunits: the catalytic subunit encoded by *Gclc*, and the modulatory subunit encoded by *Gclm*. Both *Gclc* and *Gclm* are rhythmically expressed with peak expression at night (Beaver *et al.*, 2012). The peak in *Gclc* is lost in $cyc^{01}$ mutants, while expression is constitutively high in $per^{01}$; this is typical of clock-controlled genes, due to loss of CLK-CYC activation and loss of PER-TIM repression, respectively. A previous genome-wide search for clock-controlled genes also showed cycling *Gclc* RNA, and revealed that CLK is bound periodically to the promoter region of the *Gclc* gene via E-boxes, which are the binding sites for CLK/CYC (Abruzzi *et al.*, 2011; Rodriguez *et al.*, 2013). In addition, Gene Ontology analysis found glutathione metabolism to be a category enriched in the dataset of cycling transcripts (Rodriguez *et al.*, 2013). Together, these results provide strong evidence that GSH production is clock-regulated.

The present study investigated whether GSH-related rhythms in fly heads are generated by the central pacemaker, which controls locomotor activity rhythms, or by other peripheral clocks in the fly head. The focus of the present study was on *Gclc*, because changes in *Gclc* mRNA levels alone can affect overall glutathione cysteine ligase activity (Lu, 2009), and manipulations in *Gclc* levels have been shown to have a greater effect on overall GSH levels than *Gclm* manipulations (Luchak *et al.*, 2007). Results of the current study show that in fly heads, *Gclc* rhythms do not depend on the central pacemaker, but persist cell-autonomously in peripheral clocks of the nervous system, specifically in glial cells.

## Materials and methods

### Fly rearing and strains

*Drosophila melanogaster* were raised on a standard yeast (35g $L^{-1}$), cornmeal (50g $L^{-1}$), and molasses (5%) diet at 25 ± 1°C, under a 12 h light/12 h dark (LD 12:12 h) regimen. Flies were exposed to fluorescent light of luminous energy 8 ± 2 μmol $m^{-2}$ $s^{-1}$.

To abolish clock function selectively in central clock cells, the *Drosophila* binary UAS/GAL4 system was used (Brand & Perrimon, 1993). The UAS-*cycΔ* construct encodes a dominant negative version of the CYC protein, which disrupts the clock mechanism when expressed in target cells (Tanoue *et al.*, 2004). Flies carrying UAS-*cycΔ* were crossed with *Pdf*-Gal4, driving expression in both small ventral lateral neurons (s-LNvs) and large ventral lateral neurons (l-LNvs) (Kaneko, 1998), or with *cry*-Gal4-39, driving expression in the majority of central pacemaker neurons (Grima *et al.*, 2004). These three fly lines were backcrossed for 8 generations to $w^{1118}$.

To maintain clock function specifically in central clock cells, $per^{01}$ *7.2.2d;;*$ry^{506}$ transgenic flies containing a 7.2kb section of DNA from the *per* genomic region were used. This fragment excludes most of the promotor region, the 5′ UTR, and part of the first intron of the *per* gene, yet it is sufficient for rescuing behavioural rhythmicity and clock function in lateral pacemaker neurons (Frisch *et al.*, 1994). The control line was $per^{01}$*;13.2.2e;*$ry^{506}$ flies carrying a 13.2kb genomic DNA fragment that includes the 7.2kb section mentioned above, as well as an additional 4.2kb of regulatory sequences upstream of the *per* gene. These $per^{01}$ *13.2.2e* flies have clock function rescued in all clock cells (Zerr *et al.*, 1990).

$eya^{2}$ flies without eyes were obtained from the Bloomington Stock Center (stock #2285). Two different *cyc*-RNAi lines were used in this study to reduce expression of the core clock gene *cyc*. Bloomington Stock Center stock #42563 is referred to here as *cyc*-RNAi-sh because it encodes a short hairpin of the *cyc* sequence. The other *cyc*-RNAi line from the National Institute of Genetics (stock #8727R-1) is referred to as *cyc*-RNAi-lh, as it carries a longer *cyc*-matching hairpin. To abolish clock function in glia, neurons, or both, the *cyc*-RNAi lines were crossed to the glial driver *loco*-Gal4 (Bloomington stock #26883), neuronal driver *elav*-Gal4 (Robinow & White, 1991), or all clock cell driver *tim*-Gal4 (Kaneko & Hall, 2000), respectively. These three driver lines were backcrossed for 8 generations to $w^{1118}$.

### Locomotor activity analysis

Locomotor activity was measured using Trikinetics *Drosophila* Activity Monitors DAM2 or DAM5, (Waltham, Massachusetts). Activity counts were taken in 15 min bins for 3 days in LD followed by 7 days in constant darkness (DD). A quantitative measure of rhythmicity in DD was obtained using the fast Fourier Transform (FFT) along with chi-squared periodogram analysis (ClockLab version 2.72, Actimetrics, Wilmette, Illinois). Individuals with a FFT ≥ 0.04 at a period near 24 h or 12 h and a periodogram amplitude peak breaking the 99% confidence line were deemed rhythmic.

