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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 fifth s-LNvs, and perhaps a few Dorsal Neurons (DN1s) (Cusumano et al., 2009; Grima et al., 2004; Picot et al., 2007; Stoleru et al., 2004). These cells are known as the E oscillators. The M oscillators also function as pacemaker neurons: they maintain behavioral rhythms under constant darkness (DD) and control their pace and phase (Renn et al., 1999; Stoleru et al., 2005).

Circadian rhythms are only beneficial if they are synchronized with the day/night cycle. Light is a crucial cue to entrain the circadian clock. In Drosophila, a brief light pulse in the early night, mimicking a delayed dusk, leads to a phase delay, whereas a late-night light pulse resembling an early dawn causes a phase advance (Levine et al., 1994). Light promotes rapid TIM degradation, which is critical to reset the circadian pacemaker and behavioral rhythms (Suri et al., 1998; Yang et al., 1998). Upon light exposure, the intracellular blue-light photoreceptor CRYPTOCHROME (CRY) changes its conformation, binds to TIM, and triggers its proteasomal degradation by recruiting a JETLAG (JET)-containing E3 ubiquitin ligase (Busza et al., 2004; Koh et al., 2006; Ozturk et al., 2011; Peschel et al., 2009).

Loss of CRY results in severe photoreception defects: light-induced TIM degradation and behavioral phase shifts are abolished (Dolezelova et al., 2007; Lin et al., 2001; Stanewsky et al., 1998). cry mutant flies also remain rhythmic in constant light (LL), whereas wild-type flies are arrhythmic under these conditions (Emery et al., 2000). Two jet mutants (jet-4 and jet-5) are also rhythmic in LL (Koh et al., 2006; Peschel et al., 2006). However, this and other circadian photoresponse phenotypes are only observed in flies carrying the long-short tim variant (ls-tim) (Rosato et al., 1997). The long TIM isoform encoded by this variant has reduced affinity for CRY, making flies much less sensitive to light compared to flies carrying the short tim allele (s-tim) (Sandrelli et al., 2007). Thus, although JET promotes TIM degradation, whether it is actually required for TIM degradation and circadian photoresponses remains to be determined.

Although strong evidence supports a cell-autonomous model for circadian photoreception, recent studies indicate that such a mechanism is not sufficient to explain photic resetting of circadian behavior. Indeed, TIM degradation in M oscillators appears to be neither necessary nor sufficient for phase delays (Tang et al., 2010). Based on the pattern of TIM degradation at Zeitgeber Time (ZT) 15, it was proposed that the DN1s would be important for phase delays (Tang et al., 2010). Moreover, 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 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 photo perception. Our results indicate that both cell-autonomous and nonautonomous photo receptor 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 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 LRRs was identified (Figure 1B). However, although jetc and jetr show circadian light response defects only with Is-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.

**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, I-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 jetset 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 key neurons for circadian light responses was not the result of 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 L-LNvs (Bahn et al., 2009; Grima et al., 2004) (Figures S2A and S2B), we also knocked down JET specifically in the L-LNvs with c929-GAL4. No effects on phase delays and advances were observed. Thus, JET expression in the L-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 jetset 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 jetset 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 jetset 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 significantly, TIM degradation in the E oscillators. These results indicate that JET can function nonautonomously when expressed in the M oscillators. Moreover, TIM degradation appears to be rescued in most LNds when using Mai179-GAL4, even though this driver is expressed in only three of the six LNds (Griva et al., 2004; Picot et al., 2007) (Figures 4A and S4). Indeed, the intensity of TIM signal in individual light-pulsed LNds overlapped only with that observed in 12% of LNds in nonpulsed control (Figure S4). Similar results were obtained even when Mai179-GAL4 was combined with Pdf-GAL80. This suggests that JET in the E oscillators can nonautonomously trigger TIM degradation in the three Mai179-GAL4-negative LNds. Downregulating JET in the M and E oscillators with Mai179-GAL4 attenuated TIM degradation in the E oscillators (Figures 4C, 4D, and S3B). Interestingly, TIM degradation appeared to be compromised in most LNds (Figures 4C and S4). This suggests again that the Mai179-GAL4-negative LNds, which express low or no CRY (Yoshii et al., 2008), rely predominantly on a JET-dependent nonautonomous mechanism to degrade TIM.

Importantly, downregulating JET with Mai179-GAL4 did not completely block TIM degradation in the E oscillators (Figures 4C, 4D, and S3B), whereas the jetset mutation did. Thus, the E oscillators retained residual JET activity in jet RNAi flies. This explains an apparent paradox in our behavioral results. On one hand, rescuing JET expression in M oscillators only weakly rescues phase shifts in jetset flies. On the other hand, downregulating JET specifically in E oscillators has no effect on phase shifts. In the latter case, residual JET activity in E oscillators and nonautonomous JET activity from M oscillators result in full TIM degradation in E oscillators. Hence, normal phase shifts are observed. In the former situation, nonautonomous JET activity from the M oscillators is not sufficient to trigger full TIM degradation, because there is not enough autonomous JET activity in E oscillators. Thus, phase shifts are poorly rescued. This illustrates the importance of both autonomous and nonautonomous JET activity, and the role played by interactions between M and E oscillators in circadian photoresponse.

**DISCUSSION**

Circadian photoresponse 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 L-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 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 oscillators have to communicate 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

Figure 4. Cell-Autonomous and Nonautonomous Role of JET in E Oscillators
(A) Representative confocal images showing TIM degradation in LNds 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-PER antibody (green). From left to right, fly genotypes are (1) jetmut, (2) Mai179-Gal4, jetmut/+/UAS-jet/+; (3) Pdf-Gal4, jetmut/+/UAS-jet/+; UAS-jet/Pdf-GAL80. Scale bars, 10 μm.
(B) Quantifications of TIM level. y axis shows the relative TIM level in LNds, normalized to the NLP controls. Error bars correspond to SEM. ****p < 0.0001 was determined by t test. Note that TIM is degraded in the LNds of Pdf-Gal4, jetmut/+/UAS-jet/+ flies, even though JET is only expressed in M oscillators (see also Figure S3C for additional controls).
(C) Representative confocal images showing TIM degradation in LNds when JET double-stranded RNAs are expressed in M and/or E oscillators, after a light pulse at ZT21. 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 LNds compared with the average level in three neighboring noncircadian neurons. TIM levels are normalized to NLP controls. Error bars correspond to SEM. ****p < 0.0001 was determined by t test. Note that downregulating JET in only E oscillators does not affect TIM degradation, but blocking JET expression in both M and E oscillators does. See also Figures S3 and S4.
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 activity to define the end of the light pulse and define diurnal behavior in Drosophila. Nat. Neurosci. 12, 1431–1437.

**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 LSM 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.

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