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Morning and Evening Oscillators Cooperate to Reset Circadian Behavior in Response to Light Input

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http://dx.doi.org/10.1016/j.celrep.2014.03.044
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SUMMARY

Light is a crucial input for circadian clocks. In Drosophila, short light exposure can robustly shift the phase of circadian behavior. The model for this resetting posits that circadian photoreception is cell autonomous: CRYPTOCHROME senses light, binds to TIMELESS (TIM), and promotes its degradation, which is mediated by JETLAG (JET). However, it was recently proposed that interactions between circadian neurons are also required for phase resetting. We identify two groups of neurons critical for circadian photoreception: the morning (M) and the evening (E) oscillators. These neurons work synergistically to reset rhythmic behavior. JET promotes acute TIM degradation cell autonomously in M and E oscillators but also nonautonomously in E oscillators when expressed in M oscillators. Thus, upon light exposure, the M oscillators communicate with the E oscillators. Because the M oscillators drive circadian behavior, they must also receive inputs from the E oscillators. Hence, although photic TIM degradation is largely cell autonomous, neural cooperation between M and E oscillators is critical for circadian behavioral photoresponses.

INTRODUCTION

In Drosophila, the self-sustained pacemaker that generates molecular and behavioral circadian rhythms is a negative transcriptional feedback loop: PERIOD (PER) and TIMELESS (TIM) repress CLOCK (CLK) and CYCLE (CYC), which are activators of per and tim transcription (Zhang and Emery, 2012). This mechanism is present in approximately 150 brain neurons (Nitabach and Taghert, 2008). In a standard 12-hr-light:12-hr-dark (LD) cycle, Drosophila exhibits two peaks of activity. The morning (M) peak is driven by the Pigment Dispersing Factor (PDF) positive small ventrolateral neurons (s-LNvs), also referred to as the large (l)-LNvs have been implicated in phase advances (Shang et al., 2008). Ultimately, the DN1s and the I-LNvs would...
have to communicate with the M oscillators, because these cells drive circadian behavior in DD, the condition in which phase is measured after exposing flies to a light pulse. Neuronal circuits would thus be important for circadian behavioral photoresponses. Acute TIM degradation in CRY-negative LNds also indicates the existence of nonautonomous photoreceptive mechanisms in the brain (Yoshii et al., 2008).

We used a severe jet mutant and jet RNAi to map the neuronal circuits controlling circadian photoception. Our results indicate that both cell-autonomous and nonautonomous photoreception take place within the circadian neural network, and that the M and E oscillators are crucial for sensing light and resetting circadian locomotor behavior.

**RESULTS**

**The jetmut Mutation Profoundly Disrupts Circadian Photoresponses**

In a screen for mutants affecting *Drosophila* circadian behavior, we identified a strain that remains robustly rhythmic in LL (Figure 1A; Table S1). This mutant did not complement *jetc* and *jetr* (Table S1), and a point mutation causing a threonine to isoleucine substitution in JET’s leucine-rich repeats (LRR) was identified (Figure 1 B). However, although jetmut and jet show circadian light response defects only with *ls-tim* (Koh et al., 2006; Peschel et al., 2006), our mutant carries the highly light-sensitive *s-tim* allele (Sandrelli et al., 2007). It is thus a much more severe
loss-of-function mutant, which was named jetset. Furthermore, jetset flies showed almost no behavioral phase shifts when challenged with 5 min light pulses applied early (ZT15) or late (ZT21) at night. Phase shift defects were fully rescued by expression of wild-type JET driven by tim-GAL4, a pan-circadian driver (Figure 1C) (Kaneko et al., 2000). The mutation in the jet gene is thus responsible for jetset’s defective photoresponses. TIM undergoes acute light-dependent degradation after short light pulses at night and oscillates robustly under LD cycles (reviewed in Zhang and Emery, 2012). TIM did not degrade after a light pulse at ZT21 in jetset mutants (Figure 1D). However, TIM cycling under LD was not abolished, although its amplitude was reduced (Figure 1E). This is probably because JETSET retains residual activity detectable with long exposure to light. Thus, we conclude that both molecular and behavioral circadian photoresponses are affected by jetset. JET is therefore critical for CRY-dependent circadian behavioral photoresponses and for acute TIM degradation.

**JET Expression in M and E Oscillators Controls Light-Dependent Phase Resetting**

Given its severe phase response defects, we used jetset to map the neural circuit controlling circadian entrainment. GAL4 drivers active in potentially relevant circadian neurons were used to express wild-type JET in jetset flies. When we expressed JET with Clk4.1M-GAL4 (Zhang et al., 2010) only in posterior DN1s, proposed to play a role in phase delays (Tang et al., 2010), or with c929-GAL4 (Grima et al., 2004) specifically in the l-LNvs, which are important for phase advances (Shang et al., 2008), phase responses were not rescued, suggesting that these neurons are not sufficient to reset locomotor behavior (Figure 2A). However, JET expression in both M and E oscillators with Mai179-GAL4 (Grima et al., 2004) completely restored phase shifts in jetset flies. This indicates that JET expression in these two groups of neurons is critical to phase resetting. To determine the individual contribution of the M and E oscillators, we expressed JET only in PDF-positive LNvs (M oscillators and l-LNvs) with Pdf-GAL4 (Renn et al., 1999). We could only slightly improve the phase delays. Phase advances were not rescued at all. We then combined Mai179-GAL4 with Pdf-GAL80 (Stoleru et al., 2004) to express JET only in the E oscillators. Unexpectedly, this also could not rescue phase shifts (Figure 2A). Hence, JET must be rescued in both M and E oscillators for circadian behavior to be responsive to light pulses.

