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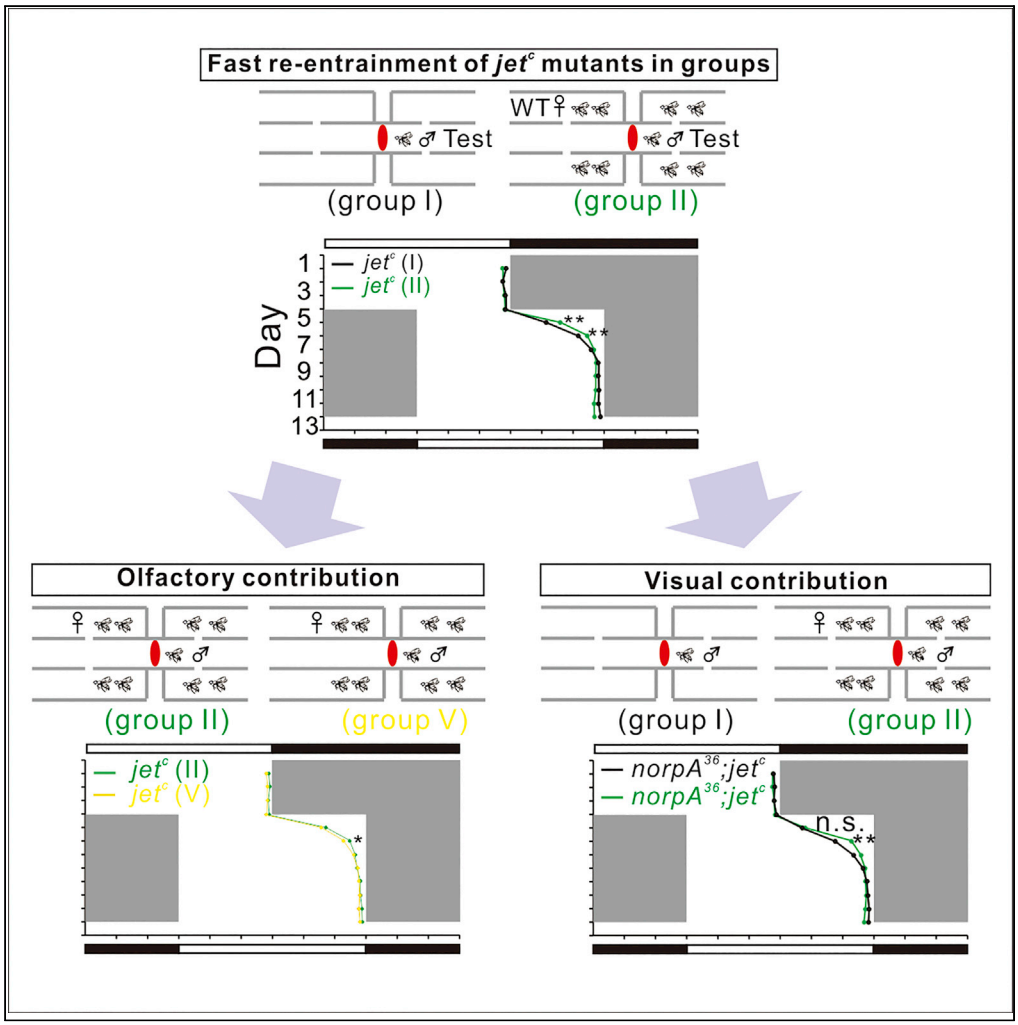

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HIGHLIGHTS

Interactions with WT females accelerates re-entrainment in *jet*^{c} and *cry*^{b} male mutants

Both visual and olfactory inputs contribute to fast re-entrainment in *jet*^{c} mutants

jet^{c} mutants in groups re-entrain faster on *per* expression rhythms than isolated one

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Contribution of Social Influences through Superposition of Visual and Olfactory Inputs to Circadian Re-entrainment

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SUMMARY

Circadian patterns of locomotor activity are influenced by social interactions. Studies on insects highlight the importance of volatile odors and the olfactory system. Wild-type *Drosophila* exhibit immediate re-entrainment to new light:dark (LD) cycles, whereas cry^b and jet^c mutants show deficits in re-entrainability. We found that both male mutants re-entrained faster to phase-shifted LD cycles when social interactions with WT female flies were promoted than the isolated males. In addition, we found that accelerated re-entrainment mediated by social interactions depended on both visual and olfactory cues, and the effect of both cues presented jointly was nearly identical to the sum of the effects of the two cues presented separately. Moreover, we found that re-entrainment deficits in *period* (*per*) expression-oscillation in jet^c mutants were partially restored by promoting social interactions. Our results demonstrated that, in addition to olfaction, social interactions through the visual system also play important roles in clock entrainment.

INTRODUCTION

Genes and environments crucially influence daily behaviors. Social influences, regarded as a subset of environmental inputs, can regulate development, physiology, and behaviors from insects to human beings (Rutter et al., 2006; Sokolowski, 2010). Previous studies have indicated that social interactions can affect a variety of behaviors, such as circadian activity patterns, sleep, aggression, mating, and learning (Beattie et al., 2015; Sokolowski, 2010). Moreover, there is substantial evidence showing social synchronization in laboratory animal models, including *Drosophila*, honeybees, and vertebrates (Bloch et al., 2013). More recent works suggest that the number of cohabiting animals is important for social synchronization in female mice and that social synchronization can even override the photic entrainment of circadian rhythms in honeybees (Fuchikawa et al., 2016; Paul et al., 2015; Simoni et al., 2014). Although *Drosophila melanogaster* is classified as a solitary species, these flies aggregate at high densities to feed and they exhibit social entrainment, aggression, and collective behavior (Levine et al., 2002; Liu et al., 2011; Ramdya et al., 2015; Schneider et al., 2012; Veenema, 2009; Wang and Anderson, 2010). Studies using *Drosophila* have begun to define the molecular and neuronal mechanisms controlling social entrainment (Bloch et al., 2013; Fujii and Amrein, 2010; Fujii et al., 2007; Levine et al., 2002; Lone et al., 2016; Lone and Sharma, 2012). In particular, volatile pheromones, the olfactory system, and some clock neurons are involved. However, social influences on clock re-entrainment to shifted light/dark (LD) cycles and the sensory inputs governing these effects have not yet been explored. We therefore set out to demonstrate whether social groups affect re-entrainment by exposing re-entrainment-deficient mutants to wide-type (WT) flies; we also sought to determine which sensory input(s) might be involved.

