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Behavioral Dissection of the *Drosophila* Circadian Multioscillator System that Regulates Locomotor Rhythms

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The fruit fly, *Drosophila melanogaster*, shows a bimodal circadian activity rhythm with peaks around light-on and before light-off. This rhythm is driven by seven groups of so-called clock neurons in the brain. To dissect the multioscillatory nature of the *Drosophila* clock system, the process of reentrainment to a reversed light cycle was examined by using wild-type flies and cry^b mutant flies that carry a strong loss-of-function mutation in *cryptochrome* (*cry*) gene. The wild-type flies showed that the morning peak dissociated into two components, while a substantial fraction of cry^b flies exhibited dissociation of the evening peak into two components that shifted in different directions. When the temperature cycle was given in constant darkness in such a manner that the thermophase corresponded to the previous night phase, the morning peak also split into two components in wild-type flies. These results suggest that both morning and evening peaks are driven by two separate oscillators that have different entrainability to light and temperature cycles. Examination of the process of reentrainment to a reversed LD in mutant flies that lack some of the four known circadian photoreceptors (compound eyes, ocelli, CRYPTOCHROME [CRY], and Hofbauer-Buchner [H-B] eyelets) revealed that these four photoreceptors play different roles in photic entrainment of the four putative oscillators.

Key words: circadian rhythm, circadian oscillators, *Drosophila*, entrainment, light, temperature

INTRODUCTION

Animals have an internal timekeeping mechanism that accurately adjusts 24-hr rhythms of physiological functions and behavior to the day-night cycle. The fruit fly, *Drosophila melanogaster*, shows a bimodal circadian activity rhythm with peaks around light-on and before light-off. This rhythm is regulated by a circadian system consisting of multiple oscillators. The oscillators are based on cyclical expression of so-called clock genes. One such gene is *period* (*per*), which is expressed in photoreceptor cells of the compound eyes, cerebral neurons, etc. (Helfrich-Förster, 2003; Kaneko and Hall, 2000; Plautz et al., 1997). Approximately 150 cerebral neurons express the PER protein and are referred to as clock neurons (Taghert and Shafer, 2006). They are classified into seven groups: three groups, dorsal neurons 1 (DN1), DN2, and DN3, are located in the dorsal part of the brain; another three groups (s-LNv, l-LNv, and LNd) are located between the optic lobe and the cerebral lobe; and the remaining group, the lateral posterior neurons (LPNs), is located near the posterior surface of the lateral brain. The s-LNv neurons are further categorized into two types, PDF-positive s-LNvs and the PDF-negative 5th s-LNv, according to expression of pigment-dispersing factor (PDF) (Kaneko et al., 1997), which is involved in the clock's output system (Lin et al., 2004; Renn et al., 1999). The circadian clock system is assumed to consist of these groups of clock neurons.

The dual oscillator model has been proposed to address the cellular organization of the clock system (Grima et al., 2004; Stoleru et al., 2004, 2005). This model assumes that the morning peak is driven by PDF-positive LNs, including l-LNvs and PDF-positive s-LNvs, whereas the evening peak is driven by LNds, the PDF-negative 5th s-LNv, and a subset of DNs. Using different strategies, other groups have reported more or less similar results (Picot et al., 2007; Rieger et al., 2006; Yoshii et al., 2004). However, it is still unclear how these neurons regulate the morning and the evening peaks and how they interact to produce temporally organized behavioral rhythms.

Light is the most powerful zeitgeber to synchronize the circadian rhythm. CRY is the principal circadian photoreceptor molecule expressed in a cell-autonomous manner, mainly in LNvs (Emery et al., 2000b). Light-activated CRY binds to the clock protein TIMELESS (TIM) and is involved in TIM's degradation, which resets the molecular oscillator (Ceriani et al., 1999). Flies overexpressing CRY are behaviorally more sensitive to short light pulses (Emery et al., 1998), while cry^b mutant flies carrying a strong loss-of-function mutation in the *cry* gene lack phase shifts after light pulses and are unable to quickly resynchronize to shifted light cycles (Stanewsky et al., 1998). However, cry^b flies are still able to entrain to light cycles, and the molecular oscillation in their LNv is still light entrainable (Emery et al., 2000a; Stanewsky et al., 1998). Therefore, other CRY-independent photoreceptors must

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exist and synchronize the LNv. The compound eyes, ocelli, and Hofbauer-Buchner (H-B) eyelets have been shown to contribute to photic entrainment (Rieger et al., 2003), but except for CRY, their roles in the entrainment mechanism remain elusive.

The present study aimed to characterize the oscillatory constituents that regulate locomotor activity, and to investigate how the four known photoreceptors contribute to the photic entrainment. We first examined the process of entrainment of the locomotor rhythm to reversed light or temperature cycles by using wild-type and cry^b mutant flies. This examination showed there are four oscillators with different entrainability to light and temperature, two for the morning peak and two for the evening peak. To clarify the role of the four circadian photoreceptors, the process of reentraimnent to a reversed LD was also examined, by using mutant flies lacking some of the photoreceptors. Each of the four photoreceptors was found to have its own role in photic entrainment of the circadian oscillators. The results are discussed in relation to the multioscillatory organization of the *Drosophila* circadian system.

MATERIALS AND METHODS

Flies and locomotor activity recordings

All experiments were performed with adult *Drosophila melanogaster* reared on standard cornmeal-glucose-yeast medium at 25°C under a light-dark cycle with 12-hr light/12-hr darkness (LD 12:12). Canton-S was used as a wild-type strain. Mutant flies used were cry^b ss^1 (aka rec6, hereafter referred to as cry^b) (Stanewsky et al., 1998) and $w;so^1;cry^b$ (provided by Dr. J. C. Hall and Dr. R. Stanewsky, respectively), and *eya* and so^1 (Pignoni et al., 1997).

For activity recordings, male flies 1 to 5 days old were used. The method for locomotor activity recordings was as described previously (Miyasako et al., 2007). Light intensity at the animal's level was approximately 900 lx, but varied slightly with proximity to the lamp. The light intensity was adjusted to desired intensities (i.e., approximately 0.1 or 20–30 lx) as necessary, by shading the lamp. The raw data were displayed as conventional double-plotted actograms in order to examine activity patterns.