### Quantitative real-time PCR

Mated flies were separated 1–2 days after emergence, and 5 day old males were used for all experiments. Flies were collected every 4 h over 24 h in LD 12:12 h. Each sample of 50 heads was separated using 710 μm and 425 μm diameter stainless steel sieves frozen with liquid nitrogen, and homogenized in TRI Reagent (Sigma-Aldrich, St. Louis, Missouri) using a Kontes handheld motor and pestle. The RNA was treated with rDNase I (Takara, Otsu, Shiga, Japan), which was removed with a phenol/chloroform extraction, followed by ethanol/sodium acetate precipitation. cDNA was synthesized using the Bio-Rad iScript cDNA synthesis kit (Hercules, California). Real-time PCR was performed with Bio-Rad iTaq SYBR Green Supermix with Rox (Hercules, California) on an Applied Biosystems Step-One Plus real-time machine. Primers were obtained from Integrated DNA Technology (Coralville, Iowa). All primers used in this study had efficiency > 96%, and their sequences are as follows: *rp49* forward 5′ GCCCAGCATACAGGCCCAAG 3′, *rp49* reverse 5′ AAGCGGCGACGCACTCTGTT 3′; *robl* forward 5′ AATCCAGAGCCACAAAGGTG 3′, *robl* reverse 5′ AGTGTTGTCCAGCGTGGATT 3′; *tim* forward 5′ GTGCTTCTGCTGAGGCGTTTCAAT 3′, *tim* reverse 5′ GGCGAATGGTTTGACATCCACCAA 3′; *Gclc* forward 5′ ATGACGAGGAGAATGAGCTG 3′, *Gclc* reverse 5′ CCATGGACTGCAAATAGCTG 3′. RNA levels were normalized to *rp49* or *robl* (Ling & Salvaterra, 2011) and analyzed using the $2^{-\Delta\Delta CT}$ method. Statistics were calculated using GraphPad Prism 6 (San Diego, California).

## Results

### Transcriptional rhythms of Gclc persist in fly heads when the central clock is disrupted

*Gclc*, the gene encoding the catalytic subunit comprising the glutathione cysteine ligase holoenzyme, has been previously shown to display significant transcriptional rhythms in heads of young CantonS (Beaver *et al.*, 2012) and $w^{1118}$ *D. melanogaster* (Klichko *et al.*, 2015). To probe the mechanism generating these rhythms, this study investigated whether they are controlled systemically by central pacemaker neurons, or in cell autonomously in peripheral oscillators.

In the first experiment, *tim* and *Gclc* mRNA was measured around the clock in heads of flies with disrupted central clock function, but intact peripheral clocks. The most important central clock neurons are small ventral lateral neurons (LNvs) expressing *Pdf*. Therefore, the *Pdf*-Gal4 driver combined with a dominant-negative version of *cyc* (UAS-*cycΔ*) was used to

disable the central clock; these flies are hereon referred to as *Pdf>cycΔ*. Locomotor activity monitoring showed that 87% of these flies became arrhythmic, whereas 94% of *cycΔ>+* control flies remained rhythmic (Table 1). Two-way ANOVA with factors being genotype and time of day show significant differences between peak and trough expression in *tim* ($P < 0.0001$, Fig. 1A) and *Gclc* mRNA levels ($P < 0.0001$, Fig. 1B) in these behaviourally arrhythmic flies, with similar phase and amplitude as in control flies. Even with a disabled central clock, high-amplitude cycling of the core clock gene *tim* is expected, because the disrupted *Pdf* clock cells consist of only 16 LNvs in the brain (Helfrich-Forster, 1998). The bulk of *tim* gene expression in fly heads comes from circadian oscillators in retinal photoreceptors and glial cells (Cheng & Hardin, 1998; Ng *et al.*, 2011); therefore the clock disruption in the central clock would not be detectable by qRT-PCR.

A second driver, *cry*-Gal4-39, was combined with UAS-*cycΔ* (*cry*-39>*cycΔ*) to disable the clock in a larger number of central clock cells. *cry*-Gal4-39 is active in additional groups of dorsal central pacemaker neurons (Klarsfeld *et al.*, 2004). Again, in both central clock disabled flies and controls, *Gclc* mRNA levels remained rhythmic in fly heads ($P < 0.0001$ between peak and trough), as well as *tim* levels ($P < 0.0001$, Fig. 2A). Locomotor activity rhythms were abolished in *cry*-39>*cycΔ* flies (Fig. 2B, Table 1), confirming that central clock neurons were not functioning. These experiments demonstrate that peripheral clocks in the head can maintain rhythm of *Gclc* mRNA in the absence of a functioning central circadian clock.

## Gclc rhythms are absent in flies with disrupted peripheral clocks but functioning central clocks

If the observed *Gclc* rhythms originate from peripheral clock cells, then abolishing clock function in these cells should cause these rhythms to disappear, even if the central clock is functional. To test this prediction, *Gclc* profiles were measured in $per^{01}$ *7.2.2d* flies, which have rescued clock function only in central pacemaker neurons and restored locomotor activity rhythms (Frisch *et al.*, 1994). For the control, $per^{01}$ *13.2.2e* flies with rescued clock function in all central and peripheral clocks were used (Zerr *et al.*, 1990). In this experiment, $per^{01}$ *13.2.2e* displayed rhythmic *tim* and *Gclc* expression as expected, while in $per^{01}$*7.2.2d* flies, expression of both genes was constitutively high and arrhythmic (Fig. 3A), similar as in non-rescued $per^{01}$ mutants (Beaver *et al.*, 2012). Locomotor activity monitoring confirmed that 91% of the $per^{01}$*13.2.2e* were behaviourally rhythmic, as well as a majority (64%) of the $per^{01}$ *7.2.2d* flies (Fig. 3B, Table 1).