RESULTS

jet^c and cry^b Mutants Show Fast Re-entrainment to New LD Cycles when Social Interactions Are Promoted

Cryptochrome (CRY) and jetlag (JET) were shown to regulate the central clock by promoting degradation of a core clock protein, timeless (TIM) in a light-dependent manner, and JET could be required for transmitting light signals from CRY to TIM (Ceriani et al., 1999; Koh et al., 2006). Previous studies have also shown slow re-entrainment to new LD cycles in jet^c and cry^b mutants (MT) compared with WT control flies (Helfrich-Forster et al., 2001; Koh et al., 2006; Yoshii et al., 2015). To investigate the re-entrainabilities of flies in different social environments, we compared the re-entrainability of the mutants in different social environments. Briefly, newly eclosed male flies were isolated for 1–3 days on 12-h:12-h LD cycles at a temperature

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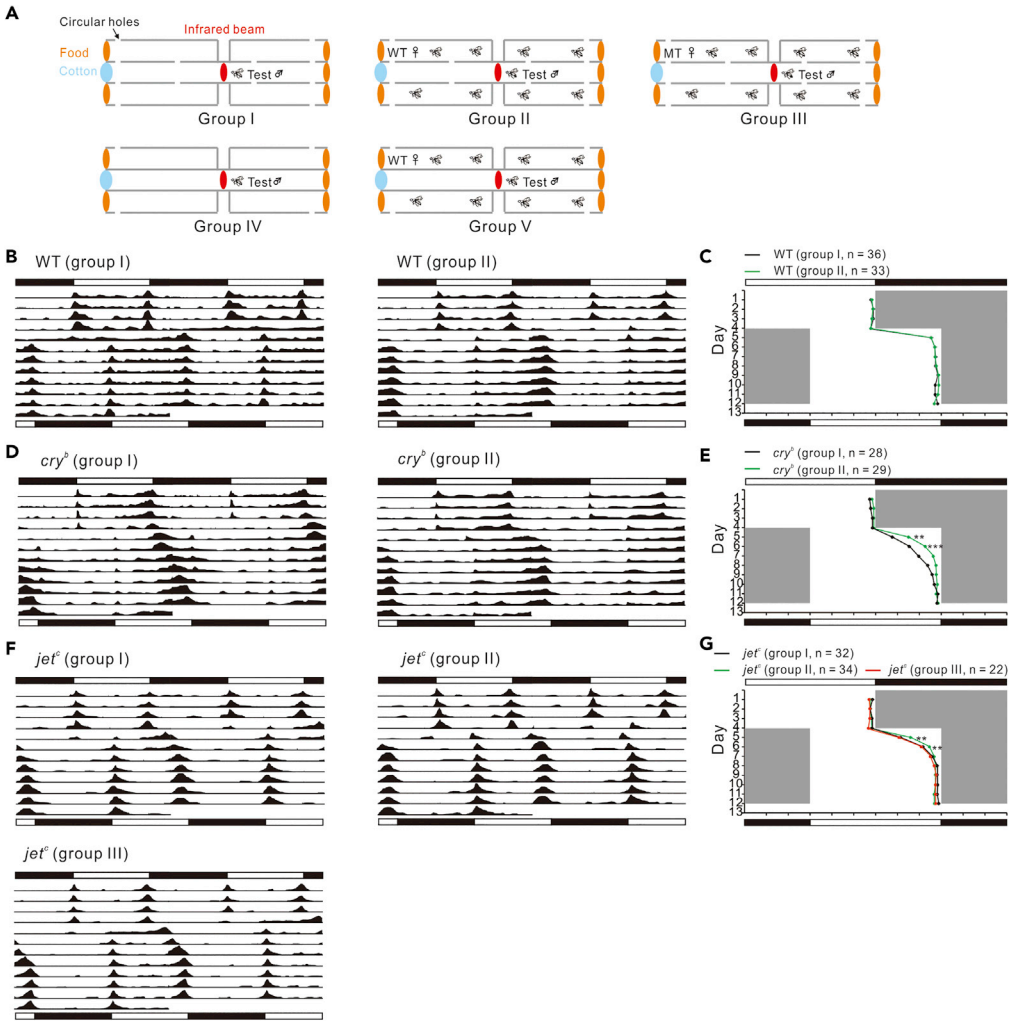

Figure 1. Social Interactions Reversed Re-entrainment Deficits in *cry*^{b} and *jet*^{c} Mutants

(A) To study social interactions between flies using the *Drosophila* Activity Monitor System (DAM2; TriKinetics), we modified the recording panels as shown in the figure. Briefly, we added two plastic cylinders to each side of the recording tube, and each contained zero or approximately 8 WT/MT flies. The experimental fly in the tube was able to contact WT or MT flies by receiving several sensory cues, such as chemosensory and visual cues (groups I, II, and III). Chemosensory signaling was abolished simply by blocking circular holes in the tube (groups IV and V).

(B, D, and F) Representative actograms of WT (B), *cry*^{b} (D) and *jet*^{c} (F) flies. Data were smoothed from 12 flies for each group. The flies were transferred from one LD cycle (indicated by the top bars) to another with 6-h delay (bottom bars) during the fourth day.

