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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, 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 non-autonomously in E-oscillators when expressed in M-oscillators. Thus, upon light exposure, the M-oscillators communicate with the E-oscillators. Since 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 ca. 150 brain neurons (Nitabach and Taghert, 2008). In a standard 12hr light: 12hr dark (LD) cycle, *Drosophila* exhibits two peaks of activity. The morning (M) peak is driven by the Pigment

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Author contributions

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

Dispersing Factor (PDF) positive small ventrolateral neurons (s-LNvs), also referred to as the M-oscillators (Grima et al., 2004; Stoleru et al., 2004). The evening (E) peak is driven by six dorsolateral neurons (LNds), two PDF negative s-LNvs called “5th 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), while wild-type flies are arrhythmic under these conditions (Emery et al., 2000). Two *jet* mutants (jet^c and jet^r) 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, since 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 non-autonomous photoreceptive mechanisms in the brain (Yoshii et al., 2008).

We used a novel, severe *jet* mutant and *jet* RNA interference (RNAi) to map the neuronal circuits controlling circadian photoreception. Our results indicate that both cell-autonomous

and non-autonomous 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 *jet*^{set} 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 *jet*^{c} and *jet*^{r} (Table S1), and a point mutation causing a Threonine to Isoleucine substitution in JET's Leucine-Rich Repeats (LRR) was identified (Figure 1B). However, while *jet*^{c} and *jet*^{r} 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 *jet*^{set}. Furthermore, *jet*^{set} 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 *jet*^{set}'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 *jet*^{set} mutants (Figure 1D). However, TIM cycling under LD was not abolished, although its amplitude was reduced (Figure 1E). This is probably because JET^{SET} retains residual activity detectable with long exposure to light. Thus, we conclude that both molecular and behavioral circadian photoresponses are affected by *jet*^{set}. 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 *jet*^{set} to map the neural circuit controlling circadian entrainment. *GAL4* drivers active in potentially relevant circadian neurons were used to express wild-type JET in *jet*^{set} 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 *jet*^{set} 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) using *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 per^{0} flies (F. Guo and M. Rosbash, personal communication). We could rescue the phase shifting defects of jet^{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* (Figure 2B, 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. Since both *Mai179-GAL4* and *DvPdf-GAL4* are expressed in l-LNvs (Bahn et al., 2009; Grima et al., 2004) (Figure S2A–B), we also knocked down JET specifically in the l-LNvs with *c929-GAL4* (Figure S2C). 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 jet^{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 non-autonomously 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^{set} mutants, TIM degradation was abolished in the M-oscillators (Figure 3A–B, 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 (Figure 3C–D, 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*^{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 (Figure 4A–B, 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 non-autonomously 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 (Grima et al., 2004; Picot et al., 2007) (Figure 4A, S4). Indeed, the intensity of TIM signal in individual light-pulsed LNds overlapped only with that observed in 12% of LNds in non-pulsed 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 non-autonomously 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 (Figure 4C–D, S3B). Interestingly, TIM degradation appeared to be compromised in most LNds (Figure 4C, 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 non-autonomous mechanism to degrade TIM.

Importantly, downregulating JET with *Mai179-GAL4* did not completely block TIM degradation in the E-oscillators (Figure 4C–D, S3B), while the *jet*^{set} 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 *jet*^{set} 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 non-autonomous JET activity from M-oscillators result in full TIM degradation in E-oscillators. Hence normal phase shifts are observed. In the former situation, non-autonomous 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 non-autonomous 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 non-autonomous 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 l-LNvs for phase shifts. The l-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 non-autonomously 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 one hour 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. Since the E-oscillators are essential for phase shifts and the M-oscillators drive circadian behavior (Stoleru et al., 2005), the formers 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 non-autonomous mechanism for TIM degradation (Yoshii et al., 2008). Our results indicate that JET's non-autonomous 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, since partial block of TIM degradation in E-oscillators is associated with compromised phase advances and delays (Figure 2, 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 non-autonomously. 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).

