Systems/Circuits

Cryptochrome-Dependent and -Independent Circadian Entrainment Circuits in *Drosophila*

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Entrainment to environmental light/dark (LD) cycles is a central function of circadian clocks. In *Drosophila*, entrainment is achieved by Cryptochrome (CRY) and input from the visual system. During activation by brief light pulses, CRY triggers the degradation of TIMELESS and subsequent shift in circadian phase. This is less important for LD entrainment, leading to questions regarding light input circuits and mechanisms from the visual system. Recent studies show that different subsets of brain pacemaker clock neurons, the morning (M) and evening (E) oscillators, have distinct functions in light entrainment. However, the role of CRY in M and E oscillators for entrainment to LD cycles is unknown. Here, we address this question by selectively expressing CRY in different subsets of clock neurons in a *cry*-null (*cry⁰*) mutant background. We were able to rescue the light entrainment deficits of *cry⁰* mutants by expressing CRY in E oscillators but not in any other clock neurons. Par domain protein 1 molecular oscillations in the E, but not M, cells of *cry⁰* mutants still responded to the LD phase delay. This residual light response was stemming from the visual system because it disappeared when all external photoreceptors were ablated genetically. We concluded that the E oscillators are the targets of light input via CRY and the visual system and are required for normal light entrainment.

Key words: circadian clock; clock neurons; Cryptochrome; *Drosophila melanogaster*; light entrainment

Introduction

Light is the most important environmental time cue [zeitgeber time (ZT)] for circadian clocks in most organisms. The light-resetting mechanism in the clock of the model organism *Drosophila melanogaster* is primarily explained by the rapid degradation of one clock protein, TIMELESS (TIM), by light (Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). Although the *Drosophila* visual system, which includes the compound eyes, ocelli, and Hofbauer–Buchner (H–B) eyelets, mediates light information to the clock, these pathways do not cause a rapid degradation of TIM (Stanewsky et al., 1998; Yang et al., 1998; Emery et al., 2000). In contrast, light-dependent TIM degradation is mainly caused by

the blue light-sensitive protein Cryptochrome (CRY), which is expressed in pacemaker clock neurons in the brain and can interact directly with TIM in these cells (Ceriani et al., 1999; Klarsfeld et al., 2004; Benito et al., 2008). After exposure to light, CRY is activated and undergoes a conformational change at the C terminus, leading to its binding to TIM (Busza et al., 2004; Dissel et al., 2004; Vaidya et al., 2013). After this, the TIM/CRY complex is ubiquitinated by JETLAG (JET) and undergoes degradation via the proteasomal pathway (Koh et al., 2006; Peschel et al., 2006).

There are ∼150 neurons expressing clock genes in the *Drosophila* brain, provisionally called "clock neurons" (Muraro et al., 2013). They are divided into nine groups: (1) dorsal neuron $(DN)_{1a}$; (2) DN_{1p}; (3) DN_2; (4) DN_3; (5) lateral posterior neuron; (6) lateral neuron $(LN)_d$; (7) fifth small type (s)-LN_v; (8) large type (l)-LN_v; and (9) s-LN_v (Helfrich-Förster, 2003, 2007). s-LN_v and l-LN_v neurons contain pigment-dispersing factor (PDF), a neuropeptide that is involved in intercellular communication between the clock neurons (Shafer et al., 2008; Yoshii et al., 2009b). Furthermore, the s-LN_v group is important for anticipatory morning activity and the pacemaker center that maintains free-running rhythms in constant darkness (DD) (Stoleru et al., 2005; Picot et al., 2007). In contrast, LN_d and fifth s-LN_v neurons are important for anticipatory evening activity. Therefore, neurons in the former group are called morning (M) cells and the latter are named evening (E) cells.

CRY is expressed in most of the important clock neurons (Yoshii et al., 2008), but several clock neurons that do not contain
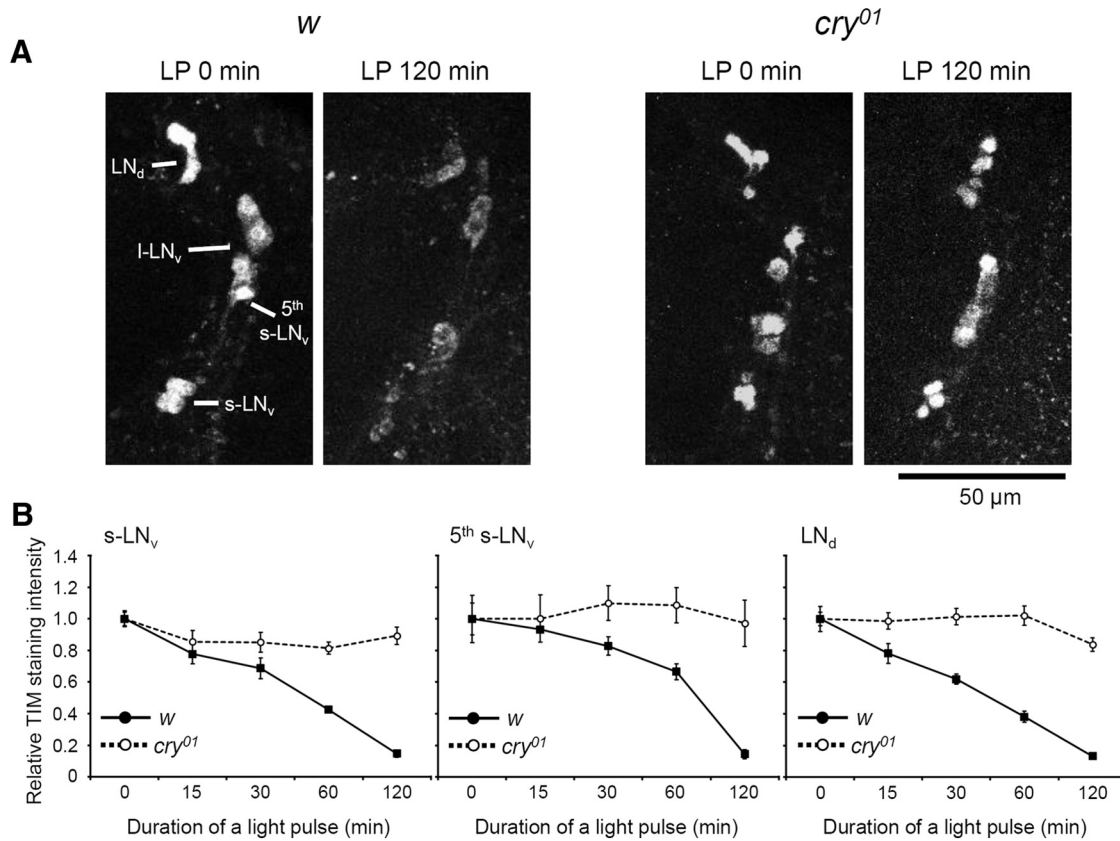
Figure 1. TIM degradation kinetics after light pulses (LP). Flies were exposed to light pulses of different durations that started at different ZTs until ZT 21, when the flies were collected. **A**, TIM immunostaining in LN cells in *w* control brains (left 2 panels) and *cry*[01] mutant brains (right 2 panels) at ZT 21. PDF costaining was also performed to distinguish the groups of LN cells (images not shown). **B**, TIM degradation kinetics in three LN groups in response to different light pulses. To normalize data, TIM staining intensity with no light pulse was set to 1.0. The mean ± SEM staining intensity was calculated from 10 hemispheres of 10 different brains. TIM staining was almost completely abolished after a 120 min light pulse in *w* control brains (solid lines), whereas it remained at a high level in *cry*[01] mutant brains (dashed lines).