Data analysis

Phase determination for the reentrainment and phase response curve (PRC) experiments was performed by three experienced researchers blind to both genotype and experimental treatment. The onsets of the morning and the evening peaks were used as the phase reference points. They were determined with the aid of histogram-type actograms in which the onset was rather easily accessed. When an activity peak showed a steady phase angle relationship with a zeitgeber for at least 5 days, the activity peak was judged to have established a steady-state entrainment. In the steady-state entrainment under the LD cycle, the phase angle difference was shown as the time difference between the onset of the morning peak and the light-on ($\Psi_{MP\text{-}L}$) or between the onset of the evening peak and the light-off ($\Psi_{EP\text{-}D}$). This value was indicated as a positive value when the peak occurred earlier than the light-on or light-off. Under the temperature cycle, the phase angle difference was shown as the time difference between the onset of the morning peak and that of the thermophase ($\Psi_{MP\text{-}TP}$), or between the onset of the evening peak and that of the cryophase ($\Psi_{EP\text{-}CP}$).

To illustrate the process of gradual phase shifts of the morning and evening peaks after reversal of the light-dark cycle, the onset of each activity peak was visually determined on individual actograms for 4 days before and for 10–20 days after the reversal. An average phase (time of day) of the activity peaks for flies examined was then calculated and plotted with SEM.

To obtain PRCs for light, flies were first entrained to LD 12:12 for 3 days. At the fourth dark phase of the cycle, flies were transferred to constant darkness (DD). During the seventh cycle in DD, a 12-hr white light pulse (approximately 900 lx) was given. To determine the magnitude of the phase shifts, we chose the activity onset and offset for the morning and evening peaks, respectively, as the phase reference, because these could be most clearly determined in the free-running conditions in DD. The activity onset of the morning peak was designated as CT0. The magnitude of the phase shifts was calculated by measuring the phase difference, on the day when the light pulse was given, between the lines fitted either to the activity onsets (morning peak) or to the offsets (evening peak) in the steady state free-running before and after the light pulse. To make the PRCs, values of phase shifts were plotted against the circadian time (CT) at which the light pulse was given.

RESULTS

Reentrainment of locomotor activity rhythms of wild-type and cry^b mutant flies to reversed LD cycles

We first examined locomotor rhythms of wild-type and cry^b mutant flies under LD cycles with light intensities of 0.1 lx, 20–30 lx, and about 900 lx. In all LD cycles, the wild-type flies showed clearly synchronized locomotor rhythms with peaks starting at light-on and before light-off (Fig. 1). The evening peak started earlier as the light intensity was lowered (Table 1, $P<0.05$, ANOVA). When the light cycle was reversed by lengthening the light phase by 12 hr, both the morning and the evening peaks resynchronized by delay phase shifts at all light intensities. The morning peaks dissociated into two components: both quickly resynchronized with only a few transient cycles (Table 2). One of them (MP1) resumed the original phase relationship, while the other (MP2) did not, establishing a new steady phase relationship a few hours earlier than light-on (Table 1). MP1 seems to be an endogenous rhythmic component rather than a simple masking effect or a startle response caused by a dark/light transition, because it often started slightly before light-on (Fig. 1). These results suggest that the morning peak consists of two components driven by separate oscillators.

Mutant flies (cry^b) also clearly synchronized to the LD cycles of the three different light intensities (Fig. 2). As in wild-type flies, the evening peak occurred earlier as the light intensity was lowered (Table 1, $P<0.05$, ANOVA). A significant delay in the morning peak was observed at 900 lux (Table 1, $P<0.05$, ANOVA followed by the Tucky test). When the light cycle was reversed, the locomotor rhythm of cry^b mutant flies resynchronized, but by a process different from that for wild-type flies. At 0.1 lx, the evening peak showed only delay shifts (Fig. 2), except for two flies (2/18, or 11%) in which the process of reentrainment could not be determined because the activity disappeared during the transient cycles. However, in flies at higher light intensities, except for a single fly that showed only delay shifts at 20–30 lx, the evening peak showed either only advance shifts or dissociation into two peaks, i.e., advancing (EPa) and delaying (EPd) peaks. The dissociation of the evening peak occurred in 60% of the flies tested at 20–30 lx and 31% at 900 lx, suggesting that the evening peak also consists of two components driven by two separate oscillators. The morning peak always showed advance shifts and synchronized signifi-