Materials and methods

Protein extraction and Western blots

Flies were entrained to a standard LD cycle and frozen on the 4th day at the indicated time points. For acute photic TIM degradation, flies were exposed to a 10-min light pulse (1500 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-minute light pulse (1500 lux) at ZT15 and 21. They were then monitored in DD for six days. The phase of their behavior was compared to non-pulsed controls. We used the off-set of subjective evening activity, as 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 post 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- JET is essential for circadian photoresponses.
- JET can function both cell-autonomously and non-autonomously in circadian neurons.
- The M- and E-oscillators are critical for circadian photoresponses.
- The M- and E-oscillators collaborate to reset circadian behavior with light inputs.

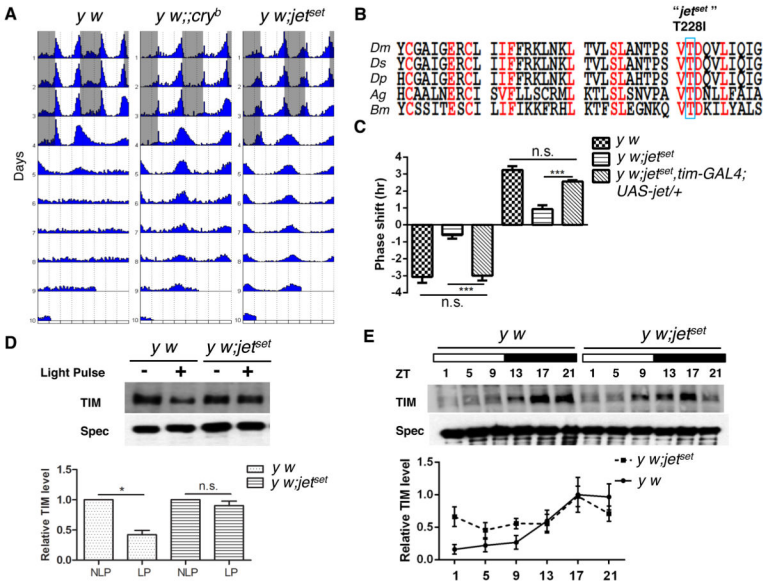

Figure 1. Identification and characterization of *jet*^{set}

(A) *y w; jet*^{set} flies are rhythmic under LL. Representative double-plotted actograms of *y w*, *cry*^{b} and *y w; jet*^{set} flies. (white indicates the light phase and gray indicates the dark phase).

(B) Sequence alignment of the LRR region of insect JET proteins. The blue box indicates the *jet*^{set} mutation.

(C) Behavioral phase shifts after short light pulses are profoundly disrupted in *jet*^{set} mutants. Phase delays and advances are plotted as negative and positive values respectively. Phase shifts were almost completely abolished compared to control (*y w*) flies. Phase shifting defects were fully rescued by expression of *UAS-jet* with *tim-GAL4*. 16 flies were used per genotype for analysis, N=3. Error bars correspond to S.E.M. ***, $p < 0.001$, n.s., not significant at the 0.05 level as determined by one-way analysis of variance (ANOVA) coupled to post hoc Tukey's test for multiple comparisons, $F(5, 12) = 121.9$ with p value < 0.0001.

(D) *jet*^{set} is defective for acute TIM degradation in response to short light pulses. Upper panel: representative Western blot showing TIM degradation after light pulse in *y w* and *y w; jet*^{set}. A light pulse (LP) was given at ZT21 and non-light pulsed (NLP) flies were used as controls. Lower panel: quantification of TIM levels. Upon light pulse, *y w* flies showed about 50% TIM degradation while *jet*^{set} did not show any obvious TIM degradation. N=3. For each genotype the LP values are normalized to their NLP control values. Data are plotted as mean ± S.E.M, *, $p < 0.05$; n.s. – not significant as determined by comparing the LP and NLP groups for each genotype by student's t test.

(E) TIM oscillations in *jet*^{set} are dampened under LD conditions. Upper panel: representative Western blots showing TIM oscillation in whole heads at indicated ZT times under a LD cycle. The white bars represent the day and the black bars represent the night. TIM levels were normalized to the SPECTRIN levels. N=5. Lower panel: quantification of TIM levels. TIM expression levels for *y w* at ZT17 were set to 1 and other values were normalized to it. Data represents mean ± S.E.M.