CRY or express only very low levels are nevertheless synchronized by light. There are additional differences in the expression levels of CRY between different clock neurons (Yoshii et al., 2008). These facts have raised the question of whether separate clock neurons have different functions in CRY-dependent light entrainment. Furthermore, several studies have shown that the responses to short light pulses are different in M and E oscillators (Shang et al., 2008; Tang et al., 2010; Lamba et al., 2014). Nevertheless, little is known about the role of M and E cells, or CRY, for entrainment to regular light/dark (LD) cycles. To address this issue, in the present study, we generated fly lines that express CRY in subsets of different neurons and tested their entrainability to LD cycles.

Materials and Methods

Fly strains. w^{1118} (*w*) flies were used as a control strain in this study. This line was used originally to create the *cry*[01] mutant; therefore, both have the same genetic background except for the replacement of the *cry* locus by a *mini-white* containing P-element transgene in the mutant (Dolezelova et al., 2007). To test the effect of eye color, *CantonS* flies were outcrossed into the *w* background for six generations to create red-eyed flies, named w^+, that have the *w* genetic background. To rescue *cry* expression in a subset of clock neurons, *cry*[01] mutants were crossed with the following transgenic flies: *uas–cry* (Emery et al., 1998), *tim(uas)–gal4* (Blau and Young, 1999), *gmr–gal4* (Freeman, 1996), *cry–gal4 #39* (Klarsfeld et al., 2004), *Pdf–gal4* (Renn et al., 1999), *npf–gal4* (Wu et al., 2003), *trpA1^SH–gal4* (Hamada et al., 2008), *c929–gal4* (Taghert et al., 2001), *mai179–gal4* (Grima et al., 2004), *r6–gal4* (Helfrich-Förster et al., 2007), *Pdf–gal80^{96A}* (Stoleru et al., 2004), *Clk4.1M–gal4* (Zhang et al., 2010), *R78G01–gal4*, and

R54D11–gal4 (Pfeiffer et al., 2008). *Clk4.1M–gal4*, *R78G01–gal4*, and *R54D11–gal4* transgenes were inserted on the third chromosome in which the *cry* gene is located; therefore, *cry*[01] strains containing the *gal4* transgenes were generated by meiotic recombination. *w;uas–GFP S65t* (#1522) flies were obtained from the Bloomington Drosophila Stock Center. Flies were reared under 12/12 h LD cycles on *Drosophila* medium (0.7% agar, 8.0% glucose, 3.3% yeast, 4.0% cornmeal, 2.5% wheat embryo, and 0.25% propionic acid) at 25°C.

Determination of s-tim and ls-tim alleles. To determine *s-tim* and *ls-tim* polymorphisms of each fly stain, PCR was performed using the following primer set: sense, TAGGTATCGCCCTCCAAG; and antisense, TAGGCAGCTCCACAATCA (Schlichting et al., 2014). The resulting PCR products were subjected to DNA sequencing. *CantonS*, *Pdf–gal80^{96A}*, and *trpA1^SH–gal4* strains possess the *ls-tim* allele. w^{1118}, *cry*[01], *uas–cry*, *tim(uas)–gal4*, *cry–gal4 #39*, *Pdf–gal4*, *npf–gal4*, *c929–gal4*, *mai179–gal4*, *r6–gal4*, *R78G01–gal4*, and *R54D11–gal4* strains possess the *s-tim* allele.

Activity recording and data analysis. Three- to 6-d-old male flies were used to record locomotor activity rhythms. Flies were confined in recording tubes containing agar/sugar food (2% agar and 4% sucrose) for the *Drosophila* Activity Monitor (DAM2; Trikinetics). The monitors were placed in an incubator (CN-40A; Mitsubishi Electric) at a constant temperature of 20°C. White LEDs were set above the monitors in the incubator, and lights on and off were controlled by an LC4 light controller (Trikinetics). The light intensity used in all experiments was 100 lux (3.2 μW/cm^{-2}). To ensure that all strains were entrained completely under 16/8 h LD, the activity rhythms of all strains were first recorded for 7 d under 16/8 h LD. Subsequently, the flies were subjected to an 8 h phase delay of LD by a prolongation of the light phase as shown in Figure 3.
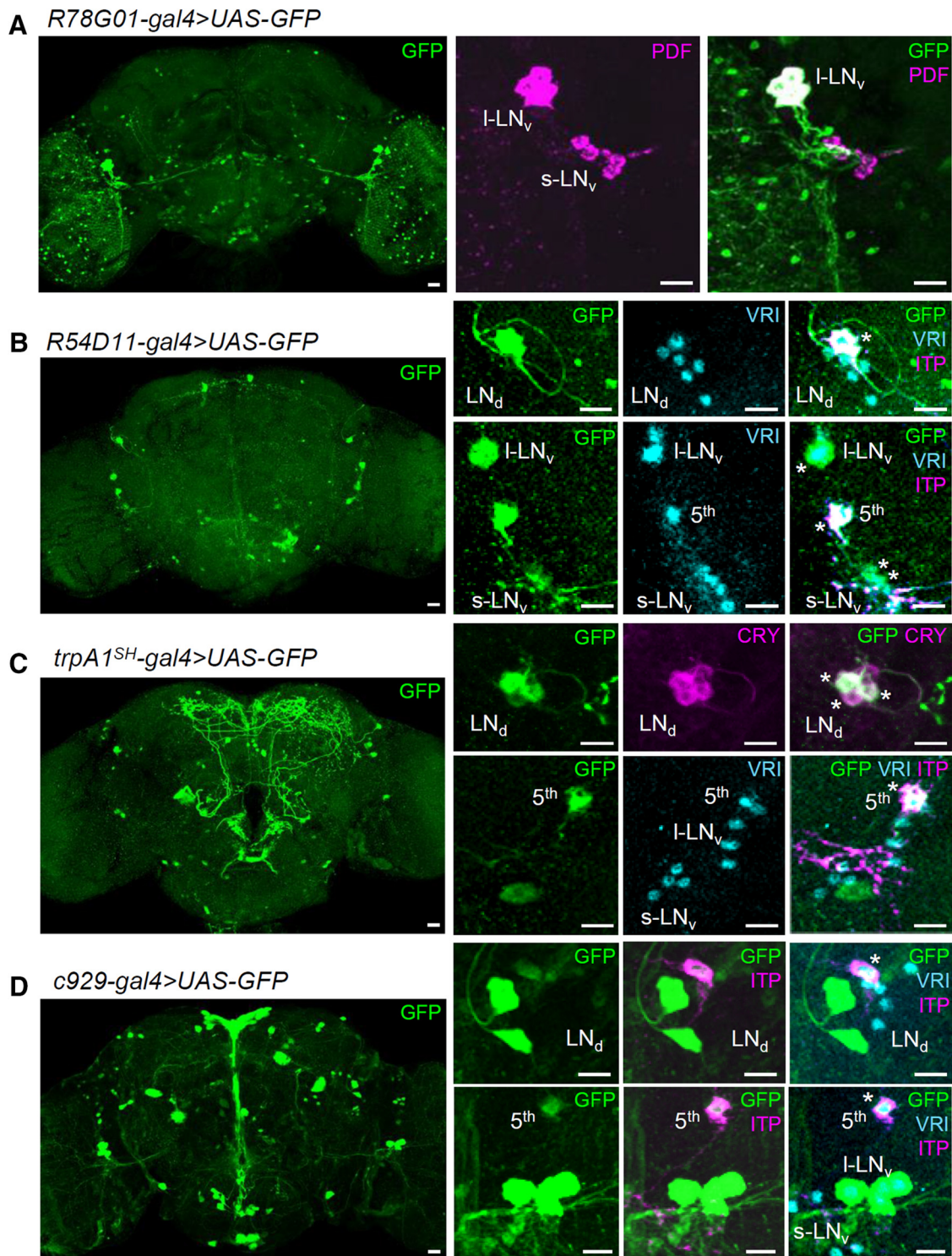
Figure 2. Characterization of *gal4* strains. *gal4* lines were crossed with *uas–GFP–S65t* to visualize the *gal4* expression patterns. Flies were entrained to 12/12 h LD for 5 d and were dissected at ZT 19. Brains were triple stained with anti-GFP (green), anti-VRI (cyan), and anti-PDF (magenta in *A*) or anti-ITP (magenta in bottom rows of *B–D*). The ITP-positive LN$_d$ and fifth s-LN$_v$ are CRY-positive neurons (Johard et al., 2009). *trpA1^{SH}– gal4/uas–GFP* flies were double stained with anti-GFP (green) and anti-dCRY (magenta) to determine whether GFP-positive LN$_d$ were also CRY positive (*C*, top row). Scale bars, 10 μm.