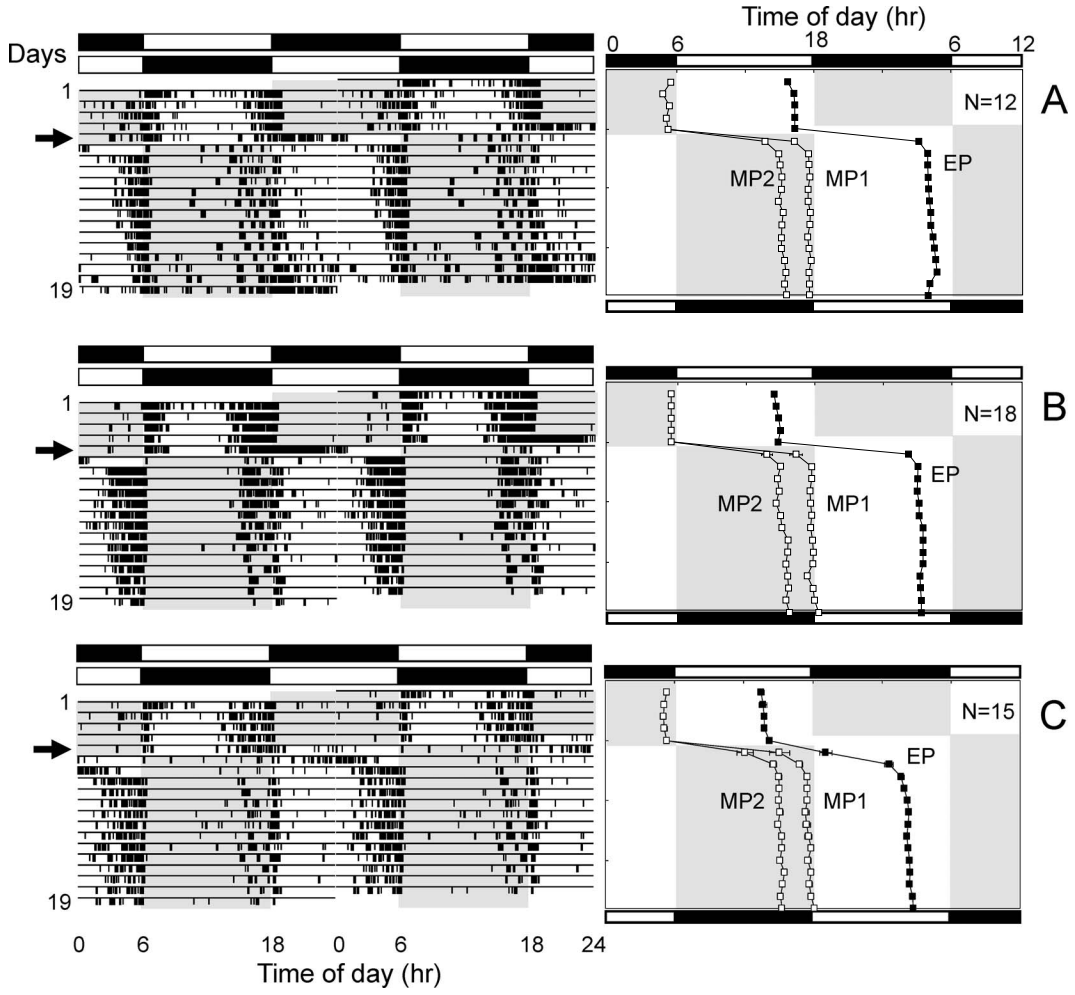

Fig. 1. Reentrainment of wild-type flies to reversed light cycles with different approximate light intensities: **(A)** 900 lx; **(B)** 20–30 lx; **(C)** less than 0.1 lx. A typical actogram at each light intensity is shown at the left, with the corresponding mean phase plot at the right. The light cycles were delayed by 12 hr by lengthening the light phase on day 5 (arrows at left). Dark phases are indicated by gray regions and by black bars above and below the mean phase plots. The bars above the actograms show the light (white) and dark (black) regimes before (upper bar) and after (lower bar) the reversal. In the mean phase plots, open squares show the morning peaks (MP1, MP2), and closed ones the evening peak (EP). N, number of individuals averaged.

Table 1. Phase angle relationship between each peak and the light cycle before and after LD reversal in wild-type (WT) and *cry*^{b} mutant flies. Phase angle differences (Ψ) were calculated between the activity onset of the morning peak and light-on ($\Psi_{MP\text{-}L}$), and between the onset of the evening peak and light-off ($\Psi_{EP\text{-}D}$).

Strain	Light intensity (lx)	N	Before LD reversal		After LD reversal			
			$\Psi_{MP\text{-}L}$ (hr)	$\Psi_{EP\text{-}D}$ (hr)	$\Psi_{MP\text{-}L}$ (hr)	$\Psi_{MP1\text{-}L}$ (hr)	$\Psi_{MP2\text{-}L}$ (hr)	$\Psi_{EP\text{-}D}$ (hr)
WT	0.1	15	0.9±0.3	5.4±0.2^a	–	0.2±0.3	2.5±0.2	3.6±0.1
	20–30	18	0.5±0.2	3.0±0.2^b	–	0.6±0.2	2.3±0.1	2.8±0.2
	900	12	0.9±0.2	1.7±0.2^c	–	0.0±0.3	2.6±0.2	1.3±0.1
cry^b	0.1	16	2.2±0.3^a	5.4±0.2^a	0.8±0.4^a	–	–	5.3±0.2
	20–30	10	2.6±0.4^a	3.4±0.2^b	3.0±0.4^b	–	–	3.5±0.4
	900	13	1.3±0.2^b	2.3±0.2^c	3.6±0.2^b	–	–	2.4±0.2

^{a,b,c}P<0.05, Tucky test following ANOVA within each category.

cantly faster than the evening peak at 0.1 and 900 lx (Fig. 2, Table 2, P<0.01 for 0.1 lx and P<0.05 for 900 lx, *t*-test). The transient cycles of *cry*^{b} mutant flies were significantly longer than those of wild-type flies both for the morning and the evening peaks at all light intensities (Table 2, P<0.01, *t*-test). The phase of the morning peak in the steady-state entrainment to the reversed LD was more advanced under brighter light (Table 1, P<0.01, ANOVA).

Table 2. Number of transient cycles (days) necessary for establishment of steady-state entrainment after the reversal of light cycles in wild-type (WT) and *cry*^{b} mutant flies.

Strain	Light intensity (lx)	Transient cycles (days) of			
		MP	MP1	MP2	EP[§]
WT	0.1	–	3.4±0.3[a]	3.6±0.3[a]	3.9±0.3[a]
	20–30	–	2.3±0.3[b]	2.2±0.2[b]	2.1±0.2[b]
	900	–	1.7±0.2[b]	2.0±0.2[b]	2.3±0.3[b]
cry^{b}	0.1	7.3±0.3**	–	–	9.4±0.3[a++]
	20–30	7.1±0.4	–	–	7.7±0.4[b++]
	900	7.3±0.3*	–	–	8.3±0.4[a,b++]

[§]EPa for *cry*^{b} mutant flies.
[a,b,c]P<0.05, Tucky test following ANOVA within each category.
*P<0.05, compared with EP by *t*-test.
**P<0.01, compared with EP by *t*-test.
[++]P<0.01, compared with WT by *t*-test.

