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timeless Plays an Important Role in Compound Eye-Dependent Photic Entrainment of the Circadian Rhythm in the Cricket *Gryllus bimaculatus*

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The light cycle is the most powerful Zeitgeber entraining the circadian clock in most organisms. Insects use CRYPTOCHROMEs (CRYs) and/or the compound eye for the light perception necessary for photic entrainment. The molecular mechanism underlying CRY-dependent entrainment is well understood, while that of the compound eye-dependent entrainment remains to be elucidated. Using molecular and behavioral experiments, we investigated the role of *timeless* (*tim*) in the photic entrainment mechanism in the cricket *Gryllus bimaculatus*. RNA interference of *tim* (*tim*^{RNAi}) disrupted the entrainment or prolonged the transients for resynchronization to phase-delayed light–dark cycles. The treatment reduced the magnitude of phase delay caused by delayed light-off, but augmented advance shifts caused by light exposure at late night. TIM protein levels showed daily cycling with an increase during the night and reduction by light exposure at both early and late night. These results suggest that *tim* plays a critical role in the entrainment to delayed light cycles.

Key words: circadian clock, cricket, compound eye, entrainment, phase shifts, RNAi, *tim*

INTRODUCTION

Entrainment to the daily environmental cycle is a fundamental property of the circadian clock. For most organisms, the most powerful entraining agent is the light-dark cycle (LD). In insects, there are two known photoreceptors for photic entrainment. One is CRYPTOCHROME (CRY), which is a blue light receptor whose sequence is similar to that of DNA photolyases (Lin and Todo, 2005), and the other is the compound eye (Helfrich-Förster, 2020). In the fruit fly *Drosophila melanogaster*, CRY is expressed in some cerebral clock neurons (Emery et al., 1998, 2000; Stanewsky et al., 1998; Yoshii et al., 2008) and activated by blue light, leading to proteasomal TIMELESS (TIM) degradation (Lee et al., 1996; Ceriani et al., 1999; Lin et al., 2001), which thus resets the clock in a phase-dependent manner. TIM reduction during early night delays the clock because TIM content must increase to the level required for TIM to enter the nucleus and prohibit its transcription by inactivating the transcriptional activator, CLOCK (CLK)/CYCLE (CYC) heterodimer. At late night, in the declining phase of TIM, the further reduction leads to an advance shift of the clock to the phase at which the TIM level is equal to the reduced level. This resetting mechanism has also been demonstrated in monarch butterflies (Zhu et al., 2008). It has also been suggested in several other species, but has not been proven (Cortes et al., 2010; Yan et al., 2013; Jiang et al., 2018).

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The compound eye is the only organ receiving photic information necessary for entrainment in some insects, including cockroaches and crickets (Tomioka and Matsumoto, 2019). Since locomotor rhythms start to free-run even under LD when optic nerves are bilaterally severed in these insects (Nishiitsutsuji-Uwo and Pittendrigh, 1968; Tomioka and Chiba, 1984), the neural information from the compound eye is required for the clock to synchronize with the LD. The underlying molecular mechanism has only been partially clarified in the cricket *Gryllus bimaculatus*. The circadian clock of this species consists of two oscillatory loops (Tokuoka et al., 2017; Tomioka and Matsumoto, 2019): the *period* (*per*)/*tim* oscillatory loop, similar to that of the *Drosophila* clock, and the *cry* loop. In both loops, CLK/CYC acts as a transcriptional activator that promotes *per* and *tim*, or *cry2* transcription. The product proteins PER and TIM, or CRY2 are thought to negatively feed back to inhibit the transcriptional activator.

Light is perceived through opsin-long wavelength, a green-light receptor molecule expressed in the compound eye (Komada et al., 2015). The light information is conveyed through the neural pathway to the clock cell in the lamina and medulla region of optic lobes (Kutaragi et al., 2018), and activates two pathways. Delayed light-off upregulates *Par domain protein 1* (*Pdp1*), which further upregulates *Clk* and *tim*, thus eventually delaying the clock (Kutaragi et al., 2016). Light exposure in the dark never upregulates *Pdp1*, but upregulates *c-fosB*, which acts on two CRYs, CRY1 and CRY2, via currently unknown pathways, and eventually resets the clock (Kutaragi et al., 2018). However, how *tim* is involved in the entrainment mechanism and its relationship

with CRYs are currently unknown.

The present study aimed to clarify the role of *tim* in the photic entrainment of the circadian clock in *G. bimaculatus*. We also examined the role of *Pdp1*, which probably works upstream of *tim* to affect CLK levels (Kutaragi et al., 2016). We performed RNA interference (RNAi) to suppress expression of *tim* and *Pdp1* and the effects were analyzed at behavioral and molecular levels. TIM protein showed cyclic expression peaking during the night/subjective night, and light induced reduction of TIM levels. RNAi of *tim* and *Pdp1* significantly reduced the TIM protein levels. Crickets treated with tim^{RNAi} failed to synchronize to a phase-delayed LD. On the basis of these findings we propose a model in which *tim* plays a critical role in entrainment to delayed light cycles.

MATERIALS AND METHODS

Animals

All experiments were performed using young adult male *G. bimaculatus* crickets within a week after the imaginal molt. They were taken from our laboratory colony maintained under controlled conditions of LD with 12 h light and 12 h darkness (light: 0600–1800, Japan Standard Time) and 25.0 ± 1.0°C. The crickets were fed laboratory chow (CA-1, CLEA Japan; Tokyo, Japan) and provided with water.