For visual inspection, raw data were displayed as actograms using ActogramJ (http://actogramj.neurofly.de/; Schmid et al., 2011). To calculate the magnitude of the phase shift, daily activity profiles of individual flies were plotted, and the phase of the evening peak of each fly was determined manually, as described previously (Rieger et al., 2003). The phase angles between the evening peak on the day of the LD phase shift and the evening peak on day 1 (or day 2) after the LD phase shift were calculated for individual flies as shown in Figure 5A, and the mean phase angles were calculated for each strain. For statistical analysis, Tukey's multiple comparison test was used after testing for a normal distribution with the Kolmogorov–Smirnov test. Statistics were calculated using EZR software, which is based on R (Kanda, 2013).

Immunohistochemistry and confocal imaging. Whole flies were fixed in 4% paraformaldehyde in PBS with 0.1% Triton X-100 for 2.5 h at room

temperature (RT). Fixed flies were washed three times in PBS, and then their brains were dissected. After washing three times with PBS containing 0.5% Triton X-100 (PBS-T), brains were blocked in PBS-T containing 5% normal donkey or goat serum for 1 h at RT and subsequently incubated in primary antibodies at 4°C for 48 h. After washing six times in PBS-T, the brains were incubated with secondary antibodies at RT for 3 h. Then brains were washed six times in PBS-T and mounted in Vectashield mounting medium (Vector Laboratories). The primary antibodies used were chicken anti-GFP (1:2000; ab13970; Abcam), mouse anti-PDF (1:1000; Developmental Studies Hybridoma Bank; Cyran et al., 2005), guinea pig anti-vrille (VRI; 1:3000; Glossop et al., 2003), rat anti-TIM (1:1000; Yoshii et al., 2008), rabbit anti-CRY (1:1000; Yoshii et al., 2008), rabbit anti-ion transport peptide (ITP; 1;10,000; Hermann-Luibl et al., 2014), and rabbit anti-Par domain protein 1 (PDP1; 1:3000; Cyran et al., 2003). We used the following fluorescence-conjugated secondary antibodies at a 1:500 concentration: Alexa Fluor antibodies (Life Technologies) of 488 nm (goat anti-chicken and goat anti-mouse), 555 nm (goat anti-rabbit), 635 nm (goat anti-mouse and goat anti-guinea pig), and goat anti-rabbit or anti-rat IgG Cy3 (Millipore).

Staining was visualized using laser scanning confocal microscopes (Fluoview300 from Olympus; and TCS SPE from Leica). For quantification of PDP1 immunostaining, the confocal microscope settings were kept constant throughout the experiments. For each time point, nine hemispheres from nine different brains were analyzed. Measurement of staining intensity was performed using NIH ImageJ (W. S. Rasband, National Institutes of Health, Bethesda, MD), as described previously (Yoshii et al., 2009a).

Results

CRY-dependent TIM degradation by a light pulse

Previous studies using whole-head extracts and S2 cells have shown that TIM is degraded rapidly by light in a CRY-dependent manner (Busza et al., 2004; Peschel et al., 2009). To test the speed of TIM degradation in the brain clock neurons under light, w control flies and cry^{01} mutants, which were entrained to 12/12 h LD, were subjected to a light pulse of different durations, starting at different ZTs, before collecting the flies at ZT 21. At ZT 21, TIM is normally at its peak level, whereas the tim mRNA level is already declined (So and Rosbash, 1997). Thus, we expected to observe relatively clear TIM degradation kinetics at this time point, unaffected by its mRNA synthesis. Our immunohistochemical analysis revealed that TIM required a 120 min light pulse to decline to a level almost undetectable by immunohistochemistry in all LN groups of the control flies, including the CRY-negative LN$_d$s (Fig. 1A,B). These data fit to TIM degradation kinetics from head extracts (Busza et al., 2004) but not to *in vitro* studies (Ozturk et al., 2014), in which TIM is more rapidly degraded by light. It is worth mentioning also that much shorter light pulses (although depending on light intensity) are sufficient for causing TIM degradation (Vinayak et al., 2013), albeit complete depletion is not reached until a few hours after the light pulse. Even after a 120 min light pulse, there was still some residual TIM detectable, especially in the cytoplasm (Fig. 1A). Therefore, light-dependent TIM degradation may be more efficient in the nucleus than in the cytoplasm.

In cry^{01} mutants, TIM levels did not change even after a 120 min light pulse, clearly indicating that TIM degradation by the light pulse was indeed mediated by CRY. The same TIM kinetics were also observed in the DN groups, including CRY-negative DN$_{1p}$ and DN$_2$ neurons (data not shown). Thus, CRY-dependent TIM degradation also occurs in CRY-negative clock neurons, as shown in a previous study (Yoshii et al., 2008). For unknown reasons, TIM staining in the DN groups in cry^{01} mutants was very weak even without a light pulse. Therefore, quantification was

Table 1. Expression patterns of different *gal4* divers in clock neurons

Gal4 driver	Expression sites in clock neurons
tim(uas)gal4	All clock neurons
cry–gal4 #39	All *cry*-positive neurons (+ 3 CRY-negative LN$_d$)
mai179–gal4	Three CRY-positive LN$_d$, fifth s-LN$_v$, ~2 l-LN$_v$, s-LN$_v$, *DN$_{1a}$
mai179–gal4/Pdf–gal80	Three CRY-positive LN$_d$, fifth s-LN$_v$, *DN$_{1a}$
R54D11–gal4	One CRY-positive LN$_d$, fifth s-LN$_v$, *one l-LN$_v$, *one to two s-LN$_v$
c929–gal4	One CRY-positive LN$_d$, fifth s-LN$_v$, l-LN$_v$
npf–gal4	Three LN$_d$ (one CRY-positive and two CRY-negative), fifth s-LN$_v$, one to two l-LN$_v$
trpA1^{SH}–gal4	Three LN$_d$ (CRY-positive), fifth s-LN$_v$, one DN$_{1a}$
Pdf–gal4	l-LN$_v$, s-LN$_v$
r6–gal4	s-LN$_v$
R78G01–gal4	l-LN$_v$
Clk4.1M–gal4	Six CRY-positive DN$_{1p}$
gmr–gal4	Eyes

*Indicates very weak expression.

not performed in these cells. Possible reasons for this weak TIM staining in the DN groups are as follows: (1) a phase shift of the TIM peak in the mutants away from ZT21, when the staining was performed, or (2) a novel role of CRY in modulating TIM stability in the dark. However, both hypotheses require additional investigation to be validated.