## Removing photoreceptors does not disrupt Gclc rhythms in the head

Having established that peripheral clocks are responsible for *Gclc* mRNA rhythms, the next step was to determine whether specific cell types are regulating rhythmic *Gclc* expression. Peripheral clocks function in retinal photoreceptor cells, glia, and some sensory neurons. When sampling mRNA levels from the whole head, a large part of clock gene expression comes from photoreceptor cells of the compound eyes (Cheng & Hardin, 1998). To determine if *Gclc* rhythms observed in whole heads are generated by retinal photoreceptor oscillators, *eyes absent* ($eya^2$) mutants, which are missing the compound eyes, were tested around the clock. Even without the peripheral oscillators in the eyes, heads of $eya^2$ flies

show rhythmic profiles of *tim*, and importantly, they also retained rhythmic expression of *Gclc* mRNA ($P<0.01$), as shown in Fig. 4.

### Disrupting clock in glial cells abolishes Gclc rhythms in the head

Since genetic removal of photoreceptors did not abolish *Gclc* rhythms in the whole head, the question of which other peripheral clocks could be responsible for this rhythm remained. It is known that not only neurons but also glial cells possess the clock mechanism (Ng *et al.*, 2011) and express several genes rhythmically (Jackson *et al.*, 2015). In addition, *Gclc* is listed as a transcript that is enriched in glia (Huang *et al.*, 2015). Therefore, it was investigated how disruption of only neuronal or only glial clocks would affect the *Gclc* expression pattern in whole heads. Since expression of *cycΔ* via *tim*-Gal4 is lethal, two different *cyc*-RNAi lines, which have been shown to be effective in cell type specific knockdown of *cyc* (Karpowicz *et al.*, 2013) were used in the following experiments. First, it was verified that the expression of the target gene *cyc* was significantly reduced when either of the *cyc*-RNAi lines were driven with *tim*-Gal4. Indeed, average *cyc* mRNA was significantly reduced by more than 50% compared to controls (Fig. 5A). This decrease in *cyc* expression also significantly reduced *tim* mRNA levels at the peak expression time point. In *tim>cyc*-RNAi-sh, peak *tim* expression at ZT16 was significantly lower ($P<0.05$) compared to both control genotypes (Fig. 5B). Similarly, peak *tim* expression was also significantly lower ($P<0.01$) in *tim>cyc*-RNAi-lh flies compared to both control groups (Fig. 5B). These data, together with significantly reduced locomotor rhythmicity (not shown) suggest that both *cyc*-RNAi lines are effective in disrupting the clock mechanism.

To investigate whether reduced levels of *cyc* in neuronal or glial clocks would affect the *Gclc* expression pattern in whole heads, *cyc* expression was reduced first in all clock cells via *tim*-Gal4. *Gclc* expression in *tim>cyc*-RNAi-sh and *tim>cyc*-RNAi-lh flies showed significantly reduced peak levels compared to respective controls (Fig. 6A), whereas trough levels were not significantly different, except between *tim>cyc*-RNAi-sh and *+>cyc*-RNAi-sh ($P < 0.05$, Fig 6A). On the other hand, reducing levels of *cyc* in neurons only via *elav*-Gal4 did not have such an effect. Both *elav>cyc*-RNAi-sh and *elav>cyc*-RNAi-lh flies did not show significantly different expression patterns in *Gclc* mRNA compared to respective controls (Fig. 6B). In contrast to what was seen with neuronal clock disruption, reducing *cyc* in all glial cells via *loco*-Gal4 resulted in a significant ($P < 0.01$) decrease in the *Gclc* expression at the peak time point of ZT20. This decrease was observed in both *loco>cyc*-RNAi-sh and *loco>cyc*-RNAi-lh flies compared to their respective controls (Fig. 6C). Both *cyc*-RNAi lines when driven by *loco*-Gal4 showed no statistical difference between the peak and trough time points providing evidence that the loss of clock function in glia abolish rhythm in *Gclc* mRNA expression to a similar degree as disruption of all clocks in the fly head.

## Discussion

Due to the power of *D. melanogaster* genetic tools (Duffy, 2002), it is possible to disable and rescue clocks in specific cells of the brain. This allows for the investigation of which clock-harbouring cells in the multi-oscillatory circadian system are responsible for the rhythm in

*Gclc* mRNA expression. The central pacemaker of the *Drosophila* circadian clock is made up of three groups of dorsal neurons, and two groups of lateral neurons (LNs) (Lin *et al.*, 2004). Ventral LNs are important for the generation of rest/activity rhythms, and also release the neuropeptide pigment dispersing factor (PDF). In *D. melanogaster* and other insects, PDF is required to maintain locomotor rhythms in constant darkness, as well as for synchronization of autonomous rhythms in many neurons expressing the PDF receptor (Renn *et al.*, 1999; Lin *et al.*, 2004; Shafer *et al.*, 2008; Im & Taghert, 2010). However, disabling the clock in *Pdf*-positive cells via expression of *cycΔ* did not impede the rhythmic expression of *Gclc*. The *cry*-Gal4-39 driver is expressed in both dorsal neurons and LNvs (Yoshii *et al.*, 2010). Expression of *cycΔ* in these cells via *cry*-Gal4-39 abolished locomotor activity rhythms; however, the rhythm in *Gclc* expression with a peak at ZT20 still occurred with no difference from the control flies. Together, these data suggest that the central clock, which controls behavioural rhythms, is not responsible for the rhythmic control of *Gclc* expression. Additional evidence supporting this idea came from investigating flies in a *per*-null background carrying constructs that rescue *per* expression in the central pacemaker (Frisch *et al.*, 1994) or all *per*-expressing cells (Zerr *et al.*, 1990). Flies with rescue of *per* in all clock cells were confirmed to have rhythmic expression of *Gclc* mRNA. However, flies with *per* rescued only in central pacemaker neurons lacked rhythmic expression of *Gclc*, despite most exhibiting rest/activity rhythms.