(C, E, and G) Phase plots showing quantification of the position of the evening peaks for the indicated genotypes for each day. WT flies in both groups I and II (C) moved their evening peak by almost 6 h during the first 2 days after the phase shift (group I: 5.1 h on day 1, 0.8 h on the second day; group II: 5.2 h on day 1, 0.7 h on the second day). *cry*^{b} mutants (E) moved the evening peak by 2.0 h on day 1 and 1.5 h on the second day in group I, whereas they moved their evening peak by 3.1 h on day 1 and 1.9 h on the second day in group II. Similarly, *jet*^{c} mutants (G) shifted their evening peak by 2.5 h on day 1 and 1.6 h on the second day in group I, whereas they shifted their evening peak by 3.4 h on day 1 and 2.2 h on the second day in group II. Note that no difference in evening peak movement was observed between groups I and III, indicating that interactions with MT female flies did not affect re-entrainment.

Mean ± SEM is shown. "**" and "***" denote $p < 0.01$ and $p < 0.001$, respectively. One-way ANOVAs followed by post hoc Tukey tests were performed for multiple comparisons. See also Figure S1.

of 25°C and humidity of ~60%, and a test male was then placed in a glass tube and separated from 16 WT virgin female flies outside the tube (Figure 1A). In some experiments (groups I, II, and III), we also include 4–6 round circles (~1 mm in diameter) on each side to allow communication between flies by means of

endogenous volatile chemical signals. Moreover, we included mutant (MT) female flies outside the recording tube as group III to test whether the presence of MT female flies would accelerate re-entrainment of MT males (MT males and females with the same genotype, Figure 1A). Studies on sexual interactions in *Drosophila* indicate that males and females exhibit entrainment of circadian locomotor rhythms (Fujii et al., 2007; Hanafusa et al., 2013; Lone and Sharma, 2012); therefore, we presumed that such interactions might affect clock re-entrainment. Mutations of genes used in this study (including cry^b and $norpA^{36}$) have been shown to play direct and indirect roles in central clock. For example, CRY serves as both a blue-light photoreceptor and a clock component and plays a crucial role in clock entrainment (Collins et al., 2006; Emery et al., 2000; Helfrich-Forster, 2019; Helfrich-Forster et al., 2001; Stanewsky et al., 1998), whereas NORPA is an essential enzyme in the canonical phototransduction cascade in photoreceptors and mediates majority of visual input to clock (Bloomquist et al., 1988; Schneuwly et al., 1991). To ensure that all mutant flies could be synchronized during the first 4 days of LD cycles and that evening activity peaks could be easily measured, we applied a 25°C(light):18°C(dark) temperature cycle in combination with the LD cycles. The flies were then exposed to a 6-h delay of the present LD cycles at a constant temperature of 25°C. cry^b and jet^c male mutants, which were shown to take longer for re-entrainment to a new schedule than WT flies (Helfrich-Forster et al., 2001; Koh et al., 2006), were used in the study as test flies to examine whether their re-entrainment could be influenced by the promotion of social interactions with WT females.

As expected, both isolated (group I) and grouped (group II) WT flies exhibited immediate re-entrainment to the new LD cycle, whereas cry^b and jet^c mutants required approximately 4–6 days in group I (Figures 1B–1G). As previously reported (Mazzotta et al., 2013), the electroretinogram (ERG) response upon illumination with bright white flashes in cry^b flies was normal, and so was the jet^c flies (Figure 3I). Moreover, cry^b flies were previously reported to have normal optomotor response (Stanewsky et al., 1998). These results indicate that both mutants have basically normal visual behavior. Interestingly, the cry^b and jet^c mutants in group II re-entrained more quickly on days 1 and 2 than did those in group I (Figures 1D–1G). Note that the jet^c mutants in group III exhibited slow re-entrainment similar to those shown in group I (Figures 1F and 1G), indicating that contacting mutant females did not accelerate re-entrainment in mutant males and fast re-entrainment in females was essential for accelerated re-entrainment in males. To clarify whether fast re-entrainment of the mutant males observed in the study was a form of masking, we released experimental flies with a 2-day phase delay or complete re-entrainment to constant darkness (DD) in the absence of WT females (Figure S1). We allowed WT females to freely move out of the tubes on the day before experimental flies were released to DD in groups I and II during zeitgeber time (ZT) 0–1 or 11–12. Our data show that jet^c flies exposed to WT females after 2 days of re-entrainment still exhibited larger phase delays in free-running behavior than isolated jet^c flies (Figures S1A and S1B). Moreover, the completely re-entrained males also maintained their evening activity peak at approximately Circadian time (CT) 17.5 (Figures S1C and S1D). These results indicate that the clock is indeed re-entrained during the process of entrainment and that masking is unlikely. These data demonstrate that promoting social interactions with WT female flies accelerates clock re-entrainment in mutants. Behavioral phenotypes in the cry^b and jet^c mutants are generally similar. cry^b mutation seems to disrupt peripheral circadian oscillators, leaving the circadian oscillator function of central pacemaker neurons almost intact, and CRY itself is blue light and even magnetic sensitive (Collins et al., 2006; Emery et al., 1998; Gegear et al., 2008; Koh et al., 2006; Krishnan et al., 2001). Because we intend to simply focus on the regulation of central clock entrainment by social interactions in the following study, we chose to focus on jet^c mutants.

Both Visual and Olfactory Inputs Influence Circadian Clock Re-entrainment

What environmental cues and sensory mechanisms underlie the effects of social exposure on re-entrainment? Sensory cues, such as signals from visual, olfactory, auditory, and mechanical inputs, may be involved. We then placed each test fly in a glass tube (without small circular holes on solid glass walls between the test and WT flies) to block potential communication between flies through the air flow by means of endogenous volatile chemical signals (Figure 2B, groups IV and V; Figure 2A, groups I and II as controls). After chemosensory signals were blocked, the jet^c mutants in group V still showed fast re-entrainment (WT female flies were present outside the recording tube; day 1, 3.3 h; second day, 1.8 h, requiring approximately 3 days) than did those in group IV (isolated; day 1, 2.4 h; second day, 1.7 h) (Figures 2A and D), and they re-entrained more slowly on day 2 than did those in group II (Figures 2A, 2B, and 2D), suggesting the olfaction preferentially affects re-entrainment on day 2. To verify that no unintended olfactory signals had reached the flies, we repeated the design with $Or83b^1/Or83b^2$ mutants, which show no behavioral or