Characterization of *gal4* lines

Before generating fly lines that express CRY only in subsets of clock neurons, we screened the Janelia *gal4* stocks (Pfeiffer et al., 2008) for strains suitable for our purpose. We found two *gal4* lines that showed rather specific GFP expression in the clock neurons when crossed with *uas–gfp* flies. In particular, GFP expression of *R78G01–gal4/uas–gfp* flies was observed in the l-LN$_v$ and few additional non-clock cells in the brain (Fig. 2A). *R54D11–gal4/uas–gfp* flies showed GFP signal in the fifth s-LN$_v$, one ITP-positive LN$_d$ (CRY-positive neuron; Johard et al., 2009), one l-LN$_v$, very weakly in two s-LN$_v$ neurons, and very few non-clock cells in the brain (Fig. 2B). We also investigated GFP expression in *trpA1^{SH}–gal4/uas–gfp* flies showing GFP signal in the fifth s-LN$_v$, three CRY-positive LN$_d$, and one DN$_{1a}$ (Fig. 2C; Hamada et al., 2008). The *c929–gal4* line had been used previously as an l-LN$_v$-specific driver (Taghert et al., 2001). Here, we found that it also shows expression in the fifth s-LN$_v$ and one ITP (and CRY)-positive LN$_d$, in addition to all l-LN$_v$ neurons (Fig. 2D). We used these lines together with well characterized clock-neuron-specific *gal4* drivers to generate the *cry*-rescue flies (Table 1).

Re-entrainment experiments to a phase-shifted LD cycle

To investigate entrainability to light, flies were subjected to an 8 h phase delay of the given LD cycle. We chose a 16/8 h LD cycle for this experiment because it is easier to discriminate the actual phase of the evening activity peak from the startle response caused by lights off (Rieger et al., 2012). In 16/8 LD cycles, the evening activity of w control flies peaked before lights off, whereas cry^{01} mutants and eyeless *grm–hid/+* mutants showed activity peaks later and earlier than the control flies, respectively (Fig. 3). After an 8 h phase delay of LD, the activity rhythms of w control flies and *grm–hid/+* mutants immediately phase shifted and established a steady phase within almost 1 d (Fig. 3). In contrast, cry^{01} mutants needed several days to completely synchronize to the new phase of LD. This
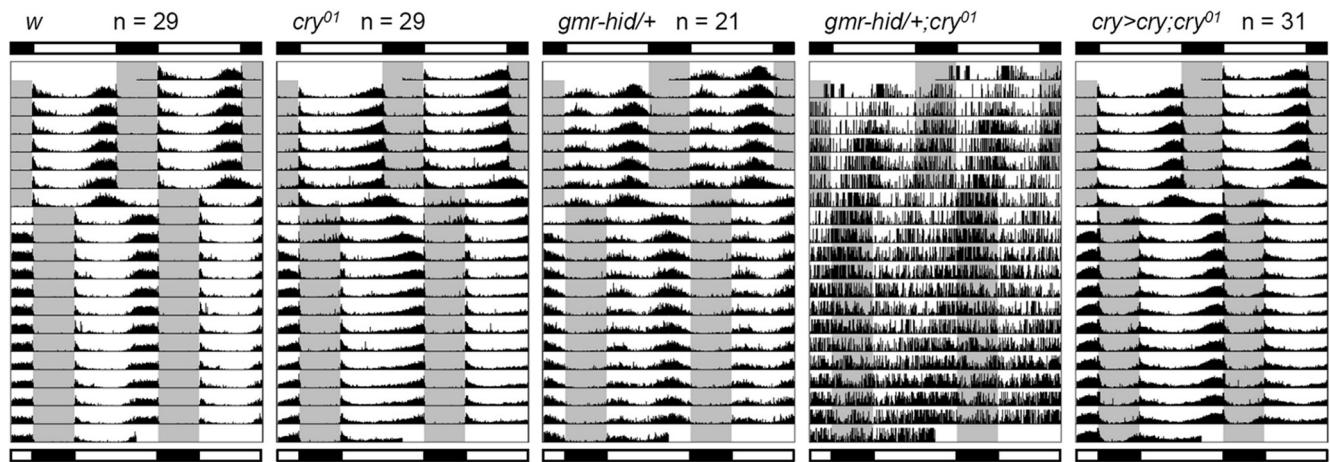
Figure 3. Activity rhythms under 16/8 h LD before and after an 8 h LD phase shift. The gray area in the actograms indicates the dark phase. Bars above and below the actograms indicate light conditions before and after the LD phase shift, respectively. Actograms show mean activity rhythms calculated from the number of flies indicated next to the strain names. Because $gmr−hid/+;cry^{01}$ flies were not able to synchronize to LD cycles, the actogram is of a representative individual fly. $cry{>}cry;cry^{01}$ indicates $w;cry−gal4\ \#39/uas−cry;cry^{01}$.

slow re-entrainment was rescued by cry expression in CRY-positive neurons in $cry−gal4/uas−cry;cry^{01}$ flies (Fig. 3). When both the eyes and CRY were absent in $grm−hid/+;cry^{01}$ mutants, the flies were not able to synchronize to LD cycles, in agreement with previous studies (Helfrich-Förster et al., 2001; Klarsfeld et al., 2004).

cry rescue in the fifth s-LN$_v$ and LN$_d$ neurons

To investigate the role of CRY in different clock neurons, we then generated 11 cry-rescue strains by crossing different clock-specific $gal4$ drivers with a $uas−cry$ line in the cry^{01} background and subjected them to the 8 h phase delay. Average actograms of all strains are shown in Figure 4. To evaluate the speed of the re-entrainment, we determined the phase delays accomplished by individual flies on days 1 and 2 after the shift (Fig. 5A). The evening activity of w control flies phase shifted by almost 7 h on day 1 after the 8 h phase shift of LD (Figs. 3, 5B), meaning that w flies almost completed re-entrainment to the new LD cycle within 1 d. w^+ flies, which have the same genetic background as w control flies but have red eyes, similarly showed fast re-entrainment, proving that eye color does not matter in this assay. CantonS wild-type flies were slightly slower than w control flies in the re-entrainment. The difference was >1 h and statistically significant. Thus, the genetic background including the tim allele (Peschel et al., 2006; w: s-tim; CantonS: ls-tim) slightly affected the speed of the re-entrainment. cry^{01} mutants phase shifted by ~2 h on both day 1 and day 2, suggesting that the phase shift mediated by visual system-dependent light entrainment is limited to ~2 h/d. In addition, rescuing cry in the eyes with a $gmr−gal4$ driver did not improve the cry^{01} phase shift.