**Figure 2. JET Expression in the M and E Oscillators Is Critical for Circadian Photoresponses**

(A) JET expression in the M and E oscillators is sufficient to rescue both phase delay and advance defects in jetset. Phase shift in response to light pulse at ZT 15 is shown on the left and the phase shift at ZT21 is shown on the right. All genotypes were compared to y w control. Note that both phase delay (ZT15) and advance (ZT21) were completely rescued only when wild-type JET is expressed in both the M and E oscillators using the Mai179-GAL4 driver. With Pdf-GAL4, partial rescue was observed at ZT15 (see also Figure S1B). Sixteen flies per genotype were used, and each experiment was repeated at least four times. Error bars represent SEM. ***p < 0.001; *p < 0.05; n.s., not significant at the 0.05 level as determined by ANOVA coupled to post hoc Tukey’s test, F(6, 33) = 24.77 for phase delay and F(6, 33) = 21.54 for phase advance with p < 0.0001. See also Figure S1 for additional controls.

(B) Knocking down JET expression in the M and E oscillators disrupts phase shifts. Phase delays are plotted on the left and advances on the right. The controls are the different GAL4 driver lines crossed to y w. All the GAL4 drivers were combined with UAS-Dcr2 to enhance RNAi (Dietzl et al., 2007). Each genotype is compared to its GAL4 driver control. ***p < 0.001; **p < 0.01; n.s., not significant at the 0.05 level, tested using Student’s t test. See Figure S2 for additional experiments.
phase shifting defects of jet
oscillators. This explains an apparent paradox in our behavioral results. On one hand, rescuing JET expression in M oscillators only weakly rescues phase shifts in jet
flies. This illustrates the importance of both autonomous and nonautonomous JET activity, and the role played by interactions between M and E oscillators in circadian photoreception.

**DISCUSSION**

Circadian photoreception is based on a cell-autonomous mechanism. However, recent studies indicate that resetting circadian behavior in response to light input requires neural interactions (Shang et al., 2008; Tang et al., 2010). Our results show that the M and E oscillators are critical for circadian photoresponses and act synergistically to shift the timing of the locomotor rhythms in response to light. Indeed JET is required in both the M and E oscillators, whereas, individually, these neuronal groups cannot, or only weakly, phase-shift locomotor rhythms. Moreover, JET promotes both cell-autonomous and nonautonomous acute TIM degradation in circadian neurons. Thus, circadian behavior relies heavily on network interactions during its photic resetting.

The identification of the E oscillators as critical cells for both phase delays and advances was unexpected. Indeed, the DN1s were proposed to be important for phase delays (Tang et al., 2010), and the I-LNvs were found to be needed for phase advances (Shang et al., 2008). However, our experiments indicate that JET is neither required, nor sufficient in DN1s and
I-LNvs for phase shifts. The I-LNvs might thus secrete a neurotransmitter in a JET-independent manner, and this only happens when the light pulse is administered late at night.

Our finding that JET in the M oscillators can nonautonomously trigger TIM degradation in the E oscillators was also unanticipated. How JET does so is unclear, but it must involve rapid communication between the M and E oscillators, because we measured TIM degradation only 1 hr after the light pulse. JET might regulate acutely neuronal activity, possibly with CRY’s help. Indeed, this photoreceptor influences neuronal activity in a light-dependent manner and is required for phase shifts in M oscillators (Fogle et al., 2011; Tang et al., 2010). Interestingly, the reverse is not true: JET in the E oscillators has no effect on TIM degradation in M oscillators. Because the E oscillators are essential for phase shifts and the M oscillators drive circadian behavior (Stoleru et al., 2005), the former must communicate with the latter through a JET-independent mechanism.

Although JET in the E oscillators cannot promote TIM degradation in M oscillators, our rescue experiments suggest that it can do so in the Mai179-GAL4-negative LNds. Indeed, JET expression restricted to the E oscillators restored TIM degradation in most LNds (Figure S4). In addition, JET expression in M oscillators promoted TIM degradation in most LNds as well. The non-E oscillator LNds are CRY negative, which