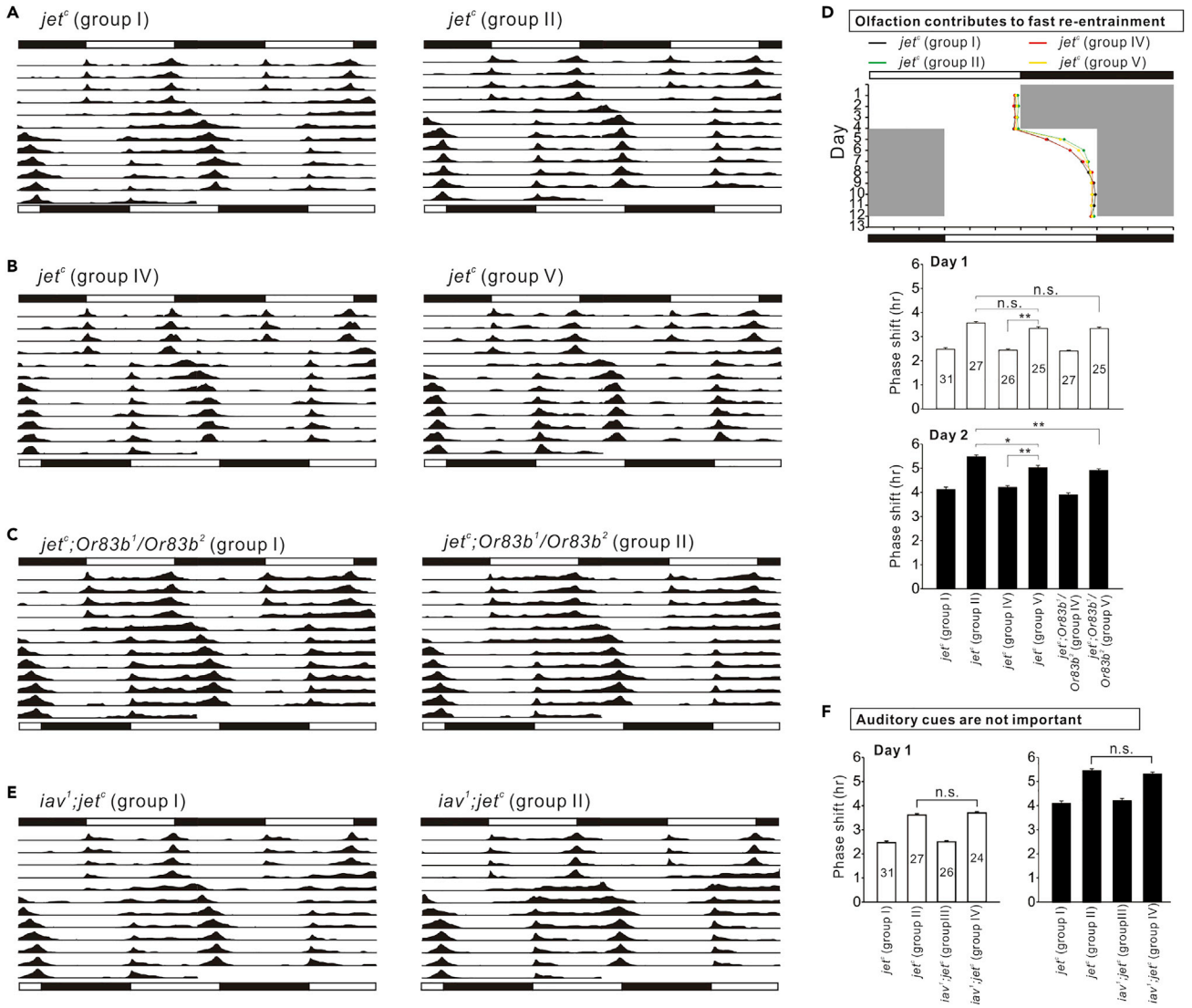

Figure 2. Olfactory Cues Contribute to Re-entrainment of *jet*^{c} Mutants Preferentially on Day 2

(A–C) The sentence "Representative actograms of 12 flies for each genotype." can be changed to "Representative actograms of *jet*^{c} (group I) (A), *jet*^{c} (group IV) (B) and *jet*^{c};*Or83b*^{1}/*Or83b*^{2} (group I) (C) flies. Data were smoothed from 12 flies for each group.

(D) Quantification of the phase shifts using phase plots (top) and bar graphs (two bottom panels) focusing on days 1 and 2 after a 6-h delay in the LD cycle. Note that *jet*^{c} mutants move their evening peak by 2.4 h on day 1 and 1.7 h on the second day in group IV, whereas they shift their evening peak by 3.3 h on day 1 and 1.8 h on the second day in group V. Similarly, *jet*^{c};*Or83b*^{1}/*Or83b*^{2} exhibited fast re-entrainment on the first day and first 2 days in group II compared with those in group I. Note that *jet*^{c};*Or83b*^{1}/*Or83b*^{2} and *jet*^{c} (group V) mutants exhibited similar phase shift on day 1 but slower re-entrainment on day 2 compared with those of *jet*^{c} in group II.

(E) Representative actograms of 12 flies for *iav*^{1};*jet*^{c} mutants.