Peripheral clocks function in many cell types in the brain including photoreceptor cells of the compound eyes, other sensory neurons, and glia. The profile of *Gclc* was measured in $eya^2$ flies, which are missing the compound eyes. These flies displayed rhythmic expression of *Gclc*, suggesting that a different clock-containing cell type in central brain may be responsible for *Gclc* expression.

Glial cells (or glia) consist of multifunctional cell types that play major roles in nervous system development, defence, and functioning. In the adult brain, glia cells provide nutrients, remove waste products, and provide neurotransmitter precursors (Jackson *et al.*, 2015). It has been shown that *Gclc* mRNA is enriched in astrocyte-like glia in adult *Drosophila* (Huang *et al.*, 2015). The present study demonstrates that disruption of the circadian clock machinery in glial cells via two different *cyc*-RNAi constructs abolished the rhythm in *Gclc* mRNA expression in whole heads, while disruption of neuronal clocks did not have this effect. It has long been known that glial cells express PER (Ewer *et al.*, 1992). A more recent study reported that glial cells exclusively express *ebony* under circadian control, which is essential for maintaining locomotor activity rhythms (Suh & Jackson, 2007). Glial cells have also been shown to play a role in daily cell morphology changes that occur in the *Drosophila* visual system (Gorska-Andrzejak, 2013). In studies of mammalian cell cultures, it was demonstrated that astrocyte glia not only generate GSH, but also release it into the extracellular space (Dringen & Hirrlinger, 2003). Neurons cultured with glial cells contain higher levels of GSH than those cultured alone, possibly through glial contribution of GSH precursors (Dringen & Hirrlinger, 2003).

Glutathione biosynthesis is the rate-limiting factor in other aspects of glutathione metabolism pathways such as glutathione-S-transferase (GST) activity, which is also rhythmic in flies (Hooven *et al.*, 2009) and mosquitos (Balmert *et al.*, 2014). The circadian

regulation of genes involved in GSH production and utilization may also explain time-of-day differences in pesticide resistance found in flies (Hooven *et al.*, 2009), mosquitos (Balmert *et al.*, 2014), cockroaches (Lin *et al.*, 2014), and other insects. Whilst many insect studies report rhythms in GST expression or activity in response to pesticide challenge, rhythmic *Gclc* expression has been reported so far in *Drosophila melanogaster* (Beaver *et al.*, 2012) and the mosquito *Aedes aegypti* (Leming *et al.*, 2014).

Glutathione biosynthesis is important for cellular defence against reactive oxygen species (ROS) inter- and extracellularly. The daily rhythms in *Gclc* may help coordinate ROS defence with increases in GSH production (Patel *et al.*, 2014). The present study demonstrates that the central clock and other neurons are dispensable for the *Gclc* expression rhythms observed in whole heads. These results suggest that the circadian clock in glia generate the daily rhythm of *Gclc* expression in the adult *Drosophila* brain.

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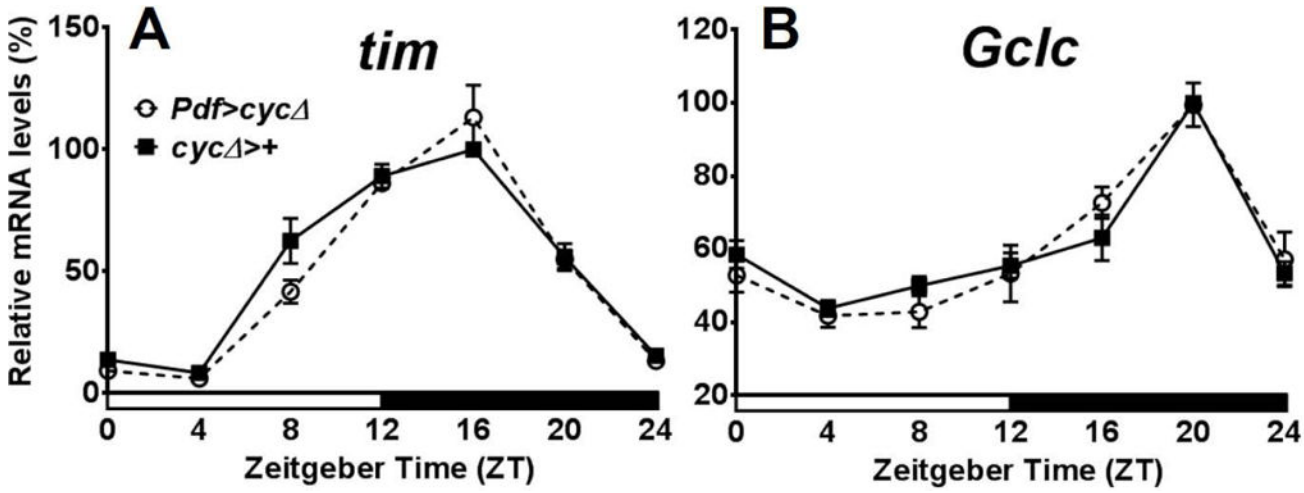


**Fig. 1.**

Expression of the genes *tim* and *Gclc* in *Drosophila melanogaster* with clock function disrupted in PDF-positive pacemaker neurons. *tim* (A) and *Gclc* (B) mRNA show similar rhythms in heads of flies with clock disrupted in PDF neurons (*Pdf>cycΔ*) as in the control (*cycΔ>+*). Levels are normalized to the reference gene *rp49*. Values are reported as percent of peak expression in the control and represent mean of 3 independent biological replicates ± SEM. Two-way ANOVA with Bonferonni post-test showed no significance difference in *tim* or *Gclc* expression between the two genotypes at each time point, but within each genotype, a significant difference between peak and trough expression ($P < 0.0001$ for both genotypes in *tim*; $P < 0.0001$ for both genotypes in *Gclc*).