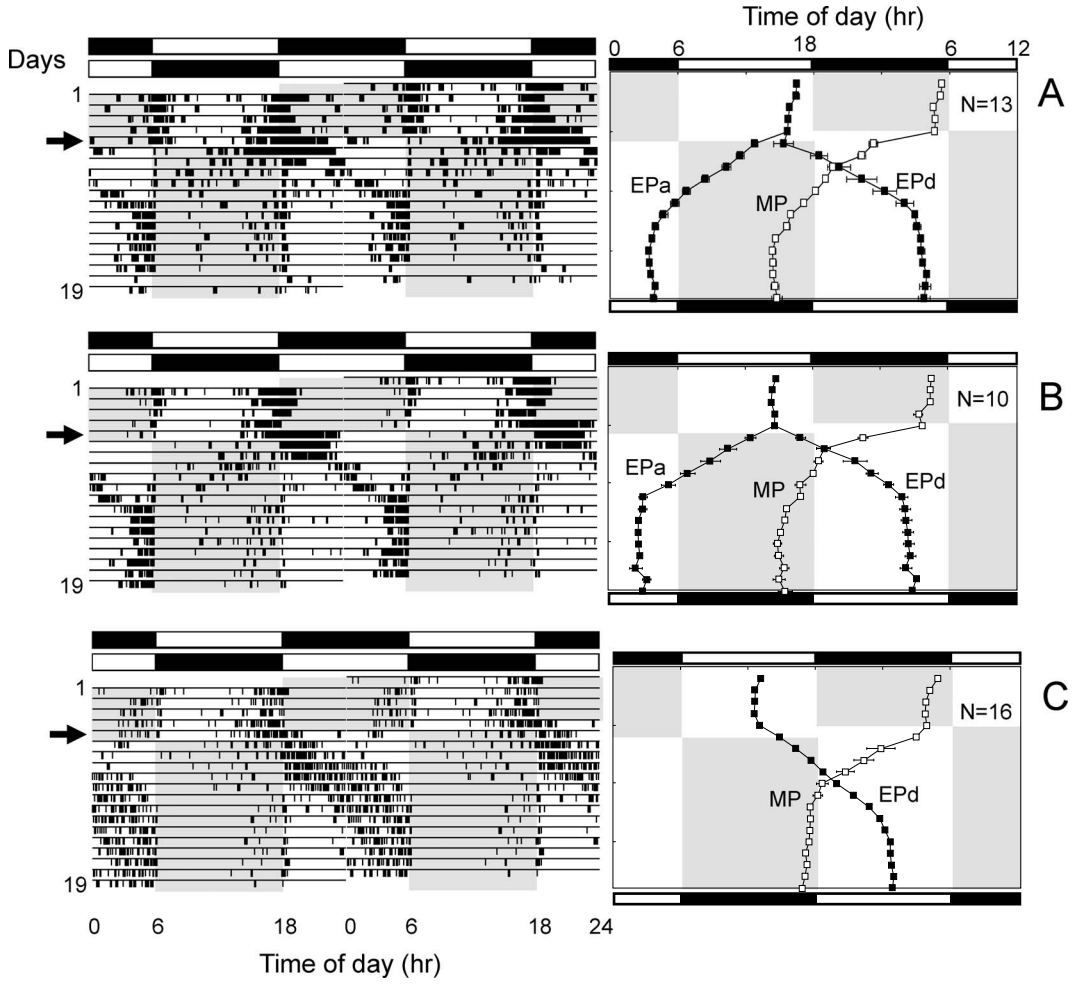

Fig. 2. Reentrainment of *cry*^{b} mutant flies to reversed light cycles with different approximate light intensities: **(A)** 900 lx; **(B)** 20–30 lx; **(C)** less than 0.1 lx. A typical actogram at each light intensity is shown at the left, with the corresponding mean phase plot at the right. Light cycles were delayed by 12 hr by lengthening the light phase on day 5 (arrows at left). Dark phases are indicated by gray regions and by black bars above and below the mean phase plots. The bars above the actograms show the light (white) and dark (black) regimes before (upper bar) and after (lower bar) the reversal. In the mean phase plots, open squares show the morning peak (MP), and closed ones the evening peaks (EPa, EPd). N, number of individuals averaged.

Entrainment of locomotor activity rhythms to temperature cycles in wild-type and *cry*^{b} mutant flies

Temperature cycles are also potent zeitgebers to synchronize the locomotor rhythm in *Drosophila* (Tomioka et al., 1998; Wheeler et al., 1993). For example, a temperature cycle of 25°C 12 hr: 30°C 12 hr synchronizes a fly's locomo-

tor rhythm both in DD and LL (Yoshii et al., 2002, 2005). To examine the process of temperature entrainment, 14 wild-type flies and 10 *cry*^{b} mutant flies were subjected to temperature cycles after transfer to constant darkness in such a manner that the thermophase corresponded to the previous dark phase.

In the wild-type flies, the locomotor rhythm synchronized by delay shifts, as in resynchronization to reversed LD (Fig. 3), with the morning peak again dissociated into two components, one (MP1) synchronizing faster than the other (MP2), suggesting that the morning peak consists of two components driven by two separate oscillators with different entrainability also to temperature cycles. MP1 nearly established a steady phase relationship ($\Psi_{MP1\text{-}TP}$ = –0.3±0.3 hr) to the given temperature cycle after long transient cycles of about 11 days (Fig.3, Table 3). MP2 showed slower and smaller phase shifts than MP1, and finally established a steady entrainment after about 13 cycles of transient cycles. Its steady phase angle difference from the onset of thermophase ($\Psi_{MP2\text{-}TP}$ = 3.2±0.2 hr) was significantly greater than that in entrainment to LD ($P<0.05$, *t*-test). The entrainment process of the evening peak was significantly slower than MP1 (Table 3, $P<0.01$, *t*-test). The shifts were initially slow for the first 3 days, but accelerated for the next three cycles after crossing the transition from cryophase to thermophase. In some flies, MP2 and the evening peak never reached steady-state synchronization during the recording period (Fig. 3B), and thus the average phase continued to shift throughout the recording period (Fig. 3C). A temperature cycle with an amplitude of 5°C may have been too weak to synchronize the rhythm in these flies. The different processes of temperature synchronization among the three peaks again suggest that the three peaks are driven by separate oscillators.

The *cry*^{b} mutant flies showed two different patterns of synchronizing process (Fig. 4); both were substantially different from those of wild-type flies. Both the morning and the evening peaks showed delay shifts in four flies, as exemplified in Fig. 4A. In the particular fly shown in Fig. 4A, the two peaks delayed for the first 5 cycles, with a similar time course by about 7 h, then stayed in this phase, showing entrainment. The remaining three flies showed a similar pattern. In the steady-state entrainment, $\Psi_{MP\text{-}TP}$ and $\Psi_{EP\text{-}CP}$ were 8.1±0.2 hr and 9.9±1.0 hr, respectively (Table 3). The offset of the evening peak sometimes became faint. In six *cry*^{b} flies, both peaks showed advance shifts, as exemplified in Fig. 4B. The morning peak showed a rapid phase advance after transfer to temperature cycles, with its onset reaching the cryophase/thermophase transition. The onset stayed in the same phase, but its offset continued to