When all clock neurons, all CRY-positive clock neurons, or the majority of the CRY-positive LN groups expressed cry by $tim−gal4$, $cry−gal4$, or $mai−gal4$, respectively, the flies were able to re-entrain at the same speed as the w and w^+ flies. $mai−gal4$ is expressed in the LN groups (except for the three CRY-negative LN$_d$ and one to two l-LN$_v$) and very weakly in the DN$_{1a}$ neurons (Picot et al., 2007; Rieger et al., 2009). Thus, this suggests that the DN groups are not major players in CRY-dependent fast light entrainment. The $Clk4.1M−gal4$ is a specific driver for CRY-positive DN$_{1p}$ neurons (Zhang et al., 2010), and the phase shift of cry-rescued flies with this line was not significantly different from cry^{01} mutants or $uas−cry/+;cry^{01}$ flies, further confirming that

CRY in the DN groups does not contribute to light re-entrainment to phase delays.

The $r6−gal4$ and $R78G01−gal4$ are specific drivers for the s-LN$_v$ and l-LN$_v$, respectively. The phase shifts of cry-rescued flies with these drivers were not statistically different from cry^{01} mutants (Fig. 5B). However, when cry was rescued in both s-LN$_v$ and l-LN$_v$ neurons with the $Pdf−gal4$ driver, the flies were able to phase shift by ~3 h/d, and this was statistically significant from the phase shift in cry^{01} mutants. In contrast, $mai−gal4/Pdf−gal80$ is a specific line for three CRY-positive LN$_d$ and the fifth s-LN$_v$. cry-rescued flies with this driver achieved ~6 h of phase shift. Other $gal4$ drivers that are expressed in both the fifth s-LN$_v$ and LN$_d$ neurons ($R54D11−gal4$, $c929−gal4$, $trpA1^{SH}−gal4$, and $npf−gal4$) also showed considerably improved phase shifts. Together, these data show that CRY expression in the fifth s-LN$_v$ and LN$_d$ neurons is particularly important for fast light entrainment. However, because cry-rescued flies with the $mai−gal4$ driver shifted at a significantly faster rate than the $mai−gal4/Pdf−gal80$ cry-rescued flies (at least on day 1), CRY expression in the PDF neurons (s-LN$_v$ and l-LN$_v$) seems to additionally support the phenotypic rescue. We should also note that $gal4$ expression in the fifth s-LN$_v$ and LN$_d$ neurons in $c929−gal4$, $trpA1^{SH}−gal4$ and $npf−gal4$ strains is relatively weak compared with the $tim−gal4$, $cry−gal4$, and $mai−gal4$ strains. In addition, the $R54D11−gal4$, $c929−gal4$, $trpA1^{SH}−gal4$ strains show $gal4$ expression only in one CRY-positive LN$_d$. Therefore, the minor differences in behavior between the E cell-specific cry-rescue strains may be attributable to the level of CRY expression and/or the number of CRY-positive E cells.

Phase shifts in clock neurons via CRY and the eyes

Next, we investigated the cycling of a core clock protein in the clock neurons under the phase shift condition. The PDP1 clock protein was chosen, because the cycling of PERIOD (PER) and TIM are thought to be influenced directly by light-mediated TIM degradation, but here we intended to show contributions from other pathways to the protein cycling, in particular pacemaker neurons. Three strains, w control flies, cry^{01} mutants, and $mai−gal4/Pdf−gal80{>}cry;cry^{01}$ flies, were entrained to 16/8 h LD for 7 d and subjected to an 8 h phase shift of the LD cycle using the same behavioral protocol that was performed in the previous experiments shown in Figure 3. The flies were collected at 3 h
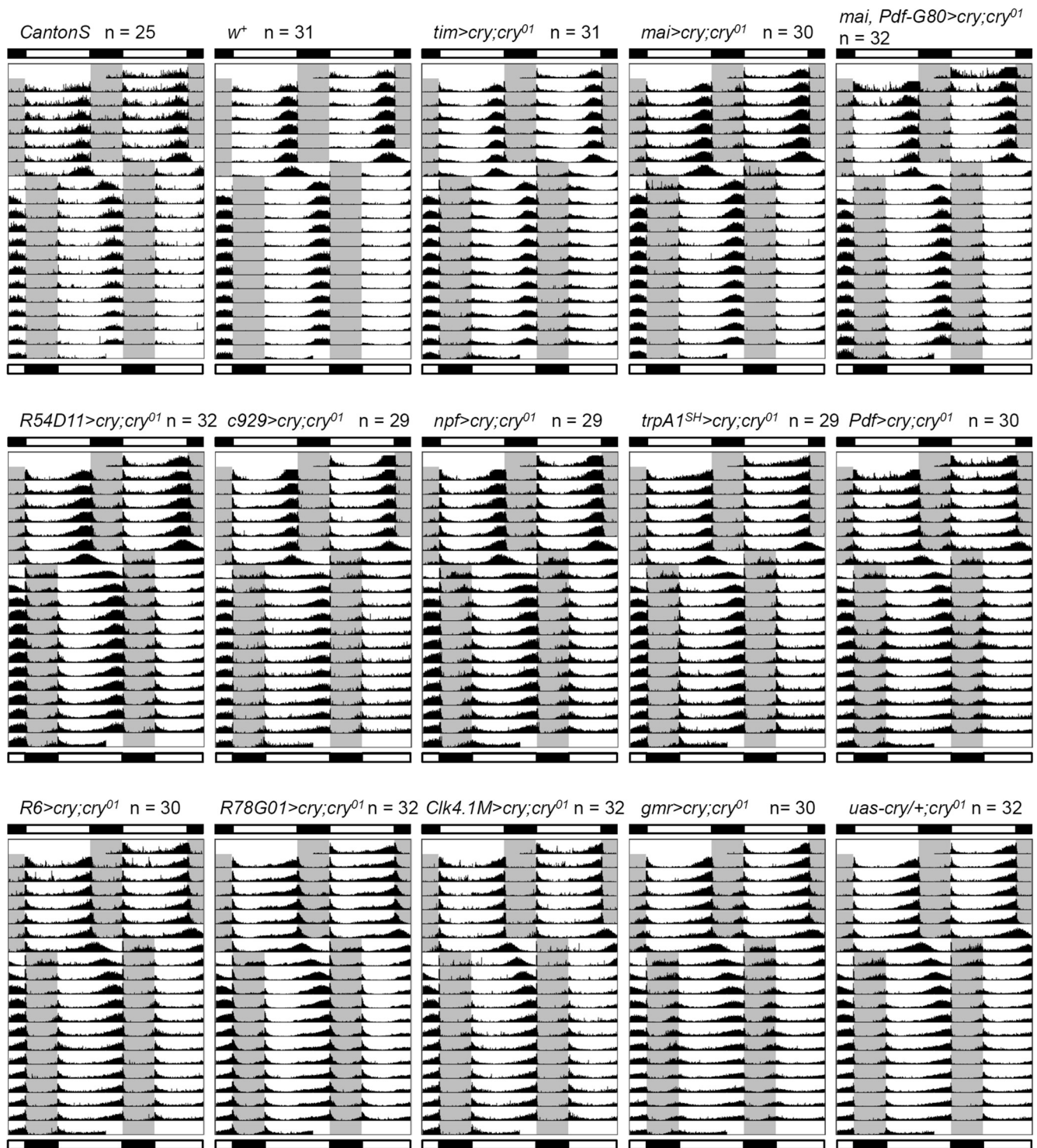
Figure 4. Activity rhythms of other strains, which were not included in Figure 3, under 16/8 h LD before and after an 8 h LD phase shift. The gray area in the actograms indicates the dark phase. Bars above and below the actograms indicate light conditions before and after the LD phase shift, respectively. Actograms show mean activity rhythms calculated from the number of flies indicated next to the strain names.

intervals for immunostaining before and after day 1 of the phase shift (Fig. 5A).