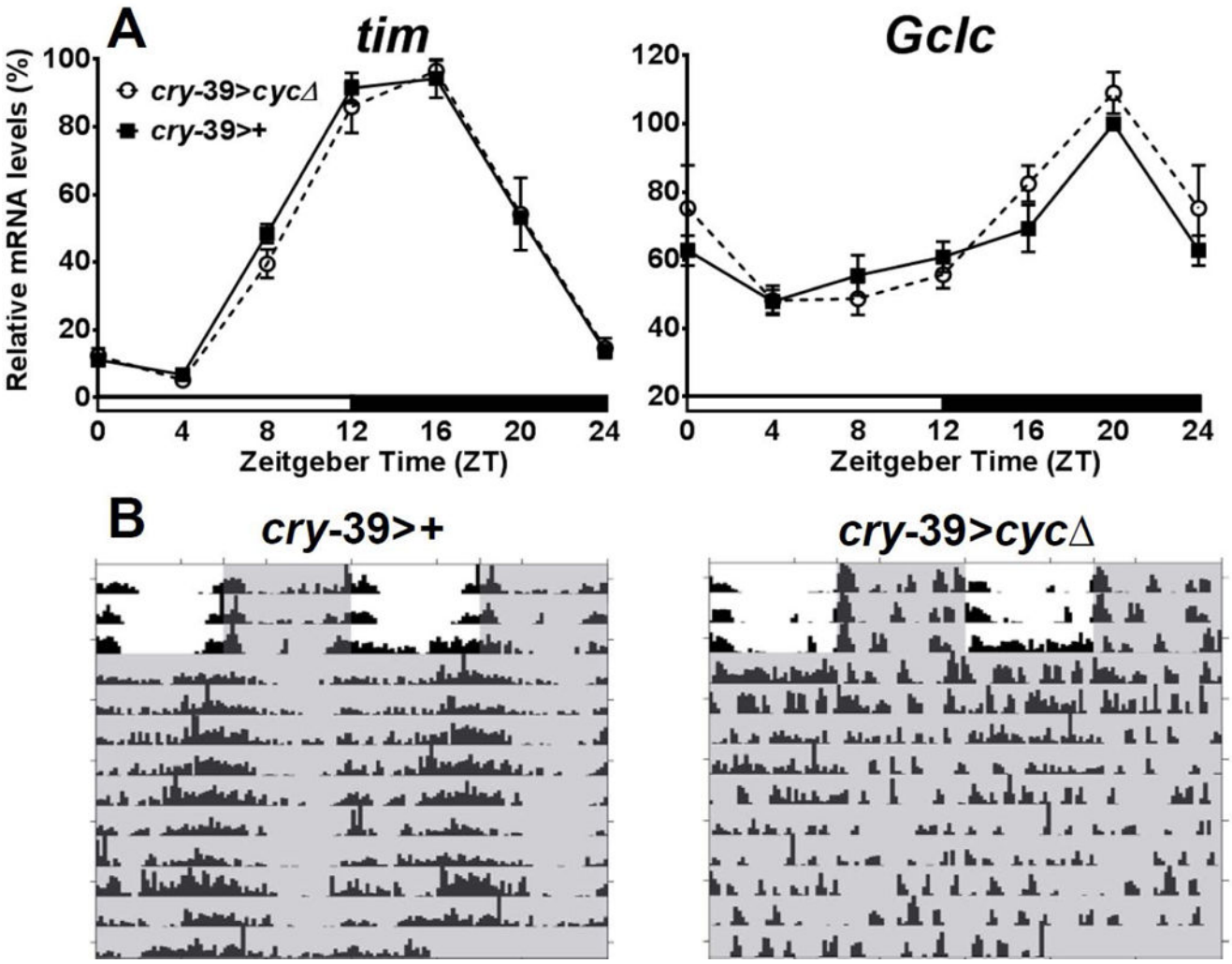


**Fig. 2.**

Expression of *tim* and *Gclc* in *Drosophila melanogaster* with a disrupted central clock. (A) Both *tim* and *Gclc* mRNA expression profiles are similar in *cry*-39*>cycΔ* and *cry*-39*>+* control. Levels are normalized to the reference gene *robl*. Values are reported as percent of peak expression in the control and represent mean of 3 biological replicates ± SEM. Two-way ANOVA with Bonferonni post-test showed no significant difference in *tim* or *Gclc* expression between the two genotypes at each time point, but within each genotype, a significant difference between peak and trough expression ($P<0.0001$ for both genotypes in *tim*; $P < 0.0001$ for both genotypes in *Gclc*). (B) Representative examples of locomotor activity in *cry*-39*>+* and *cry*-39*>cycΔ*. Shaded areas represent periods of darkness.