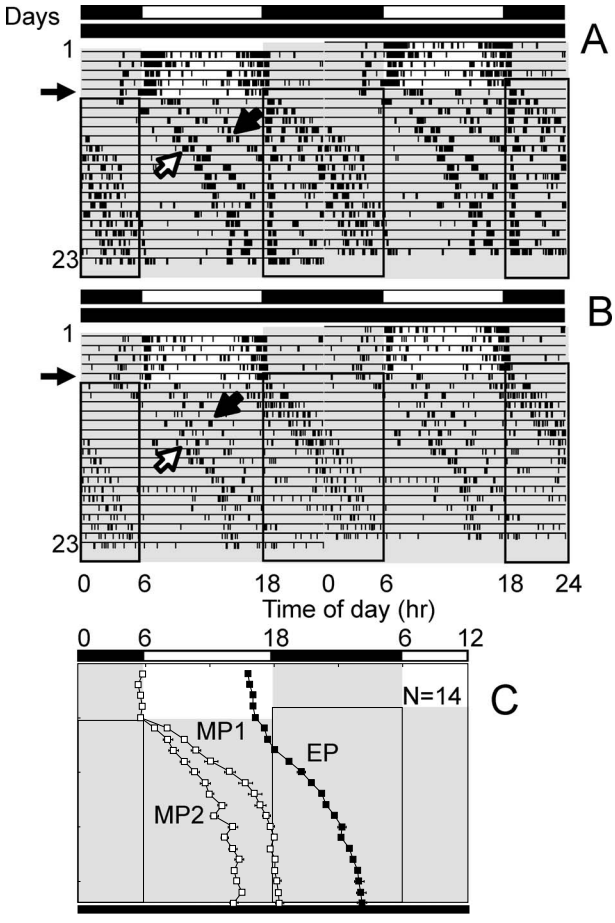

Fig. 3. Entrainment of wild-type flies to temperature cycles in constant darkness. **(A, B)** Typical actograms. **(C)** Mean phase plot. Temperature cycles were given in constant darkness in such a manner that the thermophase corresponded to the previous dark phase on day 5 (arrows at left). Dark phases are indicated by gray regions and by black bars above and below the mean phase plot. The bars above the actograms show the light (white) and dark (black) regimes. The thermophases are boxed in the actograms and mean phase plot. In the mean phase plot, open squares show the morning peaks (MPq, MP2), and closed ones the evening peak (EP). Open and closed arrows inside the actograms indicate MP2 and MP1, respectively. N, number of individuals averaged.

Table 3. Phase angle relationship between each peak and the temperature cycle, and number of transient cycles necessary for establishment of steady-state entrainment to the temperature cycle in wild-type (WT) and *cry*^{b} mutant flies. Phase angle differences (Ψ) were calculated between the activity onset of the morning peak and the onset of thermophase ($\Psi_{MP\text{-}TP}$), and between the onset of the evening peak and the onset of cryophase ($\Psi_{EP\text{-}CP}$).

Strain	N	Phase angle (hr)				Transient cycles (days)			
		$\Psi_{MP\text{-}TP}$	$\Psi_{MP1\text{-}TP}$	$\Psi_{MP2\text{-}TP}$	$\Psi_{EP\text{-}CP}$	MP	MP1	MP2	EP
WT	14	–	–0.3±0.3	3.2±0.2	4.2±0.4	–	10.6±0.8	12.6±0.7	13.5±0.5$^+$
cry^b(delay)	4	8.1±0.2	–	–	9.9±1.0	9.2±0.5	–	–	9.9±0.1
cry^b(advance)	6	5.7±1.2**	–	–	9.6±0.9	15.0±0.4**	–	–	13.3±0.3**

**$P<0.01$ compared with delay shifts in *cry*^{b} flies by *t*-test.

$^+P<0.05$ compared with MP1 by *t*-test.

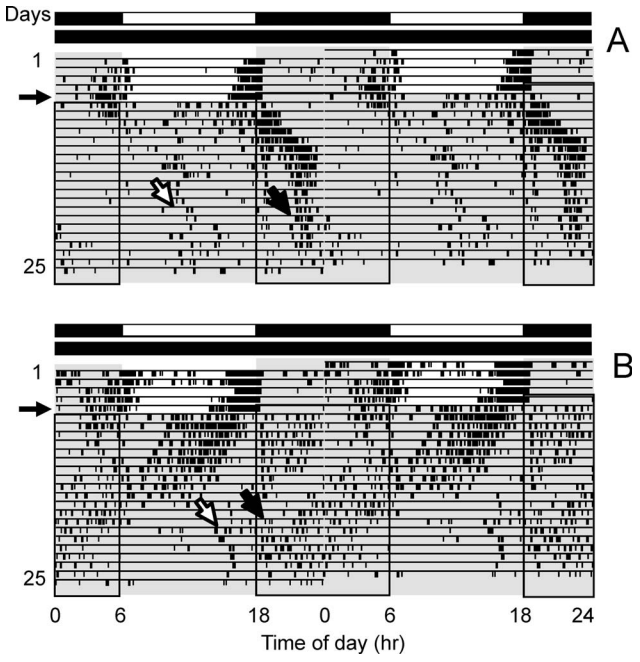

Fig. 4. Actograms indicating two processes of entrainment of *cry*^{b} mutant flies to temperature cycles in constant darkness. **(A)** Delay shifts. **(B)** Advance shifts. Temperature cycles were given in constant darkness in such a manner that the warm phase corresponded to the previous dark phase on day 5 (arrows at left). Dark phases are indicated by gray regions; thermophases are boxed; and the morning and evening peaks are indicated by open and closed arrows, respectively. The bars above the actograms show the light (white) and dark (black) regimes.