In w control flies, all LN groups displayed large phase shifts in PDP1 cycling after an 8 h phase shift of LD (Fig. 6). PDP1 began to increase at approximately ZT 12 and reached peak levels at ZT 18 before the LD phase shift in all LN groups, and these were phase shifted by 9–15 h after the LD shift. Thus,

PDP1 cycling was surprisingly shifted by >8 h in terms of the onset of the PDP1 increase and the phase of the peaks. Among the DN groups, the DN_{1a} showed similar phase shifts to the LN groups (Fig. 7). Only a subset of the DN_{1p} neurons phase shifted. The other DN groups seemed to be insensitive to the LD phase shift and showed no difference in the phase at which PDP1 peaked before or after the shift. The phase shift of the
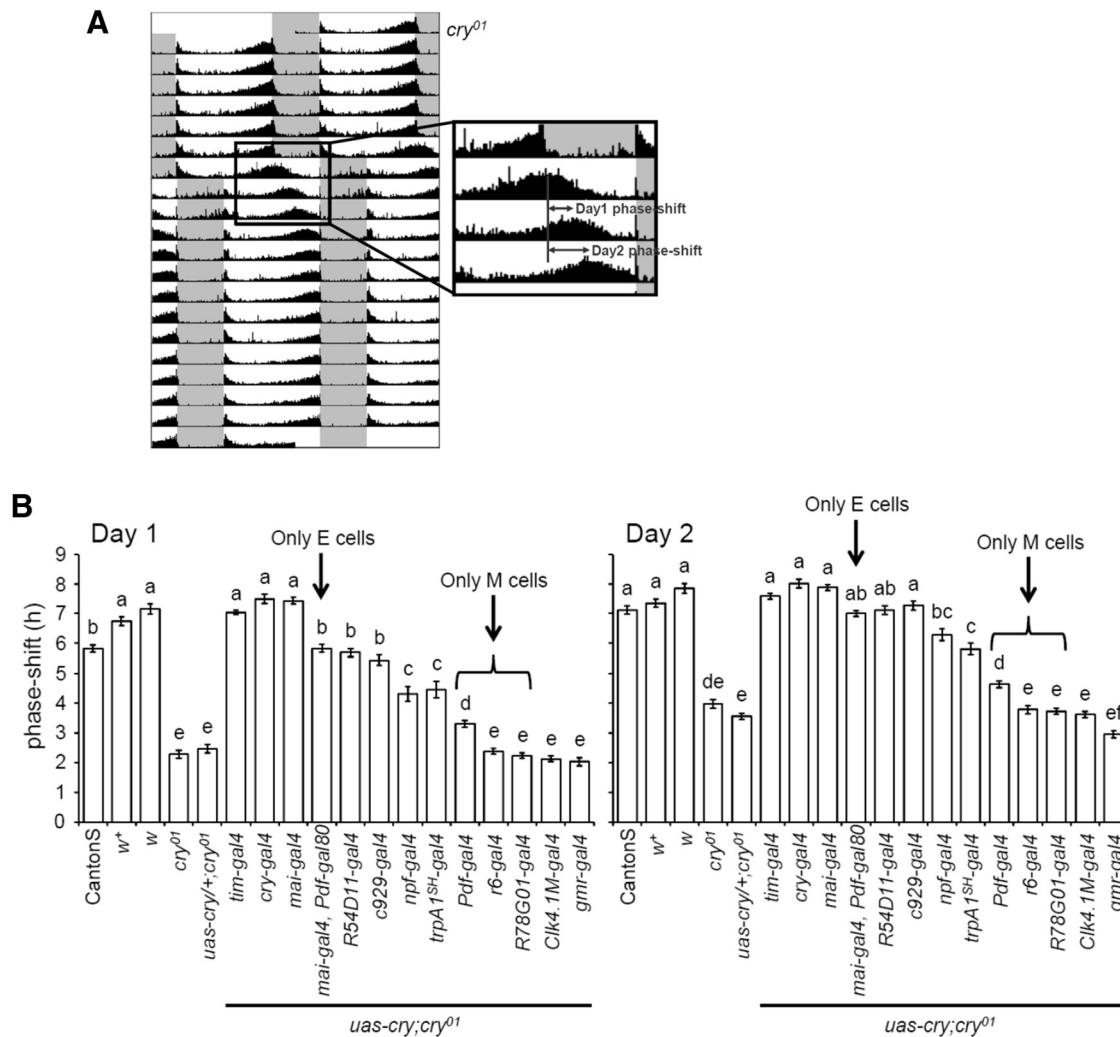
Figure 5. The magnitude of the phase shifts after an 8 h LD phase delay. **A**, Determination of the phase angle between the evening activity peak before and after the LD phase shift. The phase of the evening activity peak was determined on the day when the light phase was prolonged to induce a shift in the LD cycle. The next day was regarded as day 1, when the activity phase shift occurred. **B**, The magnitude of the phase shifts (mean ± SEM) on day 1 (left) and day 2 (right) after an 8 h LD phase delay. The control strains (CantonS, w^+, and w) were phase shifted by 6–7 h in response to an 8 h LD shift on the first day, suggesting that these flies almost finished re-entrainment within 1 d. Lowercase letters above the bars indicate significant differences revealed by Tukey's multiple comparison test ($p < 0.01$).

DN$_2$ neurons was hard to judge because these neurons seemed to resynchronize slowly.

In cry^{01} mutants, the l-LN$_v$ neurons did not show any strong PDP1 cycling before or after the phase shift; therefore, we could not reliably determine any peak (Fig. 6). The same was true for the DN neurons, although the DN$_{1p}$ showed some similarities to the controls (Fig. 7). Prominent PDP1 oscillations were present in the s-LN$_v$, fifth s-LN$_v$, and LN$_d$ neurons of cry^{01} mutants (Fig. 6). In the s-LN$_v$ neurons, PDP1 showed a narrower peak than in the controls, but, most importantly, PDP1 cycling did not follow the phase delay of the LD cycle (Fig. 6). In contrast, PDP1 cycling in the fifth s-LN$_v$ and LN$_d$ of cry^{01} mutants was reasonably phase shifted as judged from an increase in PDP1 levels. We could not determine unequivocally the phase of the PDP1 peak before the shift, because PDP1 seemed to reach its maximal level at ZT 24, when our time series was stopped. Importantly, PDP1 of the fifth s-LN$_v$ and LN$_d$ reached its peak levels later in cry^{01} mutants than in the controls, and this coincided with the late E activity peak of the mutants (Fig. 3), further providing evidence for the proposed role of the E cells in controlling this peak. The response of the molecular clock in the fifth s-LN$_v$ and LN$_d$ neurons of cry^{01} mu-

tants to the phase-delayed LD cycles shows that visual system-dependent light input pathways, in addition to CRY, entrain the E cells. The magnitude of the shifts matches the behavioral data that revealed a phase delay of 2 h/d of the E peak in cry^{01} mutants (Fig. 5B).