(F) Quantification of phase shifts on days 1 and 2 after 6-h delay in the LD cycle for the mutants in (E). *iav*^{1};*jet*^{c} mutants exhibited fast re-entrainment and no significant differences from *jet*^{c} in group II. Mean ± SEM is shown. "ns," "*," and "**" denote not significant, $p < 0.05$, and $p < 0.01$, respectively. One-way ANOVAs followed by post hoc Tukey tests were performed for multiple comparisons.

electrophysiological responses to many odorants. Similar results were obtained from *jet*^{c};*Or83b*^{1}/*Or83b*^{2} double mutants in groups I and II (Figures 2C and 2D), suggesting that olfactory cues contribute to re-entrainment preferentially on day 2 and visual cues may contribute to fast re-entrainment on day 1 in the absence of olfaction. We also tested *iav*^{1} auditory mutants crossed to *jet*^{c} mutants (*iav*^{1};*jet*^{c}) and found that they still exhibited faster re-entrainment in group II (with social exposure) than did those in group I (isolated) (Figures 2E and 2F), indicating that hearing was dispensable to the process, at least in our

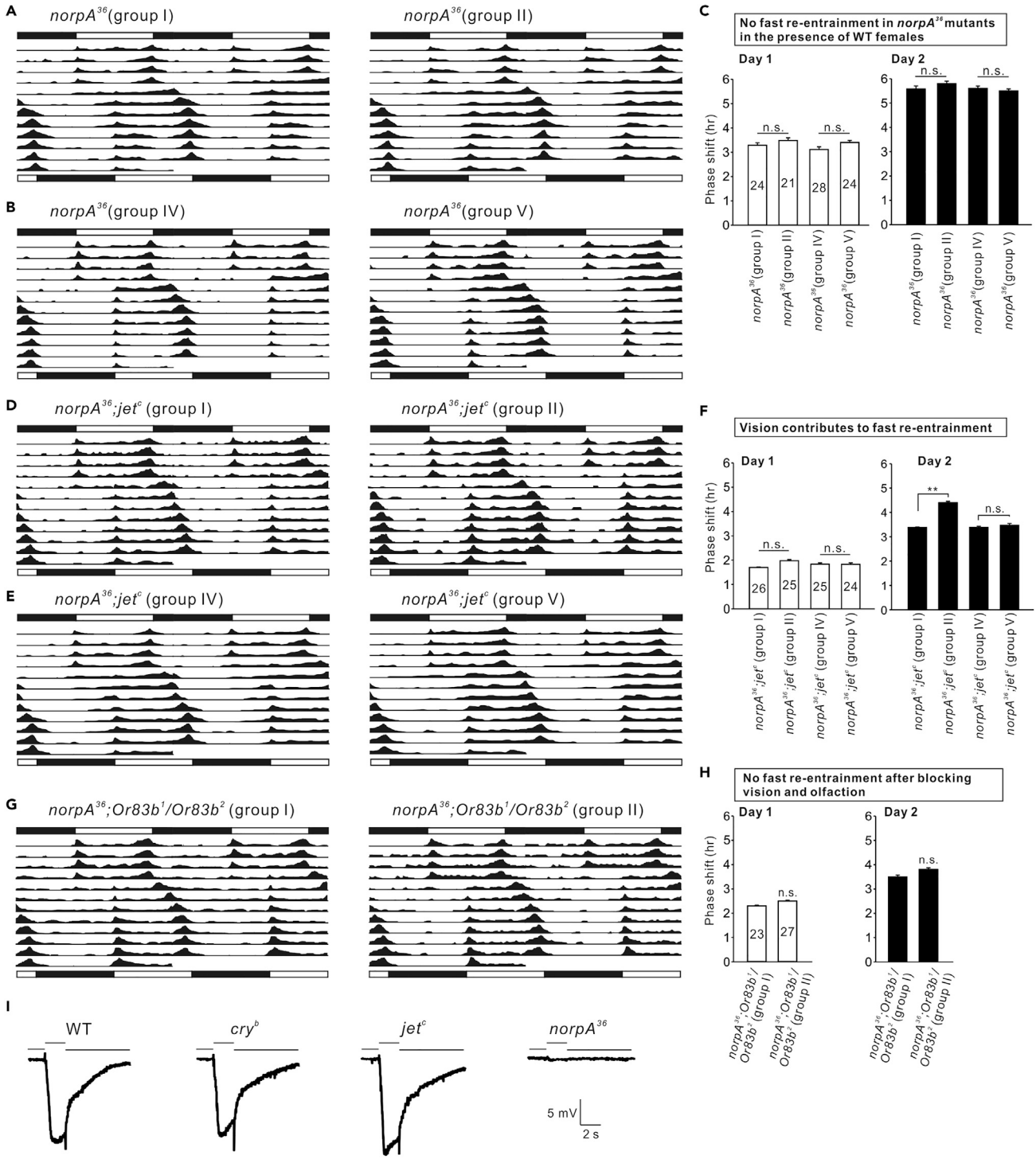

Figure 3. Visual Cues Contribute to Re-entrainment of *jet*^{c} Mutants Preferentially on Day 1

(A and B) Representative actograms of *norpA*^{36} mutants in four different groups, including group I (A, left), group II (A, right), group IV (B, left) and group V (B, right). Data were smoothed from 12 flies for each group.

(C) Quantification of the phase shifts on days 1 and 2 after 6-h delay in the LD cycle. No significant changes in phase shifts were observed in *norpA*^{36} mutants in groups I, II, IV, and V.

(D and E) Representative actograms of *norpA*$^{36;jet^{c}}$ mutants in four different groups, including group I (D, left), group II (D, right), group IV (E, left) and group V (E, right). Data were smoothed from 12 flies for each group.

Figure 3. *Continued*

(F) Quantification of the phase shifts on days 1 and 2 after 6-h delay in the LD cycle. Note that $norpA^{36};jet^{c}$ mutants moved their evening peak by 1.7 h on the first day and by 1.7 h on the second day in group I, whereas they shifted their evening peak by 1.9 h on the first day and by 2.5 h on the second day in group II. After blocking chemosensory signals (groups IV and V), flies exhibited similar phase shifts in the two groups.

(G) Representative actograms of 12 flies for $norpA^{36};Or83b^{1}/Or83b^{2}$ double mutants in groups I and II.

(H) Quantification of the phase shifts. No significant changes in phase shifts were observed after blocking visual and chemosensory cues in the double mutants.

(I) Representative ERG responses using 2-s light with an intensity of 10^{14} photons per second per cm^{2}.

Mean ± SEM is shown. "ns" and "**" denote not significant and $p < 0.01$, respectively. One-way ANOVAs followed by post hoc Tukey tests were performed for multiple comparisons.

experimental setup. Moreover, because males and females can hardly touch each other owing to small size of holes and thickness of the tubes (see Methods), other mechanical cues were not likely involved.