RNAi

Double-stranded RNA (dsRNA) of *G. bimaculatus tim* (GenBank/EMBL/DDBJ accession no. BAJ16356), *G. bimaculatus Pdp1* (LC512908), and *DsRed2* derived from a coral species (*Discosoma* sp.) were synthesized using the CUGA in vitro Transcription Kit (Nippon Gene, Tokyo, Japan). For *tim* and *Pdp1*, cDNAs were used as templates for PCR performed using KOD-Plus-Neo (TOYOBO, Osaka, Japan). The T7- or T3- containing primers used are listed in Table 1. Amplified *tim* (519 bp) and *Pdp1* (475 bp) fragments were extracted with phenol/chloroform, precipitated with ethanol, and then resuspended in Ultra Pure Water (Invitrogen, Tokyo, Japan). For preparing *DsRed2* dsRNA (659 bp), the linearized *DsRed2* fragment was amplified from pDsRed2-N1 (Clontech, Mountain View, CA, USA) using the primers listed in Table 1. With each of these linearized fragments as a template, RNA was synthesized using T7 or T3 RNA polymerase. Synthesized RNAs were extracted with phenol/chloroform and suspended in 50 μL of TE solution after isopropanol precipitation. After mixing equal amounts of sense and antisense RNA, the RNAs were denatured for 5 min at 100°C and annealed by gradual cooling to room temperature. After ethanol precipitation, the obtained dsRNA was precipitated with ethanol, suspended in Ultra Pure Water (Invitrogen), and adjusted to 20 μM final concentration. The dsRNA solution was stored at –80°C until use. dsRNA solution (760 nL) was injected with a nanoliter injector (WPI, Sarasota, FL, USA) into the abdomen of adult insects anesthetized with CO_2. Injection was performed within a week after the imaginal molt.

Measurement of mRNA levels

qPCR was used to measure mRNA levels. Total RNA was extracted and purified from two optic lobes, including only lamina and medulla region, for each adult male. The sampling was performed 1 week after dsRNA injection. TRIzol Reagent (Invitrogen) was used for RNA extraction and glycogen was added as a carrier. Total RNA was treated with DNase I to remove contaminating genomic DNA. Approximately 400 ng of total RNA from each sample was reverse transcribed with random hexamers and Oligo dT primers using the PrimeScript RT reagent kit (Takara, Otsu, Japan). Real-time PCR was performed using the Mx3000P qPCR System (Agilent Technologies, Santa Clara, CA, USA) using Thunderbird SYBR qPCR Mix (TOYOBO) including SYBR Green with primers shown in Table 1. *rpl18a* (GenBank/EMBL/DDBJ accession no. DC448653) was used as the internal control. Quantification was performed based using a standard curve obtained with a known amount of template. The results were analyzed using the software associated with the instrument. The values were normalized with the values of *rpl18a* at each time point. The results of five independent experiments were used to calculate mean ± standard error of the mean (SEM).

Anti-*Gryllus* TIMELESS antibody preparation

The *Gryllus* TIM antibody was generated in rabbits by Eurofins Genomics Inc. (Tokyo, Japan). Two different peptides of *Gryllus* TIM, VVQDKKELRRKKLV (residues 452–465) and SDSSKGSSKSSGDS (residues 323–336), were chemically synthesized and the N-terminal was coupled to KLH using a bifunctional crosslinker, 3-maleimidobenzoyl-N-hydroxy succinimide ester. Two rabbits were immunized with each of the conjugated antigens using a conventional method. The four different sera obtained were tested by Western blotting. Finally, an antiserum directed against the peptide "VVQDKKELRRKKLV" was selected for further analysis.

Western blotting

Before sampling, crickets were placed in LD with 12 h of light and 12 h of darkness (L: 06:00–18:00) for at least 7 days at 25 ± 1°C. For sampling in constant darkness (DD), crickets were transferred from the LD to DD at 18:00 1 day before sampling. Some crickets were exposed to a 2 h light pulse at ZT12, 20, or CT12. ZT stands for Zeitgeber time and ZT0 and ZT12 correspond to light-on and light-off, respectively, under a 12 h of light to 12 h of dark cycle, whereas CT stands for circadian time and CT0 and CT12 correspond to the beginning of the subjective day and subjective night, respectively. Light intensity of the light phase and light pulse was approximately 2 W/m^2. The crickets were anesthetized with CO_2 at each sampling time point, and the optic lobe-compound eye complexes of the crickets were extracted, individually placed in microcentrifuge tubes, quickly frozen in liquid nitrogen, and stored at –80°C until use. Sampling during dark phase was performed under dim red light.

Table 1. PCR primers used for quantitative RT-PCR and dsRNA synthesis. The primers tagged with T7 or T3 promoter sequences were used for PCR amplification for dsRNA synthesis. Adaptor sequences for T7 and T3 promoter are underlined.

Primers	Forward	Reverse
For qPCR		
tim	5′-TCTCTCCAATGCTGTGTGATG -3′	5′-CTGGATCAGGAACTTTAGCACTTT -3′
Pdp1	5′-CCGACTGTTGTGCAGACTAACC -3′	5′-GCTCGCCTTCCTCCTTCTT -3′
rpl18a	5′-GCTCCGGATTACATCGTTGC-3′	5′-GCCAAATGCCGAAGTTCTTG-3′
For dsRNA synthesis		
tim	5′-CTAATACGACTCACTATAGGGAGAGTAAAGAAGATAGAGAGTAT-3′	5′-CAATTAACCCTCACTAAAGGGAGATTGGAGAGAACTGAAGAGGT-3′
Pdp1	5′-CTAATACGACTCACTATAGGGAGATCAACGGACACGTCAAGGT-3′	5′-CAATTAACCCTCACTAAAGGGAGAAGCCCCATGTTCTCCTTCTC-3′
DsRed2	5′-CTAATACGACTCACTATAGGGAGATCATCACCGAGTTCATGCG-3′	5′-CAATTAACCCTCACTAAAGGGAGACTACAGGAACAGGTGGTGGC-3′

Protein extracts were prepared from the single optic lobe-compound eye complexes. Each sample was homogenized using a pestle in 100 μL sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 100 mM Tris-HCl (pH 6.8), 5% glycerol, 150 mM dithiothreitol, 0.0025% bromophenol blue), and sonicated for 1 min using a Bioruptor (Tosyo Denki, Yokohama, Japan). After sonication, each sample was heated to 95°C and then loaded on an 8% polyacrylamide–SDS Laemmli gel. The proteins were transferred to a nitrocellulose membrane using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA, USA) for 1 h at 25 V in Bjerrum Schafer–Nielsen buffer (48 mM Tris, 39 mM glycine, 20% methanol). The membrane was incubated in TBST (20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5% Tween-20) containing 5% skim milk and the antibody. We used the anti-TIM antibody (1:3000) and monoclonal anti-α-tubulin antibody produced in mouse (1:100,000, Sigma-Aldrich, Tokyo, Japan) as the primary antibody, and horseradish peroxidase (HRP)-linked goat anti-rabbit immunoglobulin G (1:5000, MP Biomedicals, Tokyo, Japan) and HRP-conjugated anti-mouse IgG (H+L) (1:5000, Promega, Tokyo, Japan) as the secondary antibody. The signals were visualized using the Immobilon Western Chemiluminescent HRP Substrate on LAS-4000 (Fuji Films, Tokyo, Japan).