When cry expression was rescued in the E cells in $mai–gal4/Pdf–gal80>cry;cry^{01}$ flies, the wild-type phase of the PDP1 peak (at approximately ZT 18–ZT 21) was restored in the fifth s-LN$_v$ and LN$_d$ neurons; in addition, these neurons also responded with a phase delay to the shifted LD cycles in a manner very similar to the wild-type neurons (Fig. 6). The increase in PDP1 levels after the phase shift occurred earlier in the rescued flies than in the controls, but the PDP1 peaks were reached at the same time (at approximately ZT 19 to ZT 22) in both fly groups, suggesting that CRY expression in the E cells was sufficient for almost normal phase delays of the E cells. Once again, this corresponds to the delay of E activity shown in the behavioral experiments (Fig. 5B). Interestingly, CRY rescue in the E cells had little effect on PDP1 cycling in the s-LN$_v$ (Fig. 6), which did not show any apparent phase delay in response to the LD shift. In addition, there was no effect on PDP1 cycling in the l-LN$_v$ (Fig. 6) or DN (Fig. 7). Thus,
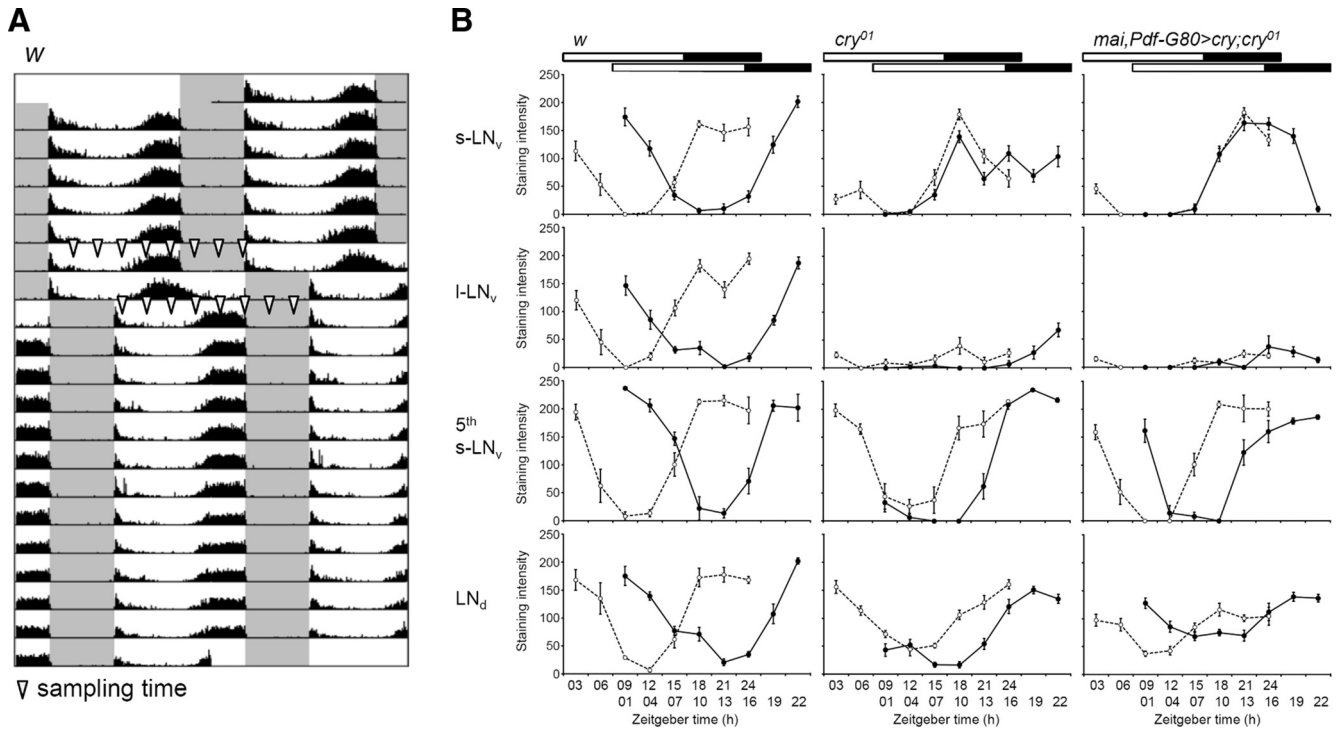
A

∇ sampling time

B

Figure 6. PDP1 cycling in LN clock neurons before and after an 8 h LD phase shift. **A**, Sampling schedule for PDP1 staining. The arrowheads on the actogram indicate when flies were sampled for immunostaining. **B**, Mean ± SEM PDP1 staining intensity was calculated from nine hemispheres of nine different brains for each time point in the course of a single experiment. The open circles with dashed lines and filled circles with solid lines indicate the PDP1 cycling before and after the LD phase shift, respectively. PDF costaining was performed on all brains to distinguish different LN groups (images not shown). The bars above the graphs indicate light conditions.

cry rescue in the E cells increased the speed of the phase shift in the E cells themselves but not in other clock neurons. Nevertheless, we emphasize that there must be crosstalk between the different clock neurons, because the shape and phase of the PDP1 oscillations in s-LN$_v$ neurons appeared more normal after CRY rescue in the E cells (Fig. 6).

Discussion

The *Drosophila* circadian clock is extremely light sensitive (Helfrich-Förster et al., 2002; Hirsh et al., 2010). Short-term exposure to a weak light pulse is sufficient for TIM degradation and a large phase shift in activity rhythms (Vinayak et al., 2013). This rapid response of the *Drosophila* clock to light is attributed to TIM degradation by the CRY-dependent pathway in the clock neurons (Stanewsky et al., 1998; Emery et al., 2000; Busza et al., 2004). In the present study, we demonstrated that the fifth s-LN$_v$ and LN$_d$ neurons, known as the E cells, play an important role in the CRY-dependent fast light entrainment. In the absence of CRY, molecular cycling in the E cells can respond to an 8 h phase shift of LD, whereas cycling in the M cells (l-LN$_v$ and s-LN$_v$ neurons) cannot (Fig. 6). Thus, visual input pathways entrain the E cells but not the M cells, at least not during a short period. The rescue of *cry* in the E cells alone increases the speed of the phase shift of activity rhythms and PDP1 cycling in these cells.
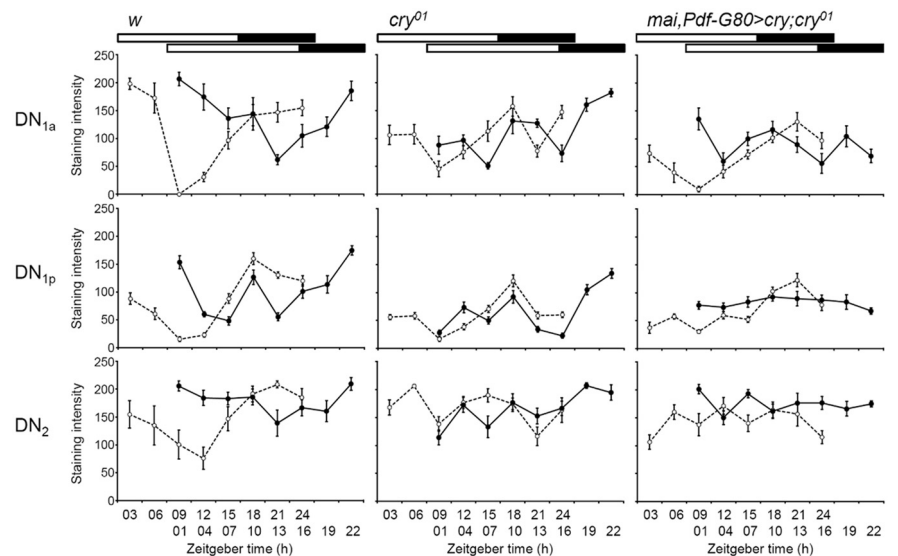

Figure 7. PDP1 cycling in DN clock neurons before and after an 8 h LD phase shift. Mean ± SEM PDP1 staining intensity was calculated from nine hemispheres of nine different brains for each time point in the course of a single experiment. The open circles with dashed lines and filled circles with normal lines indicate PDP1 cycling before and after the LD phase shift, respectively. The bars above the graphs indicate the light conditions.