Figure 3. Cell-Autonomous Role of JET in M Oscillators
(A) Representative confocal images showing TIM degradation in M oscillators of jetmut flies rescued in M- and/or E oscillators after a light pulse at ZT21. The brains were stained with anti-TIM antibody (red) and anti-PDF antibody (blue). LP represents light pulse, whereas NLP means no light pulse. From left to right, fly genotypes are (1) jetmut, (2) Mai179-Gal4, jetmut/jetmut, UAS-jet+/+, (3) Pdf-Gal4, jetmut/jetmut, UAS-jet+/+, (4) Mai179-Gal4, jetmut/jetmut, UAS-jet/Pdf-GAL80. Scale bars, 10 μm.
(B) Quantifications of TIM level. The y axis shows the relative TIM level in M oscillators, normalized to NLP controls for each genotype. Error bars correspond to SEM. n.s., no significance, ****p < 0.0001 was determined by t test.
(C) Representative confocal images showing TIM degradation in M oscillators when JET double-stranded RNAs are expressed in M and/or E oscillators. From left to right, fly genotypes are (1) Mai179-Gal4/ UAS-Dcr2, (2) Mai179-Gal4/ UAS-Dcr2; jetRNAi+/+, (3) Pdf-Gal4/ UAS-Dcr2; jetRNAi+/+, (4) Mai179-Gal4/ UAS-Dcr2; jetRNAi/Pdf-GAL80.
(D) Quantifications of TIM level. y axis shows the relative TIM level in M oscillators, normalized to NLP controls. Error bars correspond to SEM. n.s., no significance, *p < 0.05, ****p < 0.0001 was determined by t test. See also Figure S3 for the similar results obtained at ZT15.
suggests that they rely on a nonautonomous mechanism for TIM degradation (Yoshii et al., 2008). Our results indicate that JET’s nonautonomous function in TIM degradation might be critical to spread light information broadly in the circadian neural network.

Strong evidence supports the idea that acute TIM degradation is required for circadian behavioral photoresponses (Suri et al., 1998; Yang et al., 1998). However, a recent study has challenged the notion that TIM degradation in M oscillators is critical for phase shifts, or at least for phase delays (Tang et al., 2010). Our results suggest that TIM degradation is critical in E oscillators, whether it is achieved cell autonomously or not, because partial block of TIM degradation in E oscillators is associated with compromised phase advances and delays (Figures 2 and 4; Table S2). In the M oscillators, the requirement for TIM degradation remains uncertain. On one hand, JET is required in these neurons and promotes TIM degradation cell autonomously. On the other hand, this JET-dependent TIM degradation could be unnecessary for behavioral phase shifts: JET in M oscillators could contribute to phase shifts entirely nonautonomously. We note that TIM degradation is severely blocked in M oscillators when JET is downregulated, but phase delays are only partially disrupted (Table S2). This would fit with the idea that TIM degradation in M oscillators is not required for phase shifts, although

![Image](image-url)
we cannot rule out that TIM degradation occurred with a slower kinetics. In any case, we propose that after light pulses, TIM degradation in E oscillators resets their molecular pacemaker, which allows them to help the M oscillators to resynchronize their own circadian pacemaker. The M oscillators then readjust the whole circadian neural network. This bears similarities with light synchronization in mammals. The Suprachiasmatic Nucleus (SCN), the mammalian neural circadian pacemaker, receives light input through dedicated retinal ganglion cells in the retina (Hattar et al., 2006). Cells in the core of the SCN appear to be particularly sensitive to this light input. They communicate with robust pacemaker neurons of the shell, which then reset the whole circadian neural network (Yan et al., 2007).

EXPERIMENTAL PROCEDURES

Protein Extraction and Western Blots

Flies were entrained to a standard LD cycle and frozen on the fourth day at the indicated time points. For acute photic TIM degradation, flies were exposed to a 10 min light pulse (1,500 lux) at ZT21 and returned to darkness for 1 hr. Protein extraction and western blots were performed as described in Busza et al. (2004).

Behavioral Monitoring and Analysis

Behavior under LL was monitored and analyzed as previously described (Emery et al., 2000). To measure photic phase shifts, flies were entrained to a LD cycle for 5 days and exposed to a 5 min light pulse (1,500 lux) at ZT15 and 21. They were then monitored in DD for 6 days. The phase of their behavior was compared to nonpulsed controls. We used the off-set of subjective evening activity because it is the most reliable phase marker across genotypes. It is defined as the time at which the activity of a group of flies (averaged from day 2–6 after light pulse) drops to 50% of peak value.

Whole-Mount Immunocytochemistry

Whole-mount immunohistochemistry for fly brains was done as previously described (Zhang et al., 2010). All samples were viewed on a Zeiss LSM5 Pascal confocal microscope.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.044.

AUTHOR CONTRIBUTIONS

P.E. and Y.Z. supervised the project and designed the experiments. P.L., Y.Z., and D.B.-W. performed the experiments and analysis. Y.Z., P.L., and P.E. wrote the manuscript.

ACKNOWLEDGMENTS

We thank M. Freeman for providing EMS-mutagenized fly lines; D. Szydlik, J. Ling, and C. Yuan for technical support; F. Guo and M. Rosbash for communicating results before publication; the TRIP stock center for jet RNAi flies; and R. Stanewsky, C. Helfrich-Forster, and the Hybridoma Bank for PER, CRY, and PDF antibodies. This work was supported by NIH grant GM066777 to P.E.

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