Locomotor activity recording

The locomotor activities of the crickets were recorded as

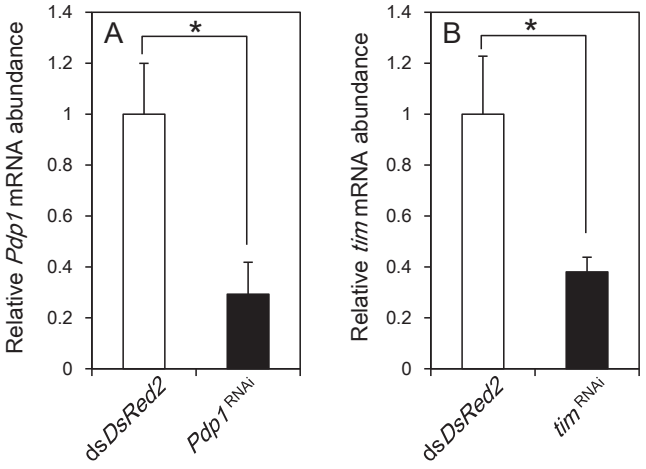

Fig. 1. Effects of *Pdp1*^{RNAi} **(A)** and *tim*^{RNAi} **(B)** on mRNA levels of the respective gene in the optic lobe in the cricket *Gryllus bimaculatus*. The mRNA levels were measured at Zeitgeber time (ZT)18 7 days after dsRNA injection. The values are normalized by the value of ds*DsRed2*-treated control. Note that *Pdp1*^{RNAi} and *tim*^{RNAi} treatment significantly reduced mRNA levels of the respective genes compared to the ds*DsRed2*-treated control. *, *t*-test, $P < 0.05$.

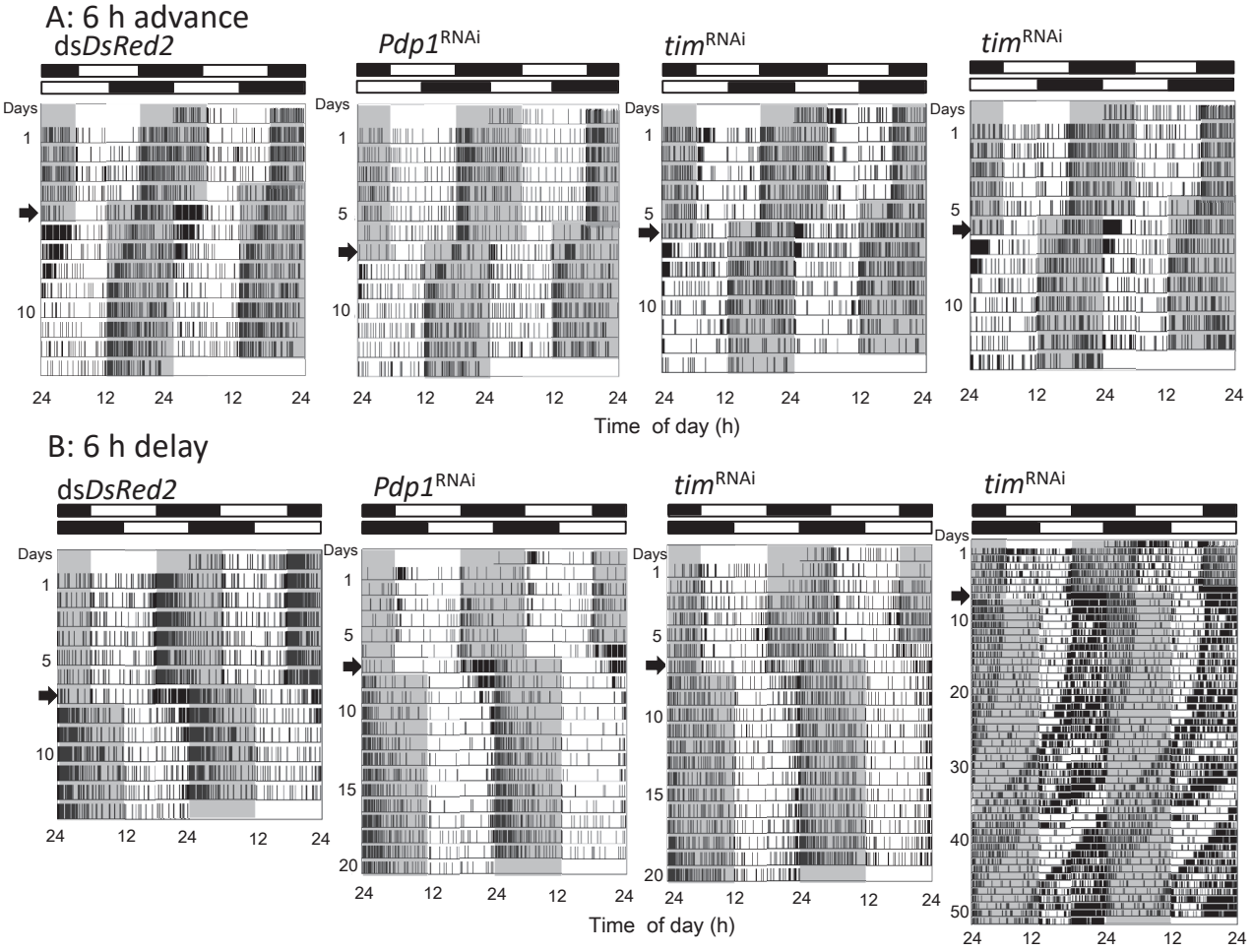

Fig. 2. Double-plotted actograms showing re-entrainment of locomotor rhythms to 6 h **(A)** advanced and **(B)** delayed light–dark cycle in ds*DsRed2*-treated control, *Pdp1*^{RNAi}, and *tim*^{RNAi} adult male crickets *Gryllus bimaculatus*. White and black bars above the actograms show light (white) and dark (black) cycles. Gray area in actograms indicates the dark phase. Arrows indicate the day when light cycle was shifted.