Together, both the visual system and CRY initially reset the E cells, enabling extremely sensitive light entrainment in the *Drosophila* clock.

Circadian network for light entrainment

To compound a hierarchical structure in the clock network, some clock neurons would dominate others to drive a coherent circa-

dian rhythm in behavior (Yoshii et al., 2012). It has been shown that the M cells are master pacemakers and maintain the molecular cycling in some of the LN$_d$ neurons in DD (Stoleru et al., 2005; Yao and Shafer, 2014). In contrast, the E cells are more important under constant light conditions. Functional E cells are able to drive free-running rhythms in constant light, but functional M cells are not (Picot et al., 2007; Stoleru et al., 2007; Rieger et al., 2009). This fact, together with our present results, suggests that the E cells are a specialized group of clock cells for light entrainment, and they may have a dominant power to control activity rhythms under light.

However, there are lines of evidence showing that the PDF neurons are also important for light resetting. The group of larger PDF neurons, the so-called l-LN$_v$, is less important for generating activity rhythms (Grima et al., 2004; Shafer and Taghert, 2009). However, flies lacking l-LN$_v$ neurons are relatively insensitive to a light pulse at dawn but not at dusk (Shang et al., 2008). Double mutants of *cry* and *Pdf* (or *Pdf-receptor*), which lack an evening activity peak but show a large morning peak under LD (Cusumano et al., 2009; Zhang et al., 2009), show damping PER oscillations in the fifth s-LN$_v$ and LN$_d$ neurons and no behavioral evidence of light re-entrainment in response to an 8 h LD phase shift (Im et al., 2011). Thus, the PDF neurons and PDF itself are also important for the light response of the clock.

To explain all the results of the present and previous studies, one can speculate that light input from the visual system may pass through the PDF neurons before arriving in the E cells. Therefore, light entrainment in *cry/Pdf* double mutants may be defective because of the lack of *cry* and the main neurotransmitter of the PDF neurons. Although all neuronal connections between the visual system and the clock neurons in the adult fly have not been identified, it is known that the neuronal projections from the H–B eyelets terminate very close to where the PDF neurons send projections into the accessory medulla (Helfrich-Förster et al., 2002, 2007; Malpel et al., 2002). The larval precursor of the H–B eyelet, the Bolwig organ, which constitutes the larval visual system, signals to the larval LN$_v$ via acetylcholine (Wegener et al., 2004; Keene et al., 2011; Yao et al., 2012). This leads to a reset of the DN$_1$ clock neurons via PDF signaling (Klarsfeld et al., 2011). Therefore, the same mechanism may also exist in the adult fly, with the PDF neurons receiving light information from the visual system and then signaling to the fifth s-LN$_v$ and LN$_d$ cells via PDF. Indeed, both the fifth s-LN$_v$ and the LN$_d$ were shown to be receptive to PDF, and there is evidence for a functional neuronal connection between the PDF neurons and the LN$_d$ (Shafer et al., 2008; Yao et al., 2012; Yao and Shafer, 2014). Guo et al. (2014) reported that temporal activation of PDF neurons by the temperature-sensitive TrpA1 channel mimics a phase response curve (PRC) in response to light pulses and that this activation of PDF neurons promotes TIM degradation in all clock neurons (Guo et al., 2014). This system is CRY independent but PDF-receptor dependent. Thus, it is possible that the PDF neurons are activated by visual input pathways and cause degradation of TIM in the E cells, resetting their molecular clocks.

One remaining question is how the M cells and PDF can contribute to the LD entrainment in the absence of CRY, although PDP1 cycling in the s-LN$_v$ and l-LN$_v$ was insensitive to an LD phase shift in *cry^{01}* and *mai–gal4/Pdf–gal80>cry;cry^{01}* flies (Fig. 6). To address this, we have to ask whether the molecular clock in

the M cells is required for LD entrainment of the E cells and of activity rhythms in future work.

PRC or LD phase shift

Two recent reports also proposed a non-cell-autonomous mechanism involved in light-dependent phase resetting of the clock (Tang et al., 2010; Lamba et al., 2014). Tang et al. (2010) revealed that the TIM degradation in M cells is insensitive to a light pulse at ZT 15, which is the phase delay zone in the PRC to light, whereas the E cells are still sensitive (Tang et al., 2010). However, CRY expression in the M cells is necessary for the phase delay, because knockdown of *cry* in the s-LN$_v$ neurons attenuates the amplitude of the phase delay. Lamba et al. (2014) further examined this using two genetic methods: (1) rescue of wild-type *jet* under a *jet* mutant background in a manner similar to the protocol performed for *cry* rescue in the present study; and (2) the knockdown of *jet* by RNAi (Lamba et al., 2014). JET is an E3 ubiquitin ligase that promotes degradation of TIM together with CRY (Koh et al., 2006; Peschel et al., 2009). Interestingly, when there is sufficient *jet* expression in the M cells, TIM degradation occurs in both the M and E cells (Lamba et al., 2014). This suggests that an interaction between the M and E cells is important for phase resetting. This is in good agreement with our data, showing TIM degradation in CRY-negative clock neurons after light exposure (Fig. 1).

Both studies showed that activity phase shifts are mediated by network interactions between M and E cells. Our data match this, because the rescue of *cry* in both the M and E cells completely restored wild-type entrainment. However, the *cry* rescue in fifth s-LN$_v$ and LN$_d$ cells consistently showed a significant improvement in the speed of re-entrainment (Fig. 5B), suggesting the importance of the E cells. One difference between our study and the above mentioned studies is the way in which we evaluated the light response of the clock. In the LD phase-shift experiment, a long-term light exposure covering the delay and advance zones of the PRC strongly resets the clock in day 1 of the LD shift. In contrast, the PRC experiment reveals finer parameters of the clock with respect to a short light response, e.g., the direction of the phase shift, the magnitude of the shift, and the sensitive phase. Therefore, it is not easy to compare our data with the PRC studies.

A longer light pulse usually causes a larger phase shift (Kistenpfennig et al., 2012). When flies receive the same number of photons, a dimmer yet longer light pulse is more effective than a brighter yet shorter pulse (Vinayak et al., 2013). Among the clock neurons, TIM degradation in the fifth s-LN$_v$ neuron is especially sensitive to a long light pulse (Vinayak et al., 2013), which may imply that the role of the E cells in light-resetting mechanisms emerges only under a long-term light exposure.

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