Phospholipase C-β (PLC-β) encoded by *no receptor potential A* (*norpA*) is an essential enzyme in the canonical phototransduction cascade operating in the visual photoreceptor cells. To verify that visual input was required for the rapid re-entraining effect by social interactions, we used $norpA^{36}$ mutants, which generally lack ERG responses (Figure 3I), indicating impaired vision in these mutants (Ogueta et al., 2018). We noticed that $norpA^{36}$ mutants required 2–3 days to be fully re-entrained to the shifted LD cycle (Figures 3A–3C) (Emery et al., 2000). Importantly, these flies exhibited re-entrainment kinetics similar to those of flies in groups I, II, IV, and V (Figures 3A–3C), indicating that visual inputs were essential for accelerating clock re-entrainment in the absence or presence of chemosensory interactions (volatile chemical signals were blocked in groups IV and V). Surprisingly, $nropA^{36}$ mutants did not re-entrain faster in group II compared with those in group V on day 2, indicating that olfactory cues did not accelerate re-entrainment in the mutants. This unexpected result could be due to almost full re-entrainment of the mutants on day 2, whereas olfactory cues preferentially accelerate re-entrainment on day 2.

Our data have shown that olfactory inputs contribute to the fast re-entrainment of jet^{c} mutants and visual cues may also be involved. Moreover, we noticed that blocking chemosensory communications (group V) delayed re-entrainment of flies compared with those in group II on day 2, but no effects were observed on day 1 (Figures 2A, 2B, and 2D), indicating that visual inputs may contribute to fast re-entrainment on day 1. Thus, we crossed jet^{c} and $norpA^{36}$ mutants and found that the $norpA^{36};jet^{c}$ double mutants did not exhibit accelerated re-entrainment in group II compared with group I (Figures 3D–3F) on day 1, indicating that visual cues are indeed essential for fast re-entrainment. Moreover, the fast re-entrainment on day 2 for the double mutants in group II was abolished by blocking volatile chemical signals in group V, confirming that olfactory cues contribute to fast re-entrainment on day 2 (Figures 3D–3F). Moreover, we bred $norpA^{36};Or83b^{1}/Or83b^{2}$ double mutants lacking both visual and olfactory signaling and found that the slow re-entrainment deficits of the mutants could not be rescued by promoting social interactions (Figures 3G and 3H). These results also confirmed our conclusion that touch does not significantly contribute to social entrainment. Unexpectedly, these flies re-entrained slower than $norpA^{36}$ mutants in groups IV and V (for example, $norpA^{36};Or83b^{1}/Or83b^{2}$, group II on day 1, 2.5 ± 0.05 h; $norpA^{36}$, group V on day 1, 3.4 ± 0.07 h). The reason for this slow entrainment is unclear, and interactions between different sensory cues and clock entrainment require further investigation. Taken together, these results demonstrate that both olfactory and visual inputs contribute to fast re-entrainment.

Oscillations of the Clock *per* Gene Expression during Re-entrainment

Next, we assayed the expression levels and oscillations of a core clock gene, *per*, in *Drosophila* heads to clarify the molecular changes that occur during re-entrainment. Before the LD shifts, all transcripts of *per* mRNA examined in all genotypes showed the expected daily oscillations with a peak at ZT 16 and a trough at ZT 4 (Figure 4). However, after a phase delay in LD cycles, *per* gene expression patterns were significantly changed in different genotypes (Figure 4). In WT flies, the peak time and amplitude of *per* oscillations completely recovered on day 2. *per* expression in isolated jet^{c} flies (group I) lost rhythmicity on day 1, oscillated with low amplitude on days 3–4, and completely recovered to the original expression pattern on day 5. In grouped jet^{c} flies (group II), per expression oscillated with low amplitude on days 1–3 and completely recovered on day 4. Flies in group II exhibit faster re-entrainment compared with those in group I, although the difference in *per* oscillation expression seemed mild. The majority of mRNA transcripts were from the eyes, not the pacemaker neurons in the heads, and eye clocks synchronized through

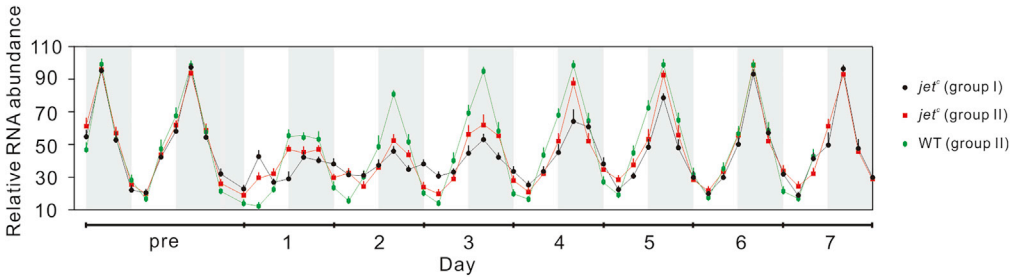

Figure 4. *jet*^{c} Mutants Subjected to LD Phase Shifting Showed Fast Re-entrainment by the Promotion of Social Interactions

Measurement by qRT-PCR of per gene expression profiles in fly heads, obtained every 4 h throughout the schedule. Each data point indicates an average of three independent experiments.

CRY, not photoreceptors (Emery et al., 2000; Zeng et al., 1994). Thus, our RT-PCR results do not reflect brain pacemaker neuron environment but mostly what happens in the eyes.