described previously (Moriyama et al., 2008). In brief, adult crickets were individually housed in a transparent plastic box (18 × 9 × 4.5 cm) with a rocking substratum immediately after dsRNA injection. Movement of the substratum caused by a moving cricket was sensed by a magnetic reed switch and recorded every 6 min using a computerized system. Food and water were provided ad libitum. The actographs were placed in an incubator in which lighting conditions were provided by a cool white fluorescent lamp connected to an electric timer, and the temperature was kept constant at 25 ± 0.5°C. The light intensity ranged from 2.04 to 9.27 W/m^2 and varied depending on the distance from the light source. The raw data were displayed as conventional double-plotted actograms to determine activity patterns.

The light cycles were shifted by 6 h in the delay or advance direction by extending or shortening the light phase, respectively, and the transient cycles for re-entrainment were estimated as the time span between the day of shift and the day when the onset restored the original phase relationship with LD.

The phase shifts caused by a 3 h single light pulse at ZT12, ZT20, and CT12 on the first day of DD were investigated. After exposure to the light pulse, the crickets were maintained under DD. The magnitude of phase shifts was estimated as the time difference between the phases of light pulse-treated and untreated animals, which were determined by extrapolation of activity onset under DD to the day of the light pulse using actogramJ (Schmid et al., 2011).

Statistics

One-way ANOVA followed by post hoc Tukey's test was used to compare the means of the differently treated groups. Student's *t*-test was used to compare the means of the two groups.

RESULTS

Re-entrainment of locomotor rhythms to 6 h advanced LD

We first examined the re-entrainment of locomotor rhythms to 6 h advanced LDs in *DsRed2* dsRNA (ds*DsRed2*)-treated control, *Pdp1*^{RNAi}, and *tim*^{RNAi} crickets. The effectiveness of RNAi was investigated in *Pdp1*^{RNAi} or *tim*^{RNAi} crickets. The *Pdp1* or *tim* mRNA levels in the optic lobe were measured at ZT18, when they were near the peak level (Danbara et al., 2010; Narasaki-Funo et al., 2020), and 7 days after the treatment. RNAi treatment significantly suppressed the mRNA levels of the respective genes (Fig. 1), as reported previously (Danbara et al., 2010; Narasaki-Funo et al., 2020). Figure 2 shows representative actograms of ds*DsRed2*-treated control, *Pdp1*^{RNAi}, and *tim*^{RNAi} crickets. Under LD, the three groups of animals showed a nocturnal rhythm similar to that of untreated animals (Tomioka and Chiba, 1982). When the light cycles were advanced by 6 h by shortening the light period, all animals resynchronized to the newly phased LD (Fig. 2A, Table 2). The transient cycles necessary for re-entrainment were approximately 3 days (3.26 ± 1.10 (mean ± SD) days) in the ds*DsRed2*-treated control crickets (Fig. 3A). *Pdp1*^{RNAi} and *tim*^{RNAi} crickets yielded results similar to those of the control crickets, with transient cycles of 3.60 ± 0.70 and 3.14 ± 1.57 days, respectively (Fig. 3A).

Re-entrainment of locomotor rhythms to 6 h delayed LD

We then tested the re-entrainment of locomotor rhythms to 6 h delayed LDs. ds*DsRed2*-treated control crickets resynchronized to the shifted LD with transient cycles of 3.00 ± 0.82 days (Figs. 2B, 3B; Table 2). *Pdp1*^{RNAi} crickets resynchronized to LD with transient cycles of 4.33 ± 1.40 days, which was slightly longer than that in the control crickets, but the difference was not significant (Figs. 2B, 3B; Table 2). In contrast, *tim*^{RNAi} prevented resynchronization in 46.7% of the treated crickets and the remaining crickets resynchronized to the shifted LD with significantly longer transient cycles (5.25 ± 1.84 days; Figs. 2B, 3B; Table 2). Figure 2B right panel shows the actogram of a representative *tim*^{RNAi} cricket showing a free-running rhythm under LD. In this particular cricket, the activity was enhanced when the active phase overlapped with the light phase, suggesting that a photoreceptive system was functioning.

Phase shifts caused by 3 h light pulses

To investigate the effect of *Pdp1*^{RNAi} and *tim*^{RNAi} on phase responsiveness to light, we examined the magnitude of phase shifts of locomotor rhythms caused by a 3 h light pulse given at ZT12–15, CT12–15, or ZT20–23. Ds*DsRed2*-treated crickets were used as controls. The pulse at ZT12–15 was given as a 3 h extension of the light phase. The light treatment was followed by constant darkness.

Table 2. Summary of results of entrainment of locomotor rhythms to light dark cycles shifted by 6 h in the cricket *Gryllus bimaculatus* treated with ds*DsRed2*, *Pdp1*^{RNAi}, or *tim*^{RNAi}.