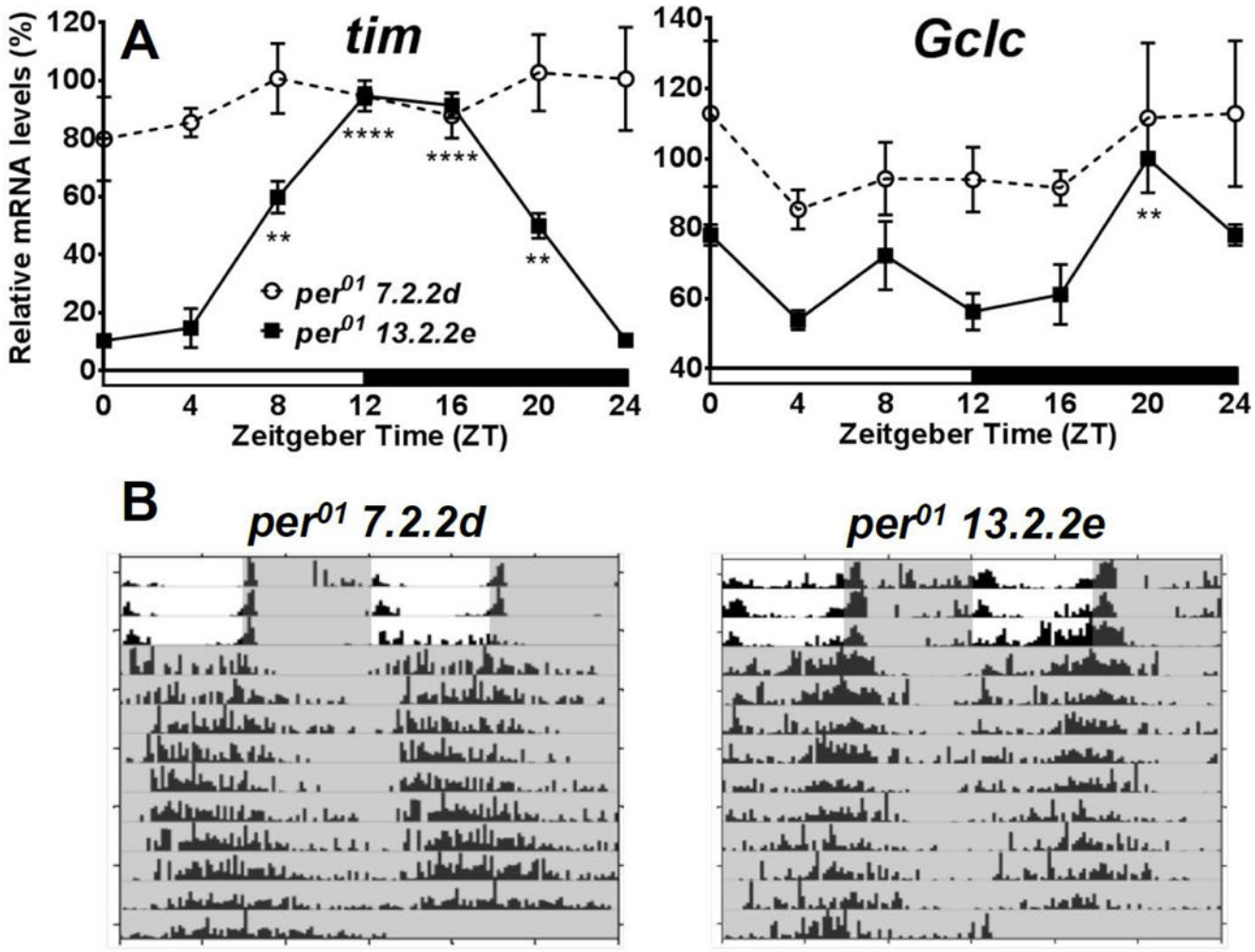


**Fig. 3.**

Expression of *tim* and *Gclc* expression in heads of *per*$^{01}$ background *Drosophila melanogaster* with rescued clock function in central pacemaker or all head clocks. (A) mRNA expression of *tim* and *Gclc* in *per*$^{01}$ *7.2.2d* flies with a rescued central clock, and *per*$^{01}$ *13.2.2e* flies with *per* rescued in all clock cells. Levels are normalized to the reference gene *rp49*. Values are reported as percent of peak expression in the control and represent mean of 3 independent biological replicates ± SEM. Stars indicate a significant difference from the trough expression of each genotype, analyzed by two-way ANOVA with Bonferonni post-test (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$). (B) Representative actograms showing rhythmic locomotor activity in both genotypes. Shaded areas represent periods of darkness.

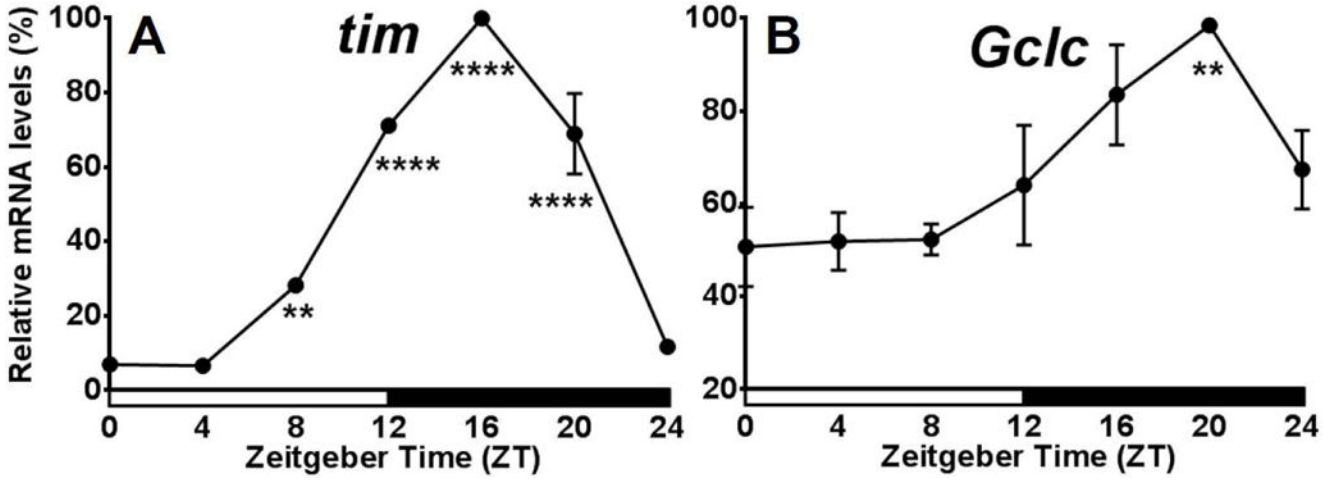


**Fig. 4.**

Expression profiles of *tim* and *Gclc* in *eya*$^{2}$ *Drosophila melanogaster*. mRNA levels of *tim* (A) and *Gclc* (B) in heads of *eya*$^{2}$ flies. Levels are normalized to the reference gene *rp49*. Values are reported as percent of peak expression and represent mean of 3 independent biological replicates ± SEM. Stars indicate a significant difference from the trough using one-way ANOVA with Bonferonni post-test (**$P < 0.01$, ****$P < 0.0001$).