In summary, we have shown that interactions with WT flies partially rescue re-entrainment deficiency in *jet*^{c} and *cry*^{b} mutants, as measured by locomotor activity. Previous works suggest that olfactory signaling mediates social entrainment in flies (Eban-Rothschild and Bloch, 2012; Fujii and Amrein, 2010; Fujii et al., 2007; Levine et al., 2002; Lone et al., 2016; Lone and Sharma, 2012). In contrast, we found that both visual and olfactory inputs significantly contributed to re-entrainment. Furthermore, the influences of both cues presented jointly were nearly identical to the sum of influences presented in isolation: visual inputs affected both day 1 and day 2 re-entrainment, whereas olfactory inputs preferentially affected that on day 2. Light is the major stimulus for the synchronization of circadian clocks with LD cycles, and light signaling through the visual pathway also contributes to proper synchronizing and phasing in flies (Helfrich-Forster, 2019; Helfrich-Forster et al., 2001; Saint-Charles et al., 2016). Here, our data indicate that visual system is important for clock re-entrainment in particular. Further studies are needed to test the nature of visual stimuli on re-entrainment; for example, whether any movement can be an entraining cue or specific for female flies? Auditory cues did not affect clock re-entrainment in our experimental setup, and this could be due to the limited interactions (no touching and small holes for communications) between MT and WT flies. Mechanical stimulus was found to entrain *Drosophila* clock by vibratory stimulus through a bass loudspeaker (Simoni et al., 2014). In summary, most environmental stimuli that lead to changes in an animal's behavior may affect clock entrainment (Simoni et al., 2014). Although *per* expression exhibited normal oscillation in *jet*^{c} mutants, they exhibit relatively slow re-entrainment of the *per* rhythm during LD shifts. Moreover, interactions with WT flies partially restored re-entrainment deficits of *per* rhythms in *jet*^{c} mutants. Note that we measured total per transcript in eyes and brain (fly head) and the transcripts were approximately three times higher in the eyes than in the brain (Zeng et al., 1994). We predict that the eye clock can be regulated by central pacemakers in subsequent days after LD shifts, although the effects could be weak. A previous study reported that early-morning exercise could accelerate re-entrainment (Mrosovsky and Salmon, 1987), indicating that anticipating locomotor activity pattern changes is beneficial for managing clock re-entrainment.

Limitations of the Study

Although our data revealed that social interactions through parallel pathways (visual and olfactory) accelerate re-entrainment in *Drosophila jet*^{c} mutants, which exhibit deficits in re-entrainability, further studies are needed to define the neuronal and molecular mechanisms governing such social re-entrainment. Whether the promotion of social interactions is beneficial for the management of circadian rhythm misalignment has not been fully determined. Further research should test regulation of clock re-entrainment by social interactions in different animal models utilizing more advanced animal activity monitoring systems.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

DATA AND CODE AVAILABILITY

The data that support the findings of this study are available from the authors on reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.100856.

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AUTHOR CONTRIBUTIONS

Y.P. directed and coordinated the study. Y.P., L.S., M.L., L.Y., and J.Z. conceived and conducted the experiments. Y.P. wrote the manuscript. All authors approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Contribution of Social Influences through Superposition of Visual and Olfactory Inputs to Circadian Re-entrainment

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Supplemental Figures

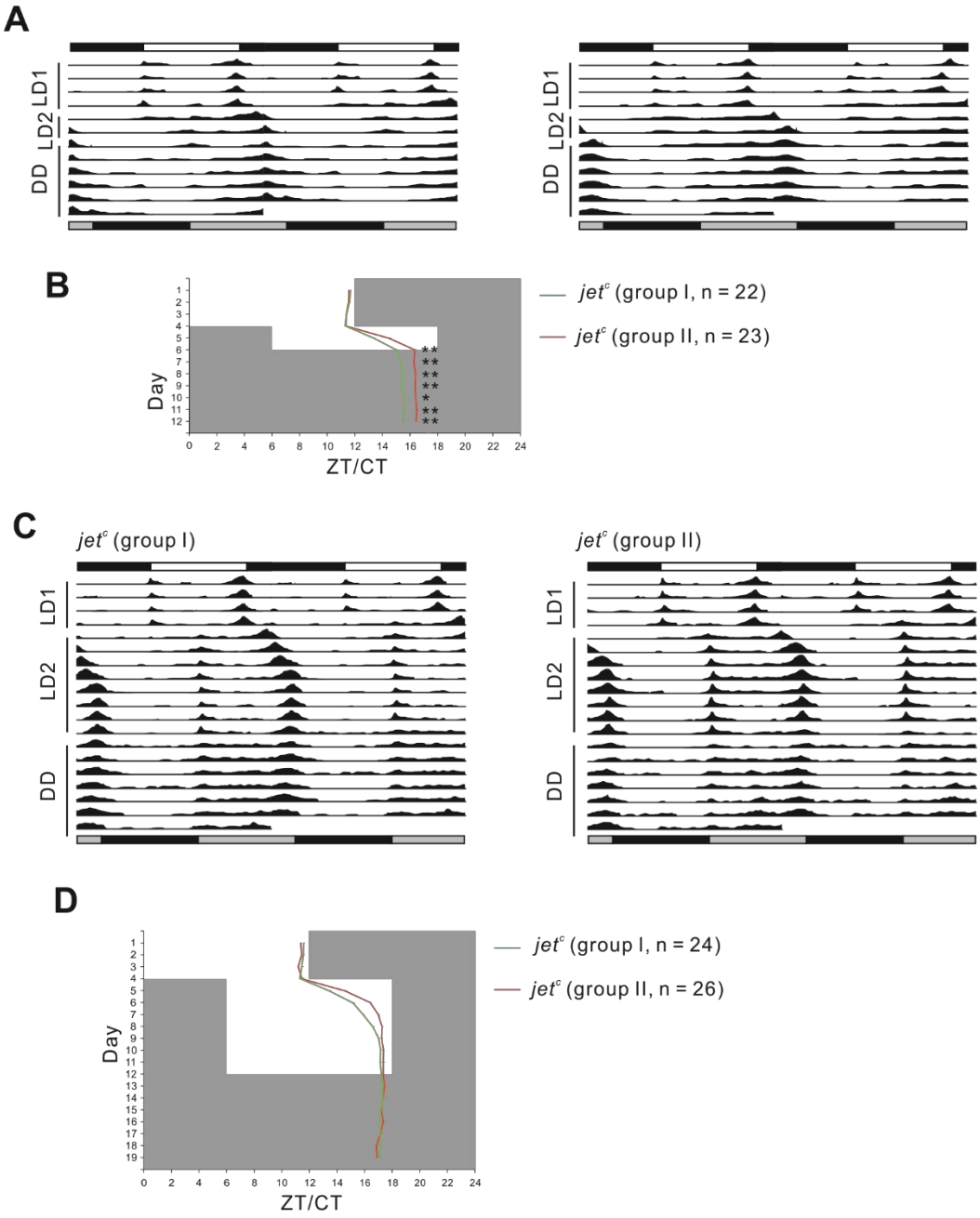

Figure S1. Determination of effects of transient phase delay (2 days) and complete re-entrainment on the biological clock by the release of experimental flies to DD without females, related to Figure 1.