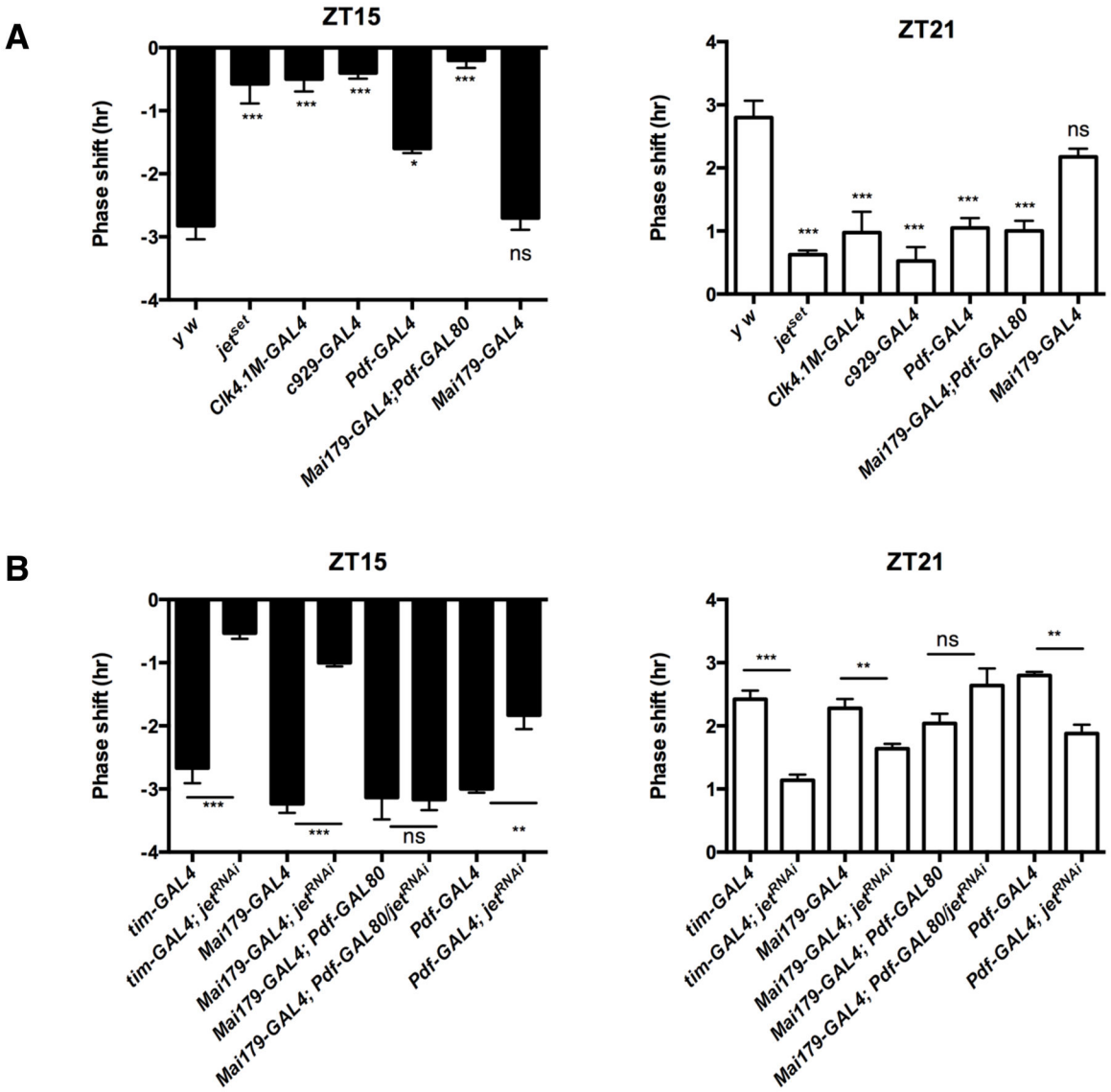

Figure 2. JET expression in the M- and E-oscillators is critical for circadian photoresponses

(A) JET expression in the M- and E-oscillators is sufficient to rescue both phase delay and advance defects in *jet*^{set}. 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). 16 flies per genotype were used and each experiment was repeated at least four times. Error bars represent S.E.M. ***, $p < 0.001$; * $p < 0.05$; 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 value < 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.