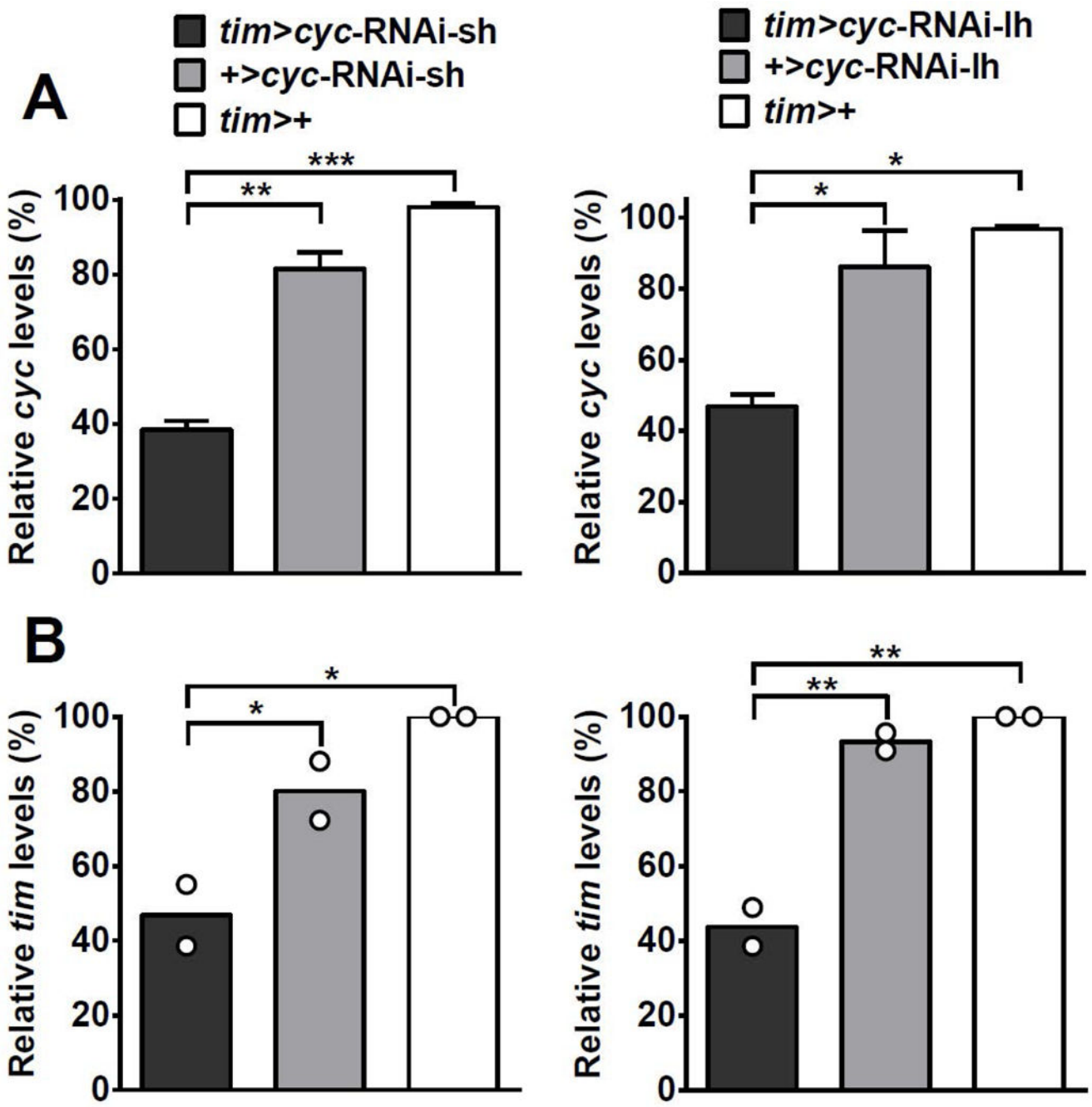


**Fig. 5.**

Verification of *cyc* mRNA knockdown in heads of *Drosophila melanogaster* expressing either *cyc*-RNAi-sh or *cyc*-RNAi-lh via the *tim*-Gal4 driver. (A) Relative expression of overall *cyc* mRNA is significantly reduced in flies expressing *cyc*-RNAi-sh or *cyc*-RNAi-lh driven by *tim*-Gal4 compared to controls. Values are reported as percent expression compared to the *tim>+* control and represent mean of 4 biological replicates ± SEM. (B) Relative expression of *tim* mRNA is significantly reduced at the peak time point in flies expressing *cyc*-RNAi-sh or *cyc*-RNAi-lh driven by *tim*-Gal4 compared to respective

controls. Values are reported as percent of peak expression in the *tim>+* control. Bars represent mean of 2 biological replicates, with dots showing individual replicate values. Levels are normalized to the reference gene *rp49*. Mean expression levels were compared using one-way ANOVA. Stars indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