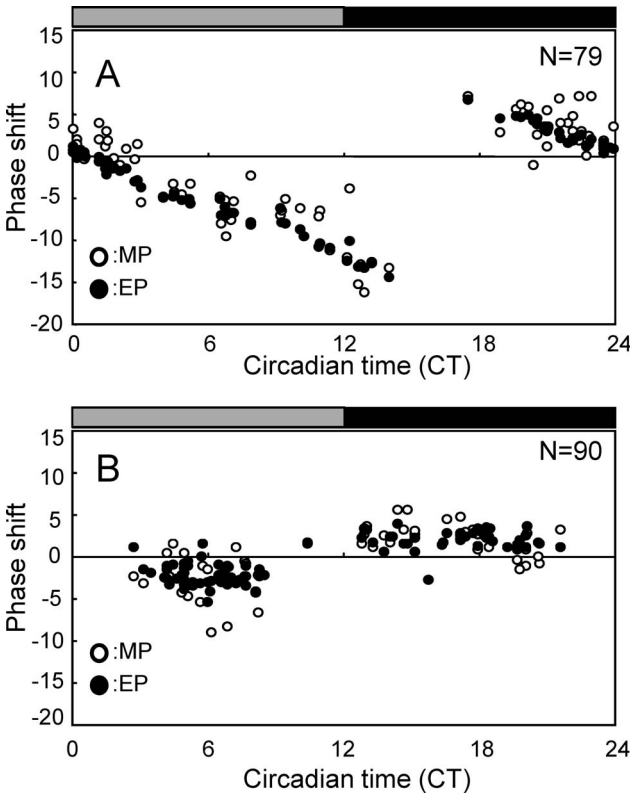

Fig. 5. Phase response curves for a 12-hr light pulse of approximately 900 lx in **(A)** wild-type and **(B)** *cry*^{b} mutant flies. The magnitude of the shift induced by the light pulse is plotted in hours, where positive values indicate phase advances and negative values indicate phase delays against the circadian time (CT) at which the pulse was given. Open and closed circles show the morning and evening peaks, respectively. Black and gray bars above the graph indicate subjective night and day, respectively. N, number of individuals.

advance. When the offset reached the transition, the morning peak jumped into the late cryophase and finally established a steady-state entrainment late in cryophase ($\Psi_{MP\text{-}TP}$ = 5.7±1.2 hr). The evening peak showed advance phase shifts slightly slower than the morning peak until it reached the thermophase/cryophase transition. It then showed a large advance shift to mid thermophase and continued slower advance shifts, eventually establishing a steady entrainment when its onset reached early thermophase. There was a significant difference in $\Psi_{MP\text{-}TP}$ of the steady-state entrainment between flies showing delay shifts and those showing advance shifts (Table 3, $P<0.01$, *t*-test). The number of transient cycles was significantly different between the two types of entrainment processes (Table 3, $P<0.01$ for both MP and EP, *t*-test).

Effects of a 12-hour light pulse on the locomotor rhythm in wild-type and *cry*^{b} mutant flies.

To obtain PRCs for the morning and evening peaks, the phase shifting effects of a long light pulse of 12 hr given in constant darkness were examined. In wild-type flies, the morning and the evening peaks showed a similar pattern of phase shifts (Fig. 5A). Delay shifts occurred from the mid subjective day to early subjective night, while advance shifts occurred during the late subjective night. The magnitude of the shifts depended on the CTs at which the light pulse was given. The maximal delay shift (ca. 15 hr) occurred around CT13, and the maximal advance shifts (ca. 8 hr) around CT18.

In *cry*^{b} flies, both the morning and the evening peaks showed phase shifts smaller than in wild-type flies (Fig. 5B). Both peaks mostly showed shifts in the same direction, as in wild-type flies. In contrast to the wild-type flies, however, advance shifts were elicited during almost the whole subjective night, while delay shifts were induced during subjective day, suggesting that CRY is required for delay shifts from late subjective day to mid subjective night. We had expected that a dissociation of the evening peaks would be induced by a light pulse given early in subjective night, since the LD reversal by extending the light phase by 12 hr caused their dissociation (Fig. 2). However, a 12-hr light pulse in this time interval in *cry*^{b} flies induced no dissociation of the evening peaks. This result suggests that the occurrence of EPd might require continuous light from late subjective day to mid subjective night or longer.

Analysis of photoreceptors for photic entrainment

To determine the roles of the known photoreceptors (compound eyes, ocelli, H-B eyelets, and CRY) in photic entrainment of the four oscillators postulated above that underlie the locomotor rhythm, we tested the process of re-