Treatment	N	Entrainment	
		Yes	No
advance			
ds*DsRed2*	16	16	0
Pdp1 ^{RNAi}	10	10	0
tim^{RNAi}	7	7	0
delay			
ds*DsRed2*	7	7	0
Pdp1 ^{RNAi}	16	16	0
tim ^{RNAi}	30	16	14

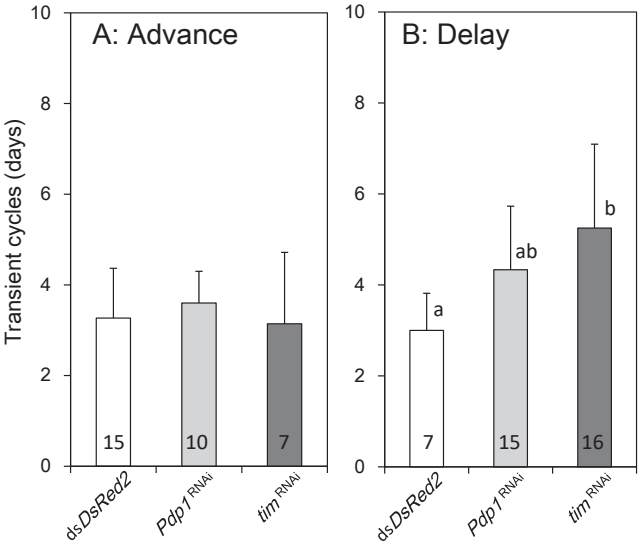

Fig. 3. Transient cycles necessary for re-entrainment of locomotor rhythms to 6 h **(A)** advanced and **(B)** delayed light–dark cycles in ds*DsRed2*-treated control, *Pdp1*^{RNAi}, and *tim*^{RNAi} adult male crickets *Gryllus bimaculatus*. Numbers in each column indicate the number of crickets used. Different symbols above the column indicate significant differences (ANOVA followed by Tukey's test, $P < 0.05$).

A light pulse at ZT20–23 caused an advance shift in all the treated crickets (Fig. 4A). The magnitudes of the shift were 1.02 ± 0.56, 1.46 ± 0.70, and 1.80 ± 0.43 h in ds*DsRed2*-treated control, *Pdp1*^{RNAi}, and *tim*^{RNAi} crickets, respectively (Fig. 5A). No significant difference was found between control and *Pdp1*^{RNAi} crickets, whereas *tim*^{RNAi} significantly increased the magnitude of the shift (Fig. 5A).

A light pulse at ZT12–15 caused a delay shift (Fig. 4B), whose magnitude was similar between ds*DsRed2*-treated control (−3.40 ± 0.44 h) and *Pdp1*^{RNAi} crickets (−3.05 ± 0.58 h) but was significantly smaller in *tim*^{RNAi} crickets (−2.71 ± 0.38 h; Fig. 5B).

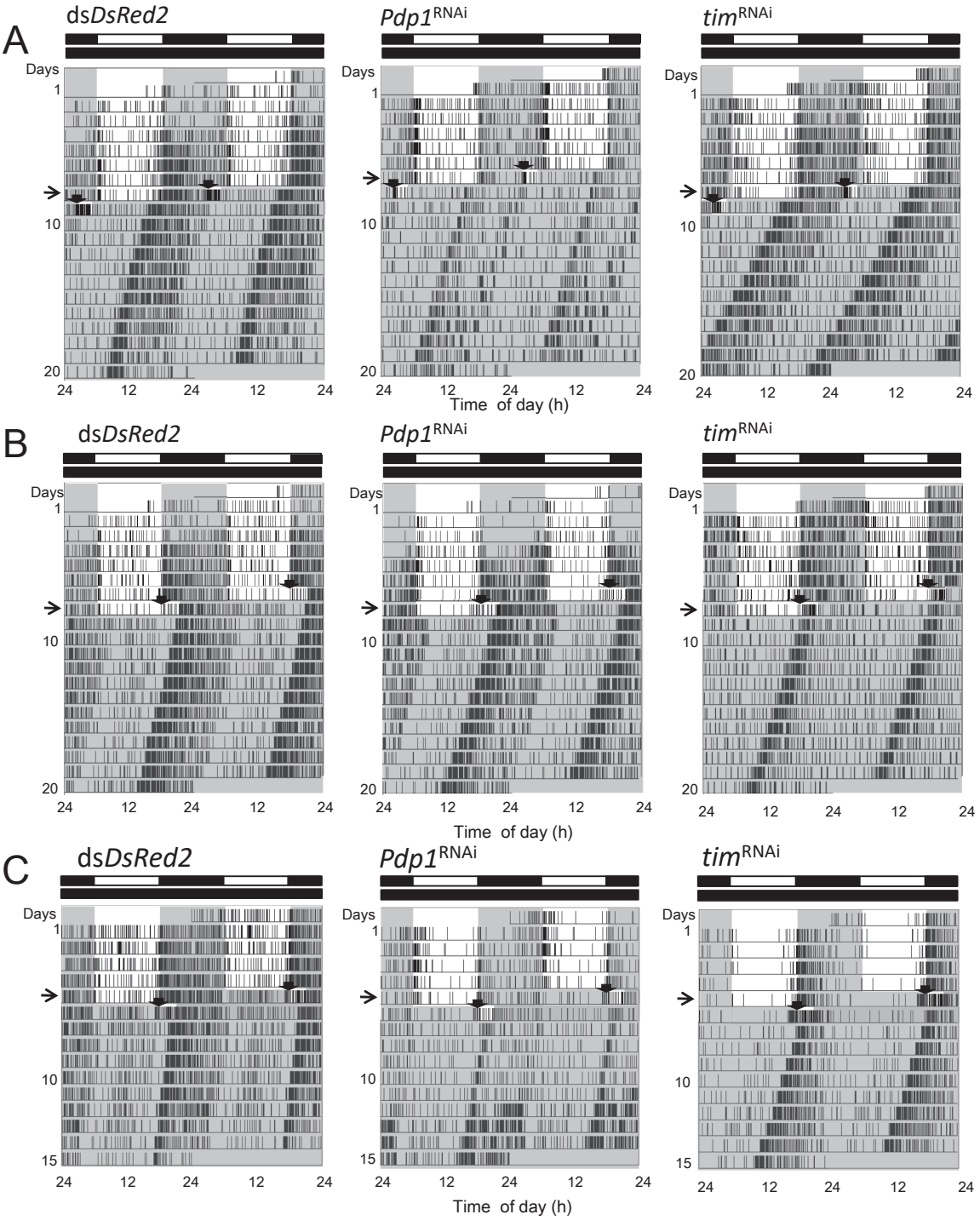

Fig. 4. Phase shifts of locomotor rhythms caused by a 3 h light pulse given at **(A)** Zeitgeber time (ZT)20–23, **(B)** ZT12–15, or **(C)** circadian time (CT)12–15 in ds*DsRed2*-treated control, *Pdp1*^{RNAi}, and *tim*^{RNAi} adult male crickets *Gryllus bimaculatus*. Thick arrows in actograms indicate the start of a light pulse. Thin arrows on the left side of actograms indicate the day of transfer to constant dark (DD). DD started at 18:00 in **(A)** and **(C)**, while at 21:00 in **(B)**. For other explanations see legend of Fig. 2.