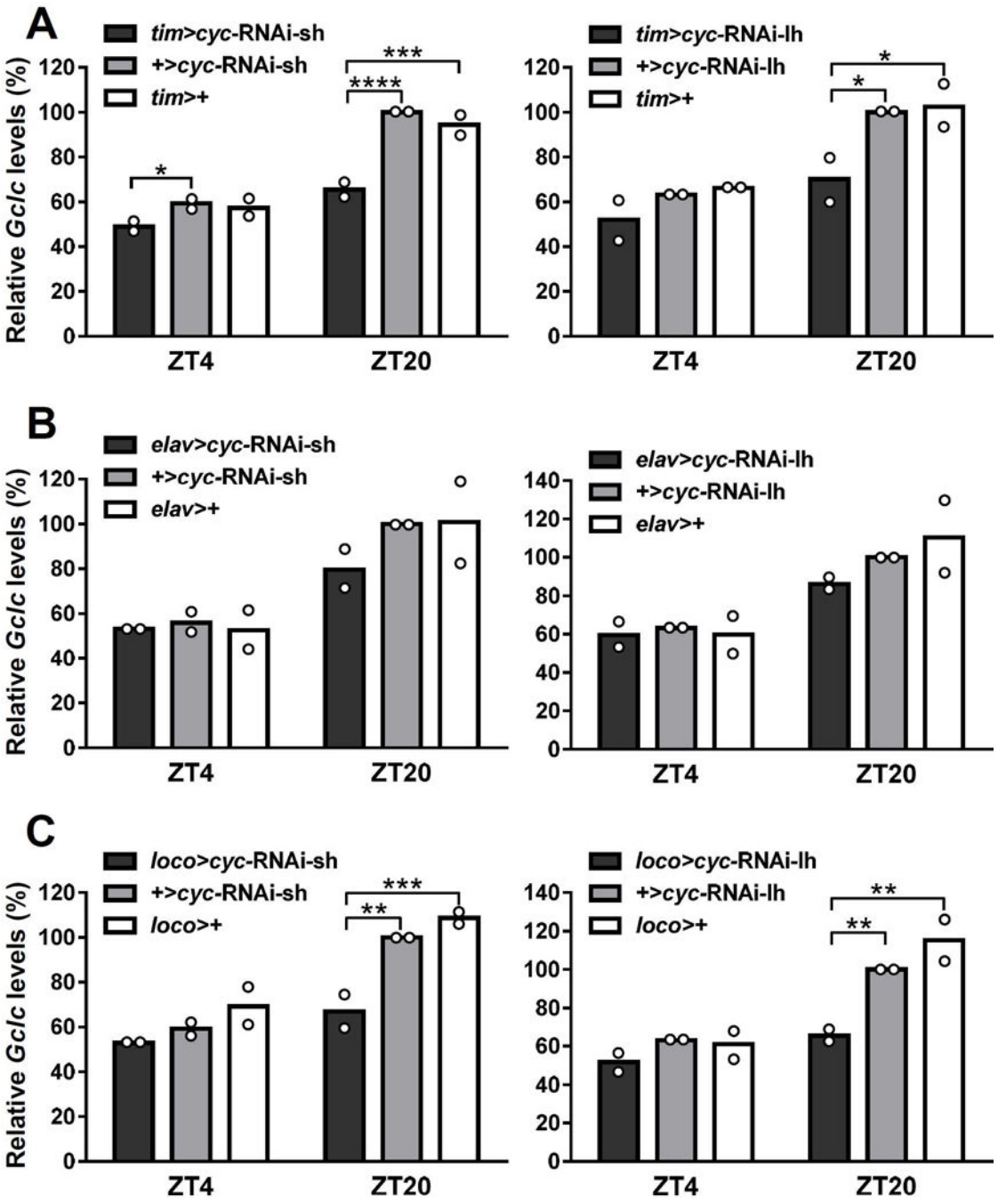


**Fig. 6.**

*Gclc* mRNA profiles in heads of Drosophila melanogaster flies with clock disrupted in all clock cells, neurons only, or glia only. Expression of *Gclc* in flies expressing *cyc*-RNAi-sh or *cyc*-RNAi-lh driven by (A) *tim*-Gal4, (B) *elav*-Gal4, or (C) *loco*-Gal4. Levels of *Gclc* mRNA are normalized to the reference gene *rp49*. Each bar represents mean of 2 biological replicates, with dots showing individual replicate values. Mean expression levels at ZT4 (trough) and ZT20 (peak) were compared between genotypes using two-way ANOVA.

Values are reported as percent of peak expression in the responder-only control. Stars indicate a significant difference (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

**Table 1**

Locomotor activity statistics in adult *Drosophila melanogaster* with genetically disrupted circadian clocks.

| Genotype | *n* | % Rhythmic | Avg Period (h) ± SEM | Avg FFT± SEM |
|---|---|---|---|---|
| *Pdf-Gal4/UAS-cycΔ* | 16 | 12.50 | 23.67± 0.00 | 0.025± 0.006 |
| *UAS-cycΔ/+* | 18 | 94.44 | 23.73± 0.03 | 0.108± 0.008 |
| *cry-Gal4-39/UAS-cycΔ* | 17 | 0 | N/A | 0.007± 0.001 |
| *cry-Gal4-39/+* | 14 | 100 | 24.21± 0.09 | 0.099± 0.010 |
| $per^{01}$ *7.2.2d;;*$ry^{506}$ | 11 | 63.64 | 24.50± 0.14 | 0.063± 0.022 |
| $per^{01}$*;13.2.2e;*$ry^{506}$ | 11 | 90.91 | 23.43± 0.10 | 0.084± 0.014 |