(A) Representative actograms of 12 flies for *jet*^{c} mutants. We allowed females to freely move out of the tubes on the day before releasing the mutant males to DD during ZT 11-12 (group II).

(B) Quantification of the position of the evening peaks. Our results show that *jet*^{c} flies exposed to WT females maintained larger phase delays in free-running behavior compared with isolated *jet*^{c} flies.

(C) Representative actograms of 12 flies for *jet*^{c} mutants. We allowed females to freely move out of the tubes on the day before releasing the mutant males to DD during ZT 0-1.

(D) Quantification of the position of the evening peaks. The evening peaks remained constant after releasing to DD.

Transparent Methods

Animals

Flies were raised at 23°C on a diet of cornmeal, yeast, and sucrose and agar food, under traditional LD cycles (Song et al., 2017). W^{1118} (#5905), jet^{c} (#27642), cry^{b} (#76023), $Or83b^{1/2}$ (#23129/23130) and $norpA^{36}$ (#9048) lines were obtained from the Bloomington Stock Center.

Drosophila activity monitoring

One to three days old male flies were individually entrained and recorded in tubes containing standard fly food using the *Drosophila* Activity Monitor System (DAM2; TriKinetics) (Song et al., 2017). To promote social interactions with WT flies without interrupting recording, we added two transparent plastic cylinders (diameter 2.0 cm; height, 1.8 cm) to both sides of each recording tube, and 8 WT female flies (groups II and V) or no flies (groups I and IV) were included in each cylinder (Figure 1A). We also added 8 MT female flies in group III as control. We also included 4-6 holes (diameter 1.0 mm) on each side of the recording tubes (thickness, 1.1 mm) to allow possible communication between flies may occur over a short distance by means of endogenous volatile chemical signals (groups I, II and III), and we presumed that the communication was terminated when the holes were blocked (groups IV and V). Eight test flies were recorded at once in each recording panel. WT or MT flies were loaded into the tube at the same time as the test flies. The flies were raised in tubes with an additional temperature cycle of 25 ℃ during lights on and 18 ℃ during lights off for the first four days. Then, the temperature was kept constant at 25 ℃ on the last day before the LD shifts. The averages, SEMs and plotting of the results were performed in GraphPad Prism 6 and R. The evening activity peaks of individual histograms from each fly were assessed in a custom-made Excel Macro by smoothing of the data using digital low-pass filter (Helfrich-Forster, 2000). The phase shift was calculated as the phase difference of the evening peaks before and after LD shifts. Double-plotted actograms were averaged from 12 entrained flies (Feng et al., 2018).

Electroretinogram recordings

Intact flies with the indicated genotypes were immobilized with low-melting-point wax in truncated

Eppendorf tips. ERG was recorded using ~8 MΩ glass microelectrodes filled with fly's normal Ringer's solution (140 mM NaCl, 5 KCl, 1.5 $CaCl_2$, 4 $MgCl_2$) inserted into the fly eye. A similar electrode was inserted into the head capsule as a reference. Signals were amplified by a DAM60 DC amplifier (WPI) and sampled and analyzed using pClamp 9.2 software. Light was delivered via an LED lamp. The light intensity of the stimulus was approximately 1.3×10^{14} photons per second per cm^2 (QE6500 spectrometer, Ocean Optics).

Quantitative RT-PCR

We measured transcripts of *per* mRNA in fly heads, including eyes and brains. Fly heads were isolated and collected at the indicated time points (at 4-hr intervals) on dry ice. Isolated heads were immersed in acetone on dry ice for 2-3 hrs, and then were kept at -20 ℃ for use. Total RNA was extracted from 10 heads using TRI Reagent (Sigma) for each sample. The samples were then purified using an RNeasy Mini Kit (Qiagen) with on-column DNase digestion (Qiagen). Quantitative RT-PCR was performed with a Step One Plus Real-Time machine (Applied Biosystems). We used default thermal cycling conditions with a dissociation curve step. The PCR products were resolved by 1% agarose gel electrophoresis. The relative quantities of gene transcripts were measured using the $2^{-\triangle\triangle C_T}$ method (Livak and Schmittgen, 2001). mRNA levels were normalized to the constitutively expressed ribosomal protein gene 49 (*rp49*, as internal control). We confirmed that rp49 transcripts do not vary with the time of day by application of the $2^{-\triangle\triangle C_T}$ method (Livak and Schmittgen, 2001). The relative mRNA amplitude was calculated with respect to the peak levels set as 100 for each group. Before the LD shifts, all transcripts of *per* mRNA examined in the genotypes showed the expected daily oscillations with peak at ZT 16 and trough at ZT 4. Thus, we compared the relative levels of per transcript at ZT 16 for determining clock entrainment on each day. Additionally, if the peak time was appeared unexpectedly during the daytime after LD shifts (including ZT0, ZT4 and ZT8), we deemed the *per* expression completely lost rhythmicity. The primer sequences used for the quantitative RT-PCR experiments were: Per-F 5'-CAGCAGCAGCCTAATCG-3' and Per-R 5'-GAGTCGGACACCTTGG-3'; Rp49-F 5'-CGACGCTTCAAGGGACAGTATC3' and Rp49-R 5'-TTACGACACCAAACGATCGA3'.

Statistical analysis

One-way ANOVA followed by post hoc Tukey tests were performed using SPSS software (RRID:SCR_002865). See the results and figure legends for specifics on statistical tests for each experiment. Statistical significance was defined as *$p < 0.05$, and data are reported as mean ± SEM.

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