A light pulse at CT12–15 also caused a delay shift in all treated crickets (Fig. 4C). The magnitudes of the shift were −2.55 ± 0.64 h, −1.31 ± 0.59 h, and −2.27 ± 0.80 h for ds*DsRed2*-treated control, *Pdp1*^{RNAi}, and *tim*^{RNAi} crickets, respectively (Fig. 5C). Thus, *Pdp1*^{RNAi} significantly reduced the magnitude of the shift compared to the shifts in control and *tim*^{RNAi} crickets. However, the magnitude was not significantly different between control and *tim*^{RNAi} crickets.

TIM cycling and light-induced reduction

By Western blotting, daily changes in TIM levels in the optic lobe-compound eye complex were examined under LD and DD conditions (Fig. 6A). TIM was detected as a protein of approximately 100 kDa, which was close to that expected from the amino acid sequence deduced from the obtained cDNA (Danbara et al., 2010). In ds*DsRed2*-treated animals, TIM showed cyclic expression, with low levels during daytime, increasing during the night to peak at the end of nighttime. The rhythmic changes in the TIM were maintained in constant darkness. TIM levels were strongly reduced by *tim*^{RNAi}. The TIM levels were also reduced by *Pdp1*^{RNAi}.

We then examined the effects of 2 h light exposure at early night (ZT12–14), late night (ZT20–22), and early subjective night (CT12–14). We used 2 h light pulses here because maximum *c-fosB* induction associated with phase shift was caused by 1 h light exposure (Kutaragi et al., 2018), and our preliminary results showed that a 2 h light pulse at ZT18-20 caused significant phase advance of locomotor rhythms: activity onset for crickets with light exposure was 16.47 ± 0.40 h (n = 13) and that for crickets without exposure 17.42 ± 0.25 h (n = 14) (t-test, $P < 0.001$). The results of light exposure are shown in Fig. 6B. In all the phases examined, TIM levels were strongly reduced 2 h after exposure. The reduction at ZT12–14 and at CT12–14 was greater than that at ZT20–22, followed by a gradual increase in the ensuing darkness (Fig. 6B).

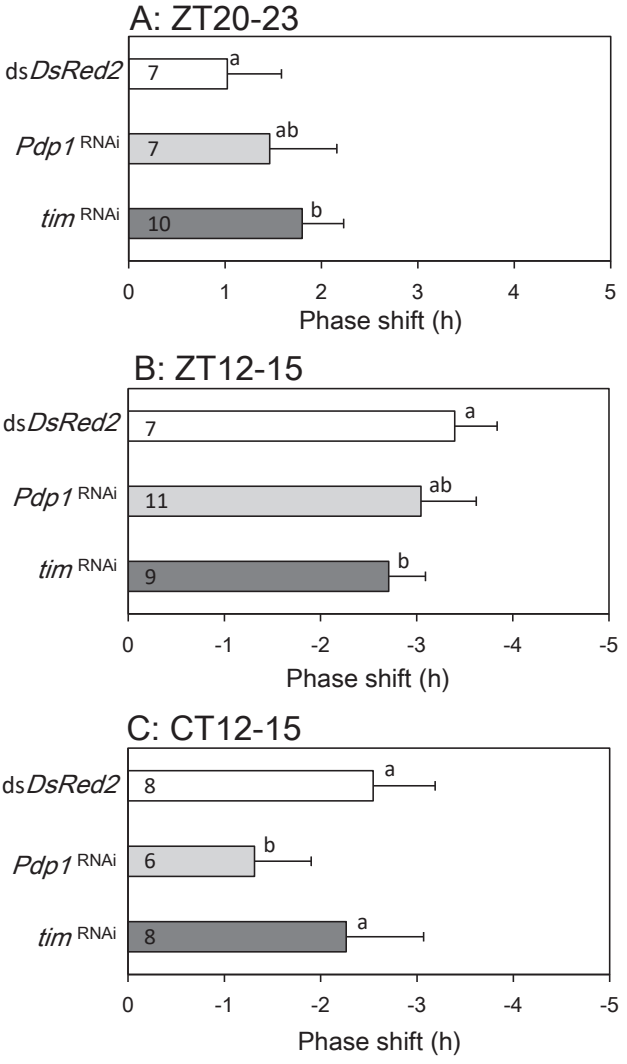

Fig. 5. Magnitude of the phase shifts of locomotor rhythms caused by a 3 h light pulse given at **(A)** Zeitgeber time (ZT)20–23, **(B)** ZT12–15, or **(C)** circadian time (CT)12–15 in ds*DsRed2*-treated control, *Pdp1*^{RNAi}, and *tim*^{RNAi} adult male crickets *Gryllus bimaculatus*. Numbers in each column indicate the number of crickets used. Different symbols indicate significant differences (ANOVA followed by Tukey's test, $P < 0.05$).

