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

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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 l-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 photoreponses. 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 photoreception. 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 jetset Mutation Profoundly Disrupts Circadian Photoreponses

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 1B). However, although jet 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 JET 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 acutely 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 Cik4.1M-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.

Mai179-GAL4 is weakly expressed in four DN1s (Picot et al., 2007) (Figure S2A). To determine if these neurons are required for phase shifts, we used DvPdf-GAL4, which is expressed in the M oscillators, l-LNvs, and a subset of Mai179-GAL4 positive E oscillators, but not in the DN1s (Bahn et al., 2009) (Figure S2B). This driver rescues the E-peak of activity in per0 flies (F. Guo and M. Rosbash, personal communication). We could rescue the
phase shifting defects of jet\textsuperscript{set} with this driver (Figure S2C). Thus, the DN1s are not required for JET-dependent phase shifts.

To ensure that our identification of the M and E oscillators as key neurons for circadian light responses was not the result of a gain of function from JET overexpression, we downregulated JET with RNAi (Figure 2B). Consistent with our rescue data, JET knockdown in both M and E oscillators severely reduced the amplitude of phase delays and advances. This was observed with Mai179-GAL4 and DvPdf-GAL4 (Figures 2B and S2C). The effects of JET downregulation were more evident at ZT15, probably because CRY levels are lower at this time point (Emery et al., 1998; Yoshii et al., 2008), and flies are thus more sensitive to JET downregulation. Because both Mai179-GAL4 and DvPdf-GAL4 are expressed in I-LNvs (Bahn et al., 2009; Grima et al., 2004) (Figures S2A and S2B), we also knocked down JET specifically in the I-LNvs with c929-GAL4 (Figure S2C). No effects on phase delays and advances were observed. Thus, JET expression in the I-LNvs is neither necessary nor sufficient for phase shifts. The M and E oscillators are therefore essential for behavioral phase shifts.

Also in agreement with our rescue experiments, knocking down JET only in PDF-positive neurons reduced the amplitude of phase shifts, although not to the same degree as knocking down JET in both groups, probably because RNAi does not reduce JET activity as efficiently as the jet\textsuperscript{set} mutation. Surprisingly, when we knocked down JET only in the E oscillators, no effect on phase responses was observed (see explanation below). Importantly however, the impact of downregulating JET in both M and E oscillators on phase shifts is greater than the sum of the effects of knocking down JET in the M and E oscillators separately. Thus, both our rescue and RNAi approaches reveal that the M and E oscillators collaborate to reset circadian locomotor behavior.

**JET Controls Photic TIM Degradation Cell Autonomously in M and E Oscillators but Also Nonautonomously in E Oscillators**

To understand our rescue and RNAi results, we measured TIM degradation after light pulses at ZT15 and 21 in the M and E oscillators. In jet\textsuperscript{set} mutants, TIM degradation was abolished in the M oscillators (Figures 3A, 3B, and S3A). JET rescue in the M oscillators with both Mai179-GAL4 and Pdf-GAL4 restored photic TIM degradation in these cells. However, expressing JET only in the E oscillators did not. JET downregulation restricted to the M oscillators inhibited TIM degradation in M cells, but E oscillator downregulation had no effect (Figures 3C, 3D, and S3B). Knocking down JET using Mai179-GAL4 also blocked TIM degradation in the M oscillators, but less severely than with Pdf-GAL4, probably because Mai179-GAL4, a weaker driver than Pdf-GAL4 (data not shown), is less effective in reducing JET activity. Taken together, these results show that JET acts cell autonomously to trigger TIM degradation in M oscillators.

In the E oscillators of jet\textsuperscript{set} flies, TIM degradation was also eliminated and rescued by JET expression in these cells, further supporting the cell-autonomous role of JET in TIM degradation (Figures 4A, 4B, and S3A). Unexpectedly, however, JET expression restricted to the M oscillators rescued partially, but signifi-
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 the M oscillators. Because the E oscillators are essential for phase shifts and the M oscillators drive circadian behavior (Stoleru et al., 2005), the former have to communicate with the latters 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...
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
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 Buza 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 from day 2–6 after light pulse) drops to 50% of peak value. 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.E., and L.P.E. wrote the manuscript.

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