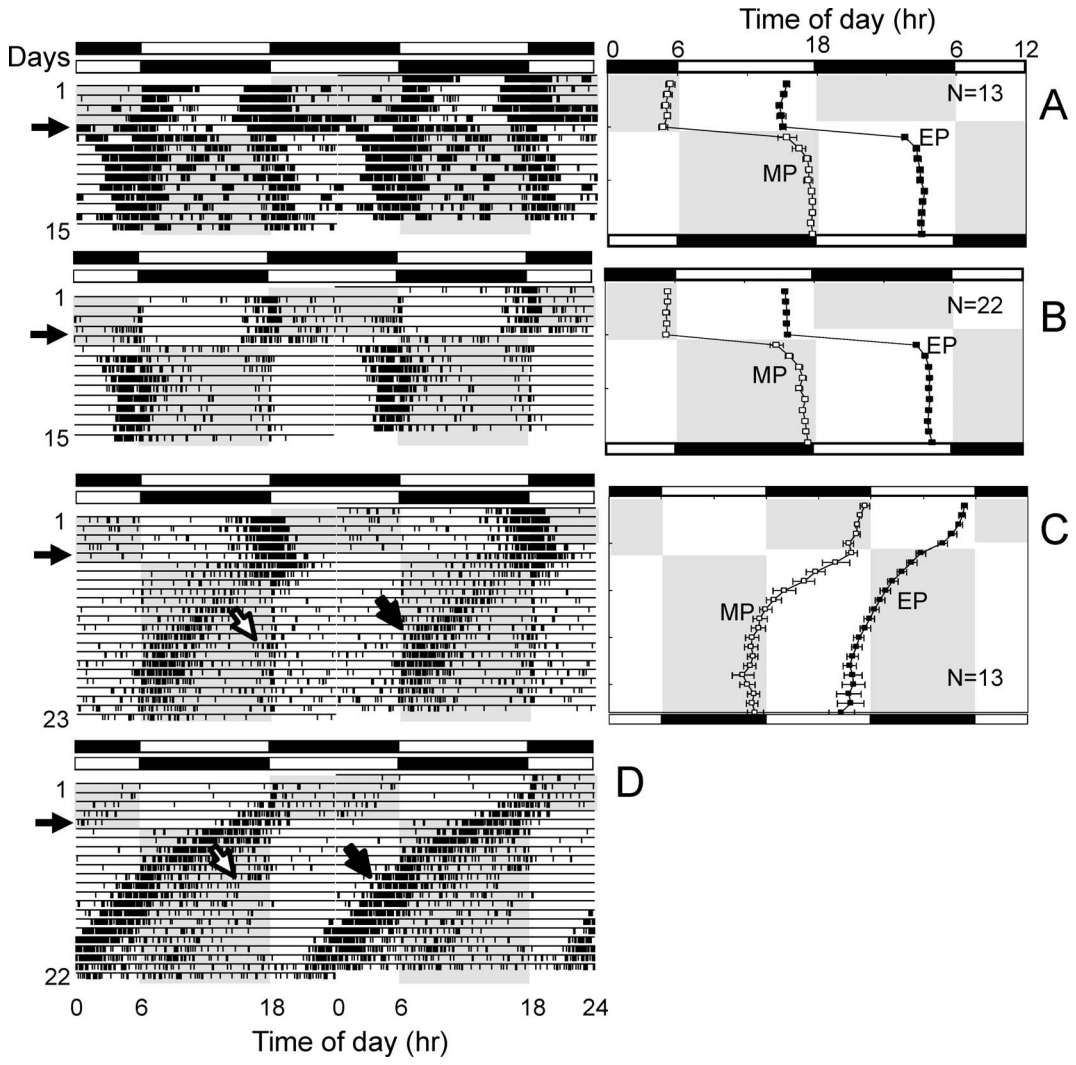

Fig. 6. Representative actograms (left) and plots of average daily onsets of the morning (MP, open squares) and evening (EP, closed squares) peaks (right) in **(A)** *eya* mutant flies, **(B)** so^1 mutant flies, and **(C, D)** $so^1;cry^b$ double-mutant flies. The LD cycle was reversed on day 5 by extending the light period for 12 hr (arrows at left). The light intensity was approximately 900 lx. Dark phases are indicated by gray regions and by black bars above and below the mean phase plots. The bars above the actograms show the light (white) and dark (black) regimes before (upper bar) and after (lower bar) the reversal. The morning and evening peaks are indicated by open and closed arrows, respectively, in the actograms for the $so;cry^b$ flies. N, number of individuals averaged.

entrainment of locomotor rhythms in reversed LD at a light intensity of approximately 900 lx in mutant flies that lacked some of the known photoreceptors: *eya* mutant flies lack the compound eyes, so^1 mutant flies lack the compound eyes and ocelli, and $so^1;cry^b$ double-mutant flies lack functional CRY in addition to external photoreceptors, except for the H-B eyelet.

The initial entrainment experiment in LD12:12 confirmed that *eya* and so^1 mutant flies synchronize to this LD cycle (Fig. 6A, B), while $so^1;cry^b$ double-mutant flies first seemed to be entrained (Fig. 6C) but then often started to free run, even under LD (Fig. 6D). In the entrained state, the evening peak of *eya* and so^1 mutant flies occurred slightly earlier than that of wild-type flies (Table 4, $P<0.01$, *t*-test). When light cycles were reversed in *eya* and so^1 mutant flies, the morning and evening peaks synchronized with delay shifts (Fig. 6A, B), and resumed the original phase angle relationship (Table 4). Although we could not judge whether or not the morning peak dissociated into two components, it resynchronized with slightly but significantly longer transient cycles than that of wild-type flies (Table 4, $P<0.05$, *t*-test). There seemed no difference in the number of transient cycles between *eya* and so^1 mutant flies (Table 4), suggesting that the contribution of the ocelli to entrainment is less remarkable. In contrast, seven of 13 $so^1;cry^b$ double-mutant flies showed that the morning and evening peaks resynchronized with advance shifts, taking about 8–12 transient cycles (Table 4, Fig. 6C); two of them showed no clear morning peak. The remaining six $so^1;cry^b$ flies showed a free-running rhythm without apparent entrainment to the reversed LD cycle. Even in these flies, slight phase-dependent modulations of the free-running period were evident (Fig. 6D); the period was shorter in the thermophase than in the cryophase.

Table 4. Phase angle relationship between each activity peak and the light cycle before and after the LD reversal at 900 lx, and number of transient cycles necessary for the establishment of steady-state entrainment in wild-type (WT) and mutant flies partially lacking the photoreceptors. Phase angle differences (Ψ) were calculated between the activity onset of the morning peak and light-on ($\Psi_{MP\text{-}L}$), and between the onset of the evening peak and light-off ($\Psi_{EP\text{-}D}$).

Strain	N	Before LD reversal		After LD reversal				Transient cycles (days)			
		$\Psi_{MP\text{-}L}$ (hr)	$\Psi_{EP\text{-}D}$ (hr)	$\Psi_{MP\text{-}L}$ (hr)	$\Psi_{MP1\text{-}L}$ (hr)	$\Psi_{MP2\text{-}L}$ (hr)	$\Psi_{EP\text{-}D}$ (hr)	MP	MP1	MP2	EP
WT†	12	0.9±0.2	1.7±0.2	–	0.0±0.3	2.6±0.2	1.3±0.1	–	1.7±0.2	2.0±0.2	2.3±0.3
cry^{b}†	13	1.3±0.2	2.3±0.2	3.6±0.2	–	–	2.4±0.2	7.3±0.3**	–	–	8.3±0.4**
eya	13	0.8±0.3	3.1±0.3**	0.5±0.2	–	–	3.0±0.1	3.0±0.4*	–	–	2.7±0.3
so	22	0.6±0.2	2.3±0.1**	0.3±0.3	–	–	2.1±0.1	3.5±0.2*	–	–	2.6±0.2
so;cry^{b}	13	1.8±0.4	2.7±0.5	2.7±1.3	–	–	2.0±1.0	8.6±0.6**	–	–	11.6±1.2**

†Data for wild-type (WT) and *cry*^{b} are those shown in Tables 1 and 2.
*P<0.05, compared with WT by *t*-test.
**P<0.01, compared with WT by *t*-test.

DISCUSSION

The results of this study suggest that *Drosophila* has at least four circadian oscillators, two for the morning peak and two for the evening peak, that have different responsiveness to light and temperature. The results also revealed that the putative four oscillators receive photic input from the known circadian photoreceptors, but respond them in different manners.

The evening oscillators

The evening peak often dissociated into two components when *cry*^{b} mutant flies were placed in a reversed LD at more than 20–30 lx (Fig. 2). This dissociation is attributable to different phase responsiveness of the two underlying oscillators to light input from the photoreceptors other than CRY, as has been suggested previously (Yoshii et al., 2004). The phase responsiveness apparently depends on the light intensity, because a substantial fraction of flies showed advance components of the evening peak, EPa, in LD at a light intensity of greater than 20 lx, while most flies showed only delay components, EPd, at 0.1 lx. EPa probably requires higher light intensity, and at low light intensity synchronizes with EPd. At 900 lx, nearly 70% of flies showed only EPa, suggesting that at this intensity EPd was often masked or synchronized with EPa.