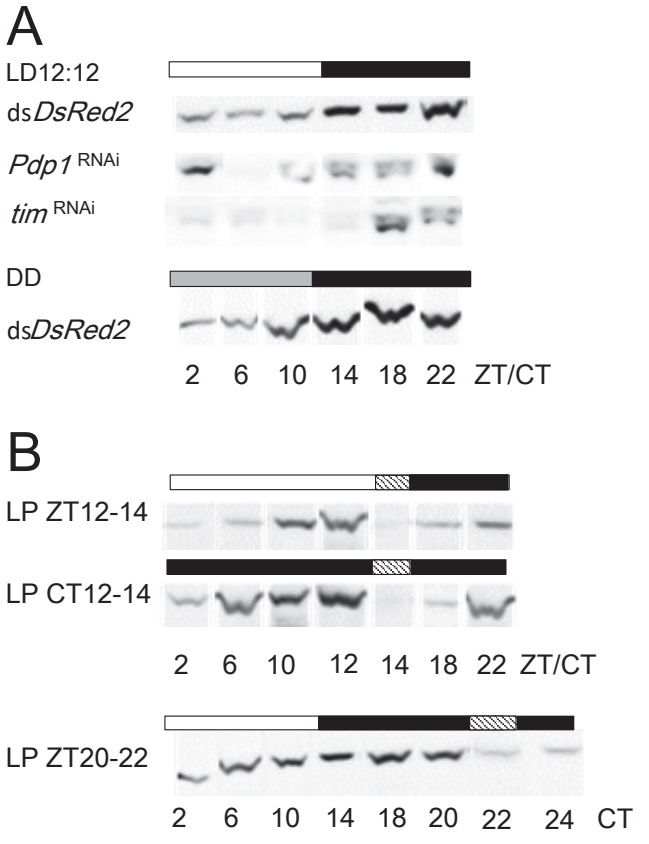

Fig. 6. TIMELESS (TIM) protein levels in the optic lobe-compound eye complex of the cricket *Gryllus bimaculatus*. **(A)** TIM expressed in a daily cyclic manner under light–dark cycle (LD) and constant darkness (DD) with increase during night or subjective night. *tim*^{RNAi} and *Pdp1*^{RNAi} suppressed TIM expression. **(B)** Light exposure for 2 h at Zeitgeber time (ZT)12–14, circadian time (CT)12–14, or ZT20–22 strongly reduced TIM levels. At early night or early subjective night the reduction followed re-increase of TIM protein levels. White, black, and gray bars indicate day, night or subjective night, and subjective day, respectively. Hatched bars indicate light exposure.

DISCUSSION

TIM cycling and effects of light on TIM

The present study revealed that TIM protein was rhythmically expressed in the optic lobe-compound eye complex with a peak at late night (Fig. 6). The cycling of TIM probably reflects the rhythmic expression of *tim* both in the optic lobe and the compound eye, lagging behind the mRNA rhythm by approximately 4 h (Danbara et al., 2010; Ohguro et al., 2020). The cycling is similar to that in *Drosophila* and the monarch butterfly, *Danaus plexippus* (Zeng et al., 1996; Zhu et al., 2008), suggesting that TIM cycling may be conserved across insect species. TIM protein levels are reduced by light exposure during both early and late night. The light-induced TIM reduction is also similar to that in *Drosophila* and the monarch butterfly (Lee et al., 1996; Myers et al., 1996; Zhu et al., 2008), suggesting that insect clock resetting, at least partially, includes TIM degradation. According to the *Drosophila* hypothesis, TIM reduction at early night delays the clock phase until a sufficient amount of TIM is produced (Lee et al., 1996). In this study, the light-induced TIM reduction at early night was followed by an increase in TIM (Fig. 6B). Light exposure at late night, however, advances the clock phase by further decreasing the TIM, which is in its declining phase (Lee et al., 1996; Myers et al., 1996). Our Western blot results also showed no clear increase in TIM levels 2 h after light exposure at ZT20–22 (Fig. 6B). Thus, crickets and flies most likely share the use of TIM for light entrainment, even if only partially, suggesting that TIM may have a common role in light entrainment of insect circadian clocks across species.

However, the mechanism underlying TIM degradation is likely to differ between crickets and flies. In *Drosophila*, TIM is degraded by light activated CRY (Hunter-Ensor et al., 1996; Lee et al., 1996; Emery et al., 1998), while *Drosophila*-type CRY (CRY1) does not seem to be involved in crickets, since light entrainment of the optic lobe circadian clock depends only on light information perceived by the compound eye in crickets (Tomioka and Chiba, 1984; Komada et al., 2015), and *cry1*^{RNAi} had no effect on light entrainment (Tokuoka et al., 2017). In the cricket, the light information perceived by the compound eye is conducted by a neural pathway toward the optic lobe and affects the clock through a neurotransmitter that activates pathways including TIM and CRYs to shift the clock (Kutaragi et al., 2016, 2018). A similar neurotransmitter-dependent photic entrainment may exist in the clock within the compound eye, because the clock in the compound eye is controlled via neural pathways from the optic lobe (Ohguro et al., 2020). This hypothesis should be investigated in future studies.

TIM reduction at early night requires TIM production to operate the negative feedback loop of PER/TIM (Tomioka and Matsumoto, 2019). TIM re-accumulation after its reduction by a prolonged light phase is consistent with the upregulation of *tim* transcription that follows *Pdp1* upregulation (Kutaragi et al., 2016). The enhancement of *tim* expression may contribute to TIM production. *Pdp1*^{RNAi} slightly increased transient cycles for re-entrainment of locomotor rhythms to 6 h delayed LD, although the increase was not significant. Western blotting in *Pdp1*^{RNAi} crickets showed that TIM levels were reduced, but there was a detectable daily TIM level oscillation. Therefore, the low-level TIM oscillation that survived *Pdp1*^{RNAi} may be sufficient for the resynchronization of the rhythm in the delay direction.

Although the mechanism of light-induced TIM degradation in this cricket is currently unknown, it is apparently neurotransmitter-dependent in the optic lobe circadian clock (Fig. 7). It is possible that CRYs are involved in this degradation because *cry1*/*cry2* double RNAi often prevents photic entrainment (Kutaragi et al., 2018). Since CRYs may be regulated by neurotransmitters light-dependently without apparent changes in *cry1* and *cry2* mRNA levels (Kutaragi et al., 2018), TIM degradation may be regulated by CRY proteins that should be modified by light through some neurotransmitters. In addition, a neurotransmitter-mediated, CRY-independent TIM degradation pathway is also known in some cerebral clock neurons in *Drosophila* (Guo et al., 2014), which should be investigated in the future.