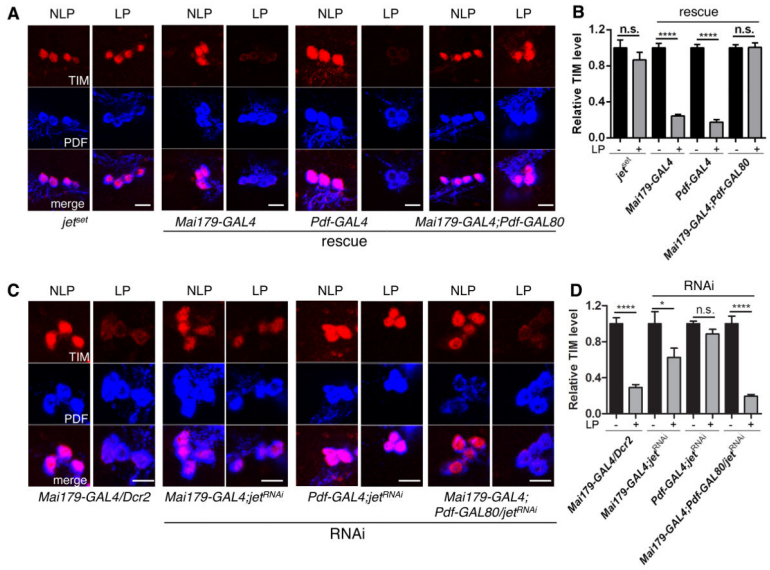

Figure 3. Cell-autonomous role of JET in M-oscillators

(A) Representative confocal images showing TIM degradation in M-oscillators of *jet*^{set} 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, while NLP means no light pulse. From left to right, fly genotypes are 1) *jet*^{set} 2) *Mai179-Gal4*, *jet*^{set}/*jet*^{set}; *UAS-jet*/+ 3) *Pdf-Gal4*, *jet*^{set}/*jet*^{set}; *UAS-jet*/+ 4) *Mai179-Gal4*, *jet*^{set}/*jet*^{set}; *UAS-jet*/*Pdf-GAL80*. Scale bars indicate 10 μm.

(B) Quantifications of TIM level y-axis shows the relative TIM level in M-oscillators, normalized to NLP controls for each genotype. Error bars correspond to S.E.M. n.s. - no significance, ****, $p < 0.0001$ was determined by t-test.

(C) Representative confocal images showing TIM degradation in M-oscillators when JET dsRNAs 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; jet*^{RNAi}/+, 3) *Pdf-Gal4*/*UAS-Dcr2; jet*^{RNAi}/+, 4) *Mai179-Gal4/UAS-Dcr2; jet*^{RNAi}/*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 S.E.M. 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.