Since the rhythm dissociation occurred when the light cycle was reversed by extending the light phase by 12 hr, we assumed that a long light pulse given early in subjective night induces advance shifts in the EPa oscillator but delay shifts in the EPd oscillator. However, this hypothesis is not supported by the results of the phase-shifting experiment using a 12-hr light pulse. When *cry*^{b} flies were subjected to a long light pulse in this time interval, they showed only advance shifts. Longer light pulses or continuous illumination are probably necessary to cause dissociation of the two oscillations, because light induced phase shifts are normally very small in *cry*^{b} mutant flies (Fig. 5B; also see Stanewsky et al., 1998). This hypothesis is also supported by a recent report that the moonlight during the night, i.e., continuous illumination throughout the night, causes a large phase delay of part of the evening peak in *cry*^{b} mutant flies under LD (Bachleitner et al., 2007).

Several studies conducted on the clock neurons that drive the evening peak have suggested that LNd cells, the 5th s-LNv cell, and DN cells are responsible for this peak (Grima et al., 2004; Stoleru et al., 2004, 2005). Besides these cells, PDF-positive s-LNv cells have recently been suggested to drive part of the evening peak in addition to the morning peak (Picot et al., 2007; Rieger et al., 2006; Yoshii et al., 2004). However, since our results showed that the two evening oscillators behave differently from those involved in the morning peak, PDF-positive cells may indirectly drive EPa or EPd. Thus, the neurons responsible for the EPa and EPd oscillators are likely among LNds, DNs, and the 5th s-LNv cells. To identify them, further critical study is required.

The morning oscillators

During reentrainment to the reversed LD, the morning peak of wild-type flies often dissociated into two components (Fig. 1). This suggests that the morning peak is also driven by two separate oscillators with different entrainability to light. Since the s-LNv cells are the only known clock neurons driving the morning peak (Grima et al., 2004; Rieger et al., 2006; Stoleru et al., 2004; Yoshii et al., 2004), it is possible that the s-LNvs consist of subgroups with different entrainability and that other clock neurons are responsible for the morning peak. The two oscillators also have different entrainability to temperature cycles. This statement is based on the results of temperature entrainment in constant darkness, where wild-type flies showed dissociation of the morning peak into MP1 and MP2 (Fig. 3). The patterns of temperature entrainment of the morning peak in *cry*^{b} mutant flies were substantially different from those in wild-type flies (Fig. 4), suggesting that CRY is somehow involved in this temperature entrainment, as has been previously suggested (Kaushik et al., 2007). CRY probably has differential roles in temperature entrainment in the MP1 and MP2 oscillators, since the dissociation was never observed in *cry*^{b} mutant flies. We recently demonstrated that the DNs and LPNs are principally temperature entrainable (Miyasako et al., 2007), but differences among cells were not examined. Identification of the cells driving the two morning peaks is a challenging issue for our future studies.

Influence from the circadian photoreceptors

This study provides information on the role of circadian photoreceptors in entrainment of the four putative oscillators. Mutant flies that lack the compound eyes or both the

compound eyes and ocelli (*eya* and so^1, respectively) showed both that the morning peaks synchronized with delay shifts and that the transient cycles took a few days longer than in wild-type flies (Figs. 1 and 6). This suggests that the compound eyes and ocelli promote the entrainment of the morning peak. In addition, these two external photoreceptors influence the EPa and EPd oscillators to cause advance and delay shifts, respectively. This view comes from the observation that in the cry^b mutant flies lacking functional CRY, the evening peak dissociated into EPa and EPd during reentrainment to reversed LDs at more than 20 lx (Fig. 2). The contribution of ocelli to photic entrainment is probably limited, as Rieger et al. (2003) have suggested, because there were no marked difference in transient cycles between *eya* and so^1. Since the morning peak always reentrained to the reversed LD by advance phase shifts in cry^b mutant flies, the compound eyes and ocelli seem to cause advance shifts in the morning oscillator.

This study revealed that CRY promotes photic entrainment, because the transient cycles were longer in all peaks in cry^b mutant flies. As to the functional role of CRY in photic entrainment, it has been suggested that CRY principally contributes to adjusting the evening activity (Emery et al., 2000b; Helfrich-Förster et al., 2001; Rieger et al., 2003). However, we found that the morning peak of cry^b mutant flies resynchronized to reversed LDs with advance shifts in contrast to delay shifts in wild-type flies (Figs. 1, 2), and that light pulses in DD caused far less shifts of the morning peak in cry^b flies than in wild-type flies (Fig. 5A, B). These findings, suggesting that CRY also regulates the phase of the morning oscillators, are consistent with the finding that *cry* is expressed in s-LNvs in addition to other clock neurons controlling the morning peak (Klarsfeld et al., 2004). CRY probably has differential effects on the light entrainment of oscillators for MP1 and MP2, because the dissociation of morning peaks was not observed in cry^b mutant flies. The H-B eyelets probably contributed to photic entrainment by inducing advance shifts in all oscillators when LD was reversed, since $so^1;cry^b$ double-mutant flies showed only advance shifts during resynchronization (Fig. 6). This hypothesis is also supported by the finding that $hdc^{jk910};cry^b$ mutants, which lacked photic input from the H-B eyelets in addition to loss of the compound eyes, ocelli, and CRY, were less entrainable to LD than $so^1;cry^b$ mutants, which retained the H-B eyelets (Rieger et al., 2003). However, photic input from the H-B eyelets seems rather weak for entrainment, because a substantial fraction of the $so^1;cry^b$ double-mutant flies in the present study failed to be entrained to the reversed LD.

In summary, the circadian photoreceptors probably contribute to reentrainment to the reversed LD as follows: the compound eyes and ocelli induce advance shifts in the two MP oscillators and the EPa oscillator, but delay shifts in the EPd oscillator, while CRY induces strong delay shifts and the H-B eyelets induce advance shifts in all oscillators. The contribution of CRY seems more potent than that of other photoreceptors. As a consequence, in wild-type flies, both the morning and evening peaks quickly complete resynchronization with delay shifts.

This study revealed that the *Drosophila* circadian system consists of four oscillators with different entrainability to light and temperature, and that these oscillators receive photic input differentially from the four known circadian photoreceptors. This complex mechanism is probably attributable to the necessity of regulating appropriately timed activity in confronting daily and seasonally changing environmental light and temperature conditions in the fly's natural habitat, as has been previously suggested (Miyasako et al., 2007).

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