Role of *tim* in photic entrainment

The results of the present study showed that *tim* is required for the phase delay of the cricket clock. Re-

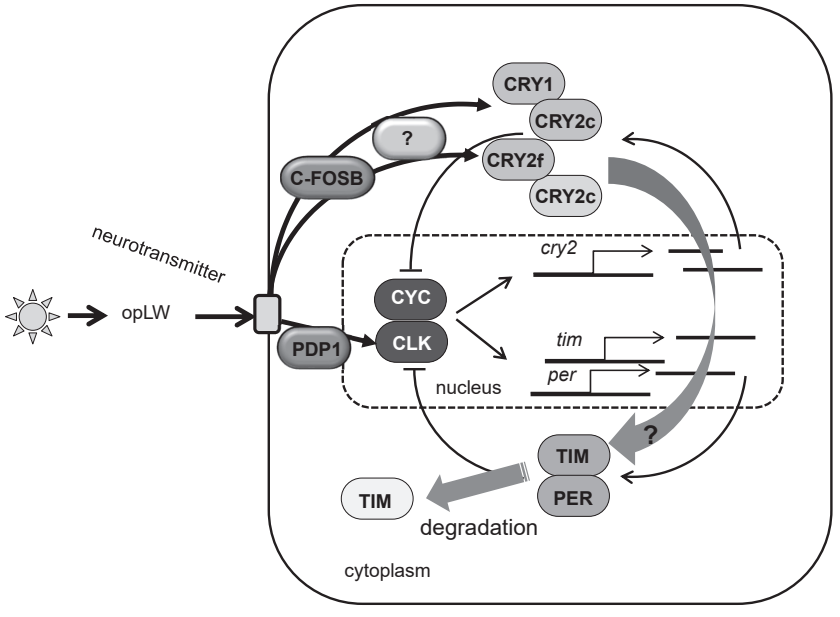

Fig. 7. Current hypothesis of the molecular mechanism of photic entrainment of the circadian clock in the cricket *Gryllus bimaculatus*. The cricket's clock consists of two major feedback loops, i.e., the *per*/*tim*-loop and the *cry2*-loop (Tokuoka et al., 2017). The latter includes CRY1 and CRY2 variants. Light is perceived by opsin-long wavelength (opLW) expressed in the compound eye, conducted to the optic lobe clock neuron via a neural pathway, and causes upregulation of *Pdp1* and *c-fosB* via neurotransmitters, increasing PDP1 and C-FOSB levels. C-FOSB subsequently modulates CRYs, which may cause TIM degradation, leading to reset of the *per*/*tim* oscillatory loop. See text for detailed explanation.

entrainment to 6 h advanced LD was not affected by either *tim*^{RNAi} or *Pdp1*^{RNAi} (Figs. 2A, 3A). Therefore, the mechanism without *tim* and *Pdp1* is sufficient for the phase advance of the rhythm. *tim*^{RNAi} prevented re-entrainment to 6 h delayed LD cycles in nearly 50% of the treated crickets and lengthened the transient cycles to re-entrainment in the rest (Figs. 2B, 3B). The disruption of re-entrainment to delayed LD in *tim*^{RNAi} crickets may be attributable, at least partially, to the reduced magnitude of phase delay caused by 3 h light phase extension (Fig. 5B). It is also likely that a greater advance shift induced by a light pulse at late night (ZT20-23, Figs.4A, 5A) and shorter free-running periods in *tim*^{RNAi} crickets (Danbara et al., 2010) caused slow re-entrainment or phase shifts in the opposite direction.

This delay shift disruption is probably because delay shift requires the *per*/*tim* oscillatory loop. *tim*^{RNAi} stops *tim* oscillation, and mRNA levels are maintained below the level of its normal oscillation (Danbara et al., 2010). The present finding that TIM protein levels were kept very low in *tim*^{RNAi} optic lobe-compound eye complexes is consistent with the previous results on mRNA (Danbara et al., 2010). However, the results of Western blotting showed that very weak TIM expression with a daily rhythm may survive *tim*^{RNAi} in some crickets (Fig. 6). This weak expression might contribute to the slow resynchronization of entrained crickets.

TIM reduction at early night should be required for the delay shift of the clock. TIM reduction is probably coupled with the *cry* oscillatory loop, because *cry1*/*cry2* double knockdown by RNAi disrupted the normal entrainment to shifted LDs in both phase advance and delay (Kutaragi et al., 2018). This also suggests that events, including TIM degradation, are most likely downstream of CRYs. To verify this, the effects of *cry1* and *cry2* RNAi on light-induced TIM degradation should be examined.

Advance shifts caused by light at ZT20-23 were greater in *tim*^{RNAi} insects than those in ds*DsRed2*-treated control crickets. This suggests that the advance shift is caused by a mechanism involving some factor(s) other than *tim* and that *tim* may weaken the advance mechanism. The advance mechanism needs to be elucidated, but it probably includes CRYs since we have previously reported that *cry1* and *cry2* double RNAi strongly disrupted entrainment to 6 h advanced LD (Kutaragi et al., 2018).

Considering the currently available data on photic entrainment, we propose the following hypothesis of light entrainment for the cricket clock (Fig. 7). Light is perceived by opsin-long wavelength expressed in photoreceptor cells in the compound eye (Komada et al., 2015). This information is transmitted to the circadian clock in the optic lobe. In this conducting pathway, the photic information from the receptor converges to some interneurons because partial removal of the compound eye reduces entrainability (Tomioka et al., 1990). The light information sent to the clock cells acts on the molecular oscillatory mechanism of the clock via neurotransmitters. The released neurotransmitter induces *c-fosB*, which acts on CRY through an unknown system (Kutaragi et al., 2018). Then, CRY degrades TIM via a system that is yet to be elucidated, thereby resetting the phase of the *per*/*tim* oscillatory loop. This hypothesis should be tested in future studies.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

YM and K Tomioka conceived and designed the study. YM, TS, K Takeuchi, and K Tomioka performed the RNAi and behavioral experiments. TY prepared antibodies, and MM and KM performed the Western blot experiments. K Tomioka analyzed the data. YM and K Tomioka wrote the original draft of the manuscript. All authors read and approved the final manuscript.

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