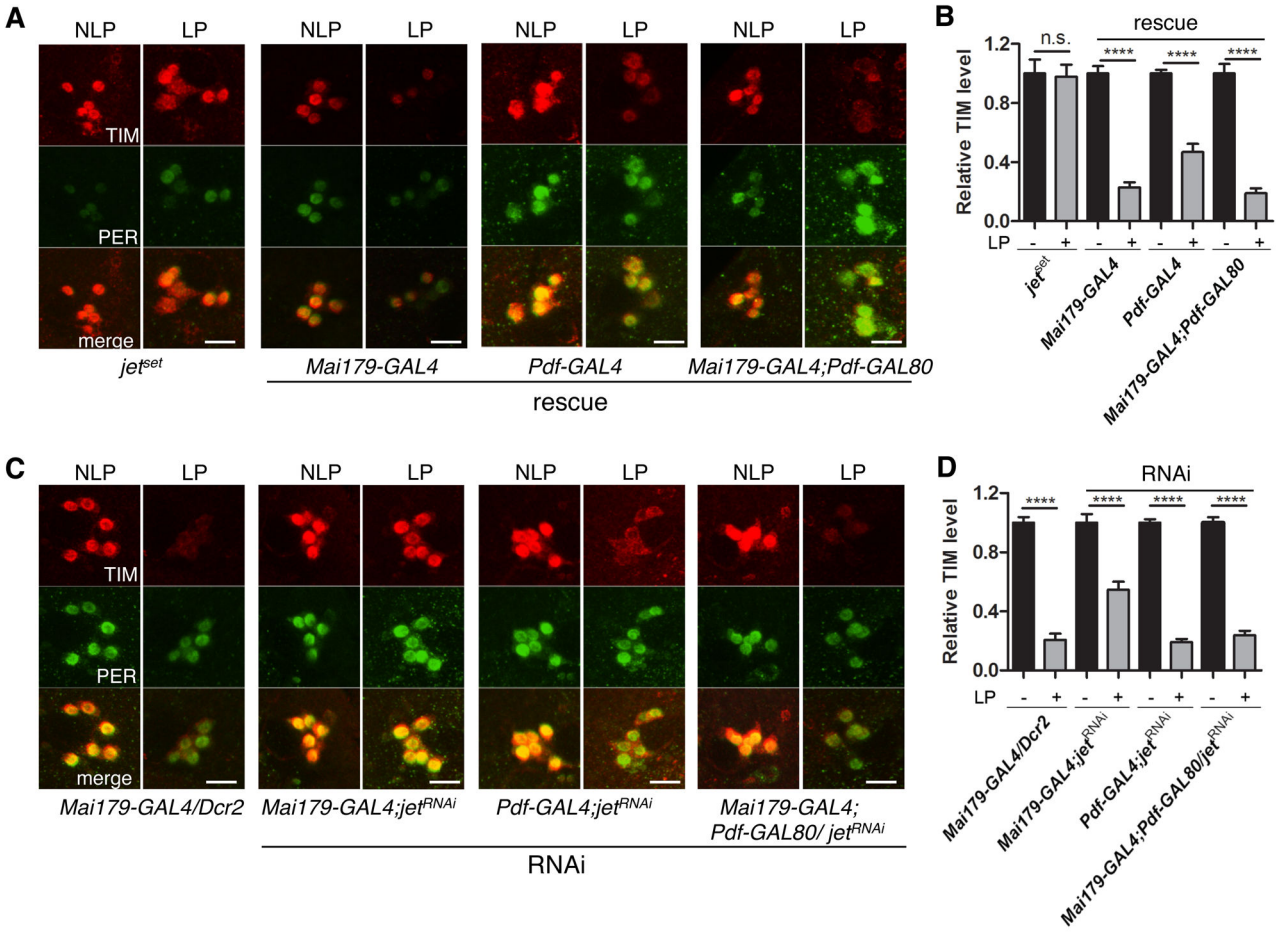

Figure 4. Cell-autonomous and non-autonomous role of JET in E-oscillators

(A) Representative confocal images showing TIM degradation in LNds of *jet*^{set} 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) *jet*^{set} 2) *Mai179-Gal4, jet*^{set}/*jet*^{set}; *UAS-jet*/+ 3) *Pdf-Gal4, jet*^{set}/*jet*^{set}; *UAS-jet*/+ 4) *Mai179-Gal4, jet*^{set}/*jet*^{set}; *UAS-jet*/*Pdf-GAL80*. Scale bars indicate 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 S.E.M. ****, $p < 0.0001$ was determined by t-test. Note that TIM is degraded in the LNds of *Pdf-Gal4, jet*^{set}/*jet*^{set}; *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 dsRNAs 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; jet*^{RNAi}/+, 3) *Pdf-Gal4*/*UAS-Dcr2; jet*^{RNAi}/+, 4) *Mai179-Gal4/UAS-Dcr2; jet*^{RNAi}/*Pdf-GAL80*.

(D) Quantifications of TIM level. y-axis shows the relative TIM level in LNds compared with the average level in three neighboring non-circadian neurons. TIM levels are normalized to NLP controls. Error bars correspond to S.E.M. ****, $p < 0.0001$ was determined by t test. Note that down-regulating JET only in E-oscillators does not affect

TIM degradation, but blocking JET expression in both M and E-oscillators does. See also Figure